ลักษณะสมบัติเชิงหน้าที่ของสเนกไลก์ซีรีนโปรติเนสจากกุ้งกุลาดำ *Penaeus monodon*

นางสาววรันธร หมอนวัน

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FUNCTIONAL CHARACTERIZATION OF SNAKE-
LIKE SERINE PROTEINASE FROM BLACK TIGER SHRIMP *Penaeus monodon*

Miss Warunthorn Monwan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
Department of Biochemistry
Faculty of Science
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วรันธร หมอนวัน : ลักษณะสมบัติเชิงหน้าที่ของสเนกไลก์ซีรีนโปรติเนสจากกุ้งกุลาดำ *Penaeus monodon* (FUNCTIONAL CHARACTERIZATION OF SNAKE-LIKE SERINE PROTEINASE FROM BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อัญชลี ทัศนาขจร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.ปิติ อ่ำพายัพ, 105 หน้า.

คลิปโดเมนซีรีนโปรติเนส (ClipSPs) มีบทบาทสำคัญในการกระตุ้นเป็นลำดับขั้นของโปรติเนสในหลายระบบภูมิคุ้มกันในสิ่งมีชีวิตไม่มีกระดูกสันหลังรวมทั้งการกระตุ้นระบบโพรฟีนอลออกซิเดส ในการศึกษาครั้งนี้ ได้จำแนกยีนคลิปโดเมนซีรีนโปรติเนส*ที่มีชื่อว่า Pm*Snake จากห้องสมุด subtractive cDNA ของเม็ดเลือดกุ้งกุลาดำ *Penaeus monodon* ที่ถูกยับยั้งการแสดงออกของยีน proPO ด้วยอาร์เอนเอสายคู่ (dsRNA) จากการศึกษาพบว่ายีน *Pm*Snake มี open reading frame ประกอบด้วยนิวคลีโอไทด์ 1,068 คู่เบส สามารถถอดรหัสให้โปรตีนที่มีกรดอะมิโน 355 ตัว ประกอบด้วย signal peptide จำนวน 22 ตัว และมีโดเมนอนุรักษ์ 2 โดเมนประกอบด้วย คลิปโดเมนที่ปลาย N และซีรีนโปรติเนสที่ปลาย C จากการวิเคราะห์ลำดับกรดอะมิโนของ *Pm*Snake พบว่ามีความคล้ายคลึงกับ Clip-SP ในกุ้งกุลาดำ *P. monodon* และ snake-like hemolymp protease 21 (HP21) ที่พบในแมลง *Manduca sexta* *Pm*Snake มีการแสดงออกเป็นจำนวนมากในเม็ดเลือดกุ้ง โดยการแสดงออกเพิ่มขึ้นหลังจากติดเชื้อแบคทีเรีย *Vibrio harveyi* ซึ่งสนับสนุนว่ายีน *Pm*Snake น่าจะเกี่ยวข้องกับยีนที่ตอบสนองระบบภูมิคุ้มกัน เมื่อยับยั้งการแสดงออกของยีน *Pm*Snake โดยการฉีดด้วยอาร์เอนเอสายคู่ dsRNA ที่เหมือนกับยีน *Pm*Snake เข้าสู่กุ้ง พบว่าสามารถลดการแสดงออกของ *Pm*Snake ในระดับทรานสคริปและระดับโปรตีน โดยมีการลดลงของกิจกรรมเอนไซม์ฟีนอลออกซิเดสในเลือดอย่างมีนัยสำคัญ (36.1%) เมื่อเทียบกับกลุ่มควบคุม นอกจากนี้เราได้ผลิตโปรตีนรีคอมบิแนนท์ *Pm*Snake ซึ่งมีขนาดโมเลกุล 37 กิโลดาลตัน ในระบบ *Escherichia coli* (BL21) และ ทำให้บริสุทธิ์โดยคอลัมน์จับจำเพาะ Ni-NTA จากผลการวิเคราะห์ด้วย Western blot โดยใช้แอนติบอดีต่อ *Pm*Snake พบโปรตีน *Pm*Snake ในเม็ดเลือดกุ้งแต่ไม่พบในน้ำเลือด ในการศึกษาการกระตุ้นในระบบโพรฟีนอลออกซิเดสและแอกทิวิตีของซีรีนโปรติเนสในหลอดทดลอง พบว่ารีคอมบิแนนท์โปรตีน *Pm*Snake สามารถเพิ่มแอกทิวิตีของฟีนอลออกซิเดสและซีรีนโปรติเนสในเลือดกุ้ง บงชี้ว่า r*Pm*Snake สามารถกระตุ้นระบบโพรฟีนอลออกซิเดสได้โดยผ่านการกระตุ้นเป็นลำดับขั้นของโปรติเนส

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Clip domain serine proteinases (ClipSPs) play critical roles in the activation of proteolytic cascade in invertebrate immune systems including the prophenoloxidase (proPO) activating system. In this study, we characterized a clip domain serine proteinase, namely *Pm*Snake, which has previously been identified from the shrimp *Penaeus monodon* based on subtractive cDNA library of proPO double-stranded RNA (dsRNA) treated hemocytes. An open reading frame of *Pm*Snake contains 1,068 bp encoding a predicted protein of 355 amino acid residues. A putative signal peptide of 22 amino acids and two conserved domains (N-terminual clip domain and C-terminal trypsin-like serine proteinase domain) were identified in *Pm*Snake. Sequence analysis of deduced amino acids revealed that *Pm*Snake shared similarity with ClipSPs in *P. monodon* and the insect *Manduca sexta* snake-like hemolymph protease 21. *Pm*Snake is mainly expressed in shrimp hemocytes and up-regulated after systemic *Vibrio harveyi* infection supporting that it is an immune-responsive gene. Suppression of *Pm*Snake transcripts by injection of dsRNA corresponding to the *Pm*Snake gene into shrimp, reduced both transcript and protein levels leading to a reduction of the hemolymph phenoloxidase (PO) activity (36.1%), compared to the control, suggesting that the *Pm*Snake functions as a clip-SP in shrimp proPO system. *Pm*Snake with predicted molecular mass of 37 kDa was successfully produced in *Escherichia coli* BL21 cells and purified by Ni-NTA chromatography. The Western blot analysis using anti-*Pm*Snake showed that *Pm*Snake was detected in hemocytes but not cell-free plasma. *In vitro* PO activity assay and serine proteinase activity showed that adding r*Pm*Snake could increase PO activity and serine proteinase activity in shrimp hemolymph, suggesting that r*Pm*Snake can activate the proPO system via serine proteinase cascade.

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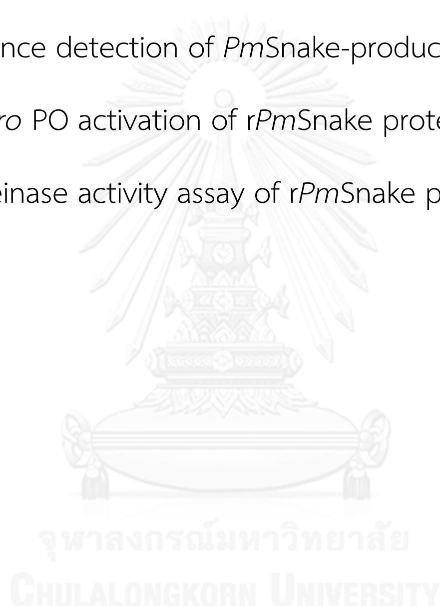
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LIST OF ABBREVIATIONS

°C	degree celcius
μM	micromolar
μg	microgram
μl	microlitre
A	Absorbance
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFU	Colony forming unit
Clip-SPs	Clip domain serine proteinases
C-terminal	Carboxy terminal
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
D	aspartate
dsRNA	double stranded ribonucleic acid
EF1-α	elongation factor 1 alpha
EMS	Early mortality syndrome
hr	hour
HLS	hemocyte lysate

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IPTG	isopropyl-beta-D-thiogalactopyranoside
H	histidine
kb	Kilobase
kDa	kilodalton
L-dopa	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
M	molar
mg	milligram
m	minute
ml	mililitre
mM	milimolar
ng	nanogram
O.D.	optical density
ORF	open reading frame
Pm	*Penaeus monodon*
PO	phenoloxidase
proPO	prophenoloxidase
RNA	ribonucleic acid
TSB	tryptic soy broth

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RNAi	ribonucleic acid interference
RNase	ribonuclease
S	serine
s	second
SP	serine proteinase
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PAP, PPAE	prophenoloxidase activating enzyme
r*Pm*Snake	recombinant *Penaeus monodon* snake mature protein
SPH	serine proteinase homolog
WSSV	white spot syndrome virus
YHV	Yellow head virus

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CHAPTER I
INTRODUCTION

1.1 General introduction

Shrimp aquaculture is a very important activity and considered as a large exchange earner today. Shrimp farming has been developed for more than a century for food and become the livelihood of coastal people. The farming can be classified by stocking densities (the number of seedstock per hectare). First, the low stocking density is called traditional or extensive, seedstock normally come from the wild and supply is season dependent. Semi-intensive culture operation is medium stocking density that the improvement over the traditional approach is in the introduction of a systematic pond configuration. Intensive culture operation is high stocking density, more sophisticated requiring very high financial and technical inputs. Super-intensive is the highest stocking density, greater control of the environment and the technology gets more sophisticated. The several countries in Asia such as Thailand, China and Japan have some super-intensive shrimp farms (Food and Agriculture Organization, FAO 1986).

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World shrimp production increased over the last decades reaching close to 7.5 million tons in 2012. Asian countries contributed 80% of the world shrimp production of 3.5 million tons and the top producers are China, Thailand, Vietnam, Indonesia and India. According to FAO data, the global shrimp production increased

approximately at the rate 4.4 percent per year on average from 2006 to 2012. Unfortunately, disease outbreaks have caused significant dropped of shrimp production in Asia especially in 2013.

Shrimp production in China and Thailand was significantly declined in 2013 due to the deadly disease named Early Mortality Syndrome (EMS) and the production was recovered slightly in 2014 in China but not in Thailand. It is expected that China could be recovered by 2016 while it would take at least 3-4 years for Thailand to recover (Figure 1.1). Nevertheless, from the Global Outlook on Aquaculture Leadership (GOAL) conference 2014, they are expected that the production from 2014 to 2016 will recover and reach a growth rate of approximate 8 percent per year.

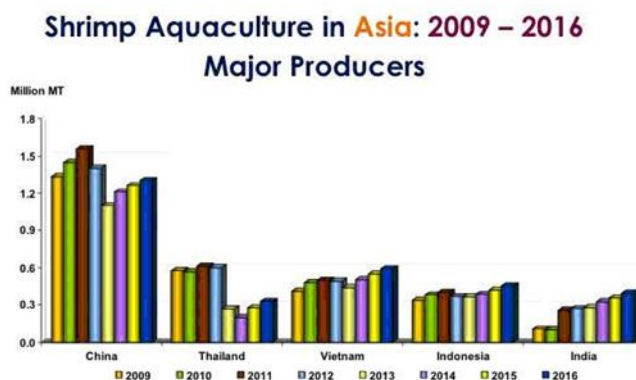

Figure 1.1 Shrimp aquaculture by major producing regions during 2009-2016 (Source: FAO (2013) for 2009-2012; GOAL (2014) for 2013-2016 (the presentation that Dr. James Anderson from World Bank delivered during the recent GOAL 2014 conference in Ho Chi Minh City).

1.2 Black Tiger Shrimp

1.2.1 Taxonomy

Phylum *Arthropoda*

Class *Crustacea*

Subclass *Malacostraca*

Order *Decapoda*

Suborder *Natantia*

Infraorder *Penaeidea*

Superfamily *Penaeoidea*

Family *Penaeidae* Rafinesque, 1815

Genus *Penaeus* Fabricius, 1798

Subgenus *Penaeus*

Species *monodon*

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Scientific name: *Penaeus (Penaeus) monodon* Fabricius,1798.

It has four synonyms: *Penaeus carinatus* Dana, 1852, *P. caeruleus Stebbings*, 1905, *P. monodon* var. manillensis Villaluz and Arriola, 1938, *P. bubulus Kubo*, 1949

The FAO names are giant tiger prawn (English), crevette geante tigree (French), and camaron tigre gigante (Spanish) (Noel B. Solis, 1988).

Figure 1.2 Black tiger shrimp (*Penaeus monodon*)

(http://www.visualfoods.co.uk/sites/default/files/imagecache/guide/Black%2520Tiger_0.jpg)

Black tiger shrimp or giant tiger prawn (*Penaeus monodon*) is one of the most important species of *Penaeus* currently being cultured commercially in many countries. It was found in some Asian countries, such as Indonesia, the Philippines, Taiwan Province of China, Thailand and Viet Nam (FAO 2013). In 2012, worldwide aquaculture realized over 850,000 tonnes of black tiger shrimps (almost all produced in Asia) (CBI Market Information Database). *Penaeus monodon* was originally cultivated with other shrimp species including *Fenneropenaeus indicus*, *Litopenaeus vanamei* and *Fenneropenaeus chinesis*. Thailand is the leader of shrimp production, the world's leading producer of farm-raised *P. monodon* in 1988 (FAO) and currently being an important exporter in the international market. The location of shrimp farms in Thailand are in the central (e.g., Samutsakhorn, Samutsongkharm provinces), the eastern (e.g., Chanthaburi, Chachoengsoa provinces) region and also in the southern region along coasts (e.g., Nakhornsrithammarat, Suratthani provinces).

Recently, the giant tiger shrimp production ranked the second of all shrimp species in Thailand while the pacific white shrimp (*L. vannamei*) has become more popular since 2005 The country has experienced fluctuations in *P. monodon* production, due primarily to the impact of disease, smaller size and the high mortality of shrimp. Black tiger shrimp is highly susceptible to pathogen infection and the production is still reduced. The genetic selection of white shrimp was successfully performed to tolerate diseases and also gain high survival rate (Figure 1.3). Thus, the white shrimp are easy to domesticate and become the main aquaculture shrimp species instead of the black tiger shrimp. Additionally, the genetic information of black tiger shrimp has been studied less than the white shrimp (Wyban 2007). To promote the black tiger shrimp to intensive farming again, the knowledge of shrimp immunity is necessary to find the new proficient strategies for diseases control and further development of shrimp farming.

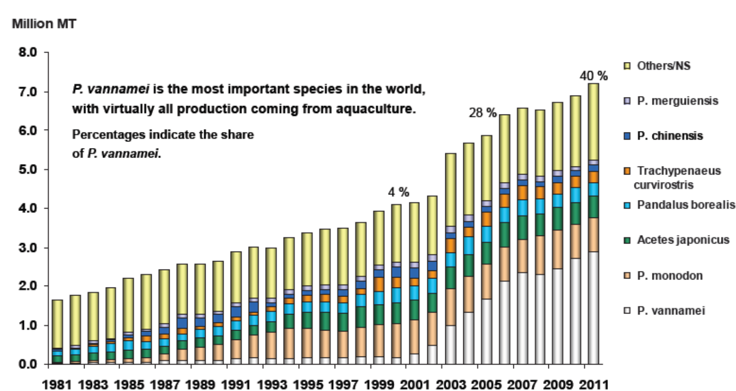

Figure 1.3 The world production of shrimp by species (Source: FAO 2013)

1.3 Diseases

The production of black tiger shrimp in all countries was still decreasing and seriously affected by diseases (Figure 1.4). Shrimp diseases can be classified into two groups, infectious and noninfectious etiologies (Lightner and Redman 1998). Diseases are one of the major problems for shrimp aquaculture caused by various pathogens for example fungi, bacterial, parasites and virus. Bacteria and virus are major serious causes of diseases in shrimp farming.

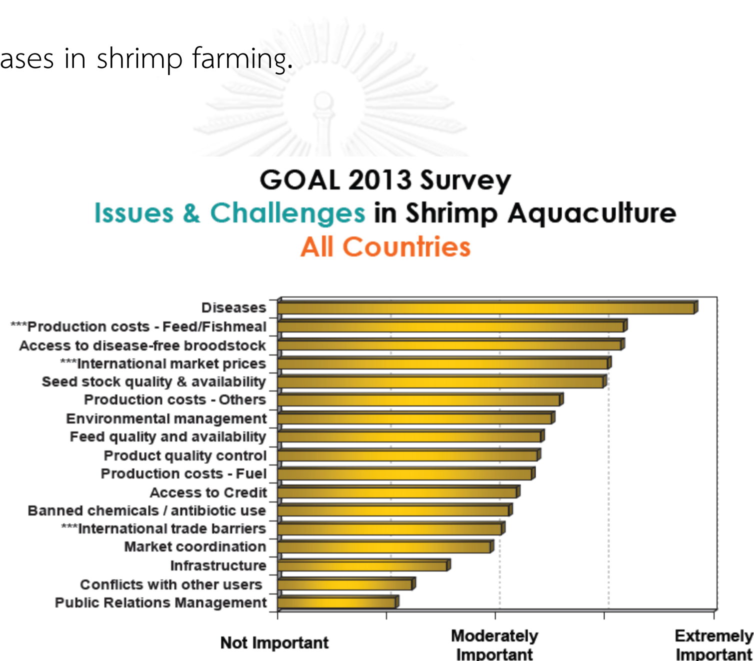

Figure 1.4 Factors affecting shrimp aquaculture in all countries

(Source: GOAL 2013)

1.3.1 Viral diseases

The first virus was discovered in penaied shrimp named *Baculovirus penaei*. At present, several pathogens have been found in shrimp aquaculture in Thailand including white spot syndrome virus (WSSV), yellow head virus (YHV), taura syndrome

virus (TSV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV), infectious hypodermal and hemotopoeitic virus (IHHNV) and laem singh virus (LSNV) (Lightner 1999; Lightner 2011). The WSSV and YHV are two major viral pathogens in black tiger shrimp.

1.3.1.1 White spot syndrome

The white spot syndrome has spreaded to most farmed shrimp species. The major targets of infection are tissues of ectodermal, mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues , (Momoyana et al. 1994; Wonteerasupaya et al. 1995), gills, stomach, hematopoietic tissues and lymphoid organ (Tan et al. 2001; Durand and Lightner 2002; Escobedo-Bonilla et al. 2007). Most shrimp in the ponds are rapidly dead by this disease, and these shrimp show the white spots on their carapaces. The disease outbreaks have caused 80-100% mortalities within 2-10 days (Lo et al. 1996; Flegel 1997; Flegel and Alday-Sanz 1998; Flegel 2006). Shrimp occur a loose cuticle with white spots of 0.5 to 2.0 mm in diameter, which are more apparent on the inside surface of the carapace (Lightner 1996). The other symptoms are the body surface and appendages turning into red or pink, loosing shell, lower food consumption and slow lethargic behaviors (Liu et al. 2009).

This disease is caused by the virus namely white spot syndrome virus (WSSV) which is very large double-stranded DNA virus and enveloped rod-shaped particle with a single filamentous like tail one end of the nucleocapsid (Yang et al. 2001; Lightner 2011) (Figure 1.6). WSSV assigned as the only member of the genus *Whispovirus* (family *Nimaviridae*). The virions are large rod-shape to elliptical and with trilaminar envelope (Lightner 2011). The virus infects only crustaceans and it is not related to any other known viruses. In crustaceans, including shrimp, lobsters and crabs from marine, brackish or freshwater environment, are considered susceptible to infection. However, the disease has mainly been a problem in farmed shrimps.

The penaeid shrimp aquaculture worldwide was affected by the white spot syndrome disease especially in Asian countries (Kim et al. 1998). White spot viral disease emerged in East Asia in 1992-1993 and it was quickly dispersed to South East Asia and India. The first reported from farmed *Marsupenaeus japonicus* in Japan in 1993 (Inouye et al. 1994; Inouye et al. 1996; Nakano et al. 1994). The diseases have caused severe damage to the shrimp culture industry in China (Huang et al. 1994), Thailand (Wonteerasupaya et al. 1995), Japan (Takahashi et al. 1994), Taiwan (Huang et al. 1995), Korea (Kim et al. 1998) and India (Karunasagar and Otta 1998).

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Figure 1. 5 White spot syndrome disease. The cuticle of shrimp occur white spot at the late phase of infection (Thai Agricultural Standard, TAS 10451-2007)

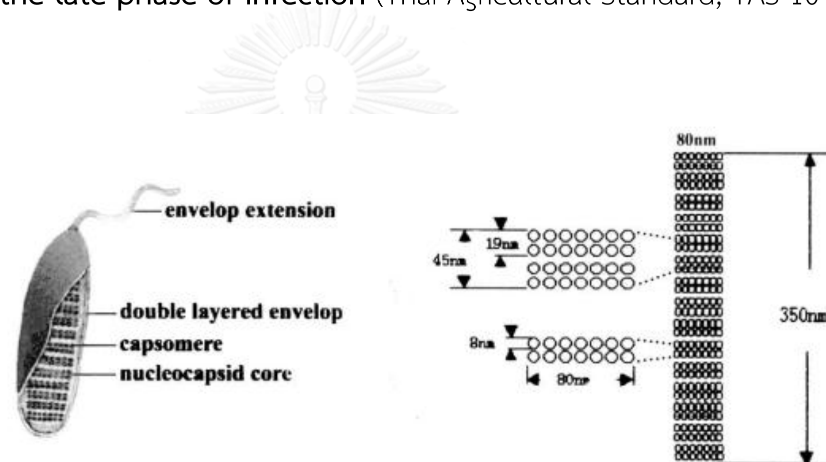

Figure 1.6 The WSSV nucleocapsid structure encapsidated within the double-layered envelope. Each spiral helix has two striations composed of seven pairs of globular capsomers (Huang et al. 2001)

1.3.1.2 Taura syndrome

Taura Syndrome is caused by taura syndrome virus (TSV) that can be transmitted by horizontal transmission or by contaminated water. Shrimps have a general pale red colouration with the tail fan and pleopods appearing hyperpigmented due to the expansion of chromatophores. Typically, the cuticle is

soft and the gut is empty, and infected shrimp may not resist ecdysis. Moribund shrimp accumulates at the pond surfaces and edges. The disease due to TSV has ranged 40-90% mortality in shrimp populations.

In 1992, Taura syndrome was reported on the epizootiology of the disease in Ecuador. The principal host for TSV is the Pacific white shrimp *L. vannamei* although other species can be infected. TSV also infects a number of penaeid shrimp species (Overstreet et al. 1997; Lightner and Redman 1998) and has been largely and geographically distributed in the Americas (Hasson et al. 1999a; Hasson et al. 1999b), which was later spreaded to Southeast Asia, where it is responsible for acute mortalities of farmed penaeid shrimp in Taiwan (Yu and Song 2000).

Taura syndrome syndrome virus is a small single RNA virus. The particle non-enveloped icosahedral in shape, virion is a 32 nm diameter. The genome of TSV consists of a linear, positive sense single-stranded RNA, poly-A tail, and it contains two large open reading frames (ORFs) (Lightner 2011). The virus replicates in the cytoplasm of host cells. TSV has been assigned by the International Committee on Taxonomy of Viruses (ICTV) to the newly created genus *Cripavirus* in new family *Dicistroviridae* (in the superfamily of *Picoranviruses*).

Figure 1.7 Taura syndrome diseases in shrimp (European Community Reference Laboratory for Crustaecean Diseases leaflet, 2008)

1.3.1.3 Yellow head disease

The symptom of Yellow Head Disease (YHD) can induce up to 100% mortality in infected shrimp within 3-5 days (Lightner 1996) of the first appearance, abnormally high rate feeding, yellow light cephalothorax and hepatopancreas (Chantanachookin 1993).

YHD is considered to be an infection of the Yellow Head Virus (YHV). YHV is an enveloped, rod-shaped, positive sense single-stranded RNA (ssRNA) virus in the genus *Okavirus*, family *Roniviridae* and order *Nidovirales*. Virus replicates within the cytoplasm of infected host's cells. The major target tissue for viral replication is the lymphoid organ (Kanobdee et al. 2000). To analyze the YHV infection, the immune-histochemistry, the monoclonal antibody aggregated with a surface glycoprotein and the nucleocapsid protein were examined YHV virus infection (Sánchez-Barajas 2009).

In 1991, the disease occurred as an epizootic in the Thai shrimp farms (Limuswan 1991), and the subsequent outbreaks have been reported from other black tiger shrimp and white shrimp farming countries in Asia. A closely related strain of YHV, named Gill-Associated virus (GAV), has been reported from Australian shrimp farms (Walker et al. 2001).

Figure 1.8 Yellow head disease in shrimp

(Source: AGDAFF-NACA (Photo D. V. Lightner))

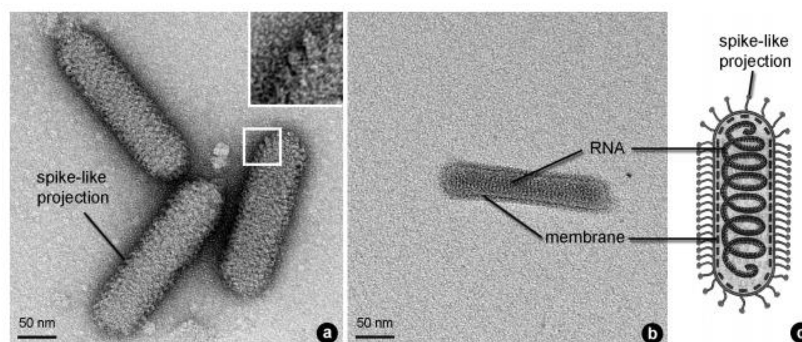

Figure 1.9 TEM micrographs and a schematic diagram of intact virus and nucleocapsid of the yellow head virus. (Duangsuwan et al. 2011)

1.3.2 Bacterial diseases

1.3.2.1 Vibriosis

Vibriosis, one of the major problems in shellfish and finfish aquaculture, causes the mortality of cultured shrimp worldwide (Lightner and Lewis 1975; Austin and Zhang 2006). The disease causes high mortality in shrimp up to 100% which usually occurred in post-larvae and young juvenile shrimp (Lightner et al. 1983). The symptoms of vibriosis are vasculitis, eye-lessions, gastro-enteritis and luminous vibriosis.

Vibriosis, a bacterial disease caused by gram-negative bacteria species, are widely distributed in culture facilitates throughout in the family *Vibrionaceae*, including *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. penaeicida* (Brock and Lightner 1990; Ishimaru et al. 1995). There have been occasional reports of vibriosis caused by *V. damsela*, *V. fluvialis*and and other undefined Vibrio species (Lightner 1996). The species that can affect to penaeid shrimp is *V. harveyi* (Austin and Zhang 2006).

V. harveyi, a gram-negative marine bacterium, is one of the important pathogens of mass mortalities of fish and invertebrates, including shrimp, seabass, seahorses, sharks and lobster. *P. monodon* larvae suffered mortalities within 48 hr of immersion challenge with strains of *V. harveyi* and *V. splendidus* (Lavilla-Pitogo et al. 1990). Adult shrimps suffering vibriosis may appear hypoxic and show reddening of

the body with red to brown gills, reduce feeding and may be observed swimming lethargically at the edges and surface of ponds (Anderson et al. 1988).

Figure 1.10 Vibriosis in shrimp farming.

(http://mail-enaim.espol.edu.ec/noti/cursos_material/curso19/ligthner/Photo4_2.htm)

1.3.2.2 Early mortality Mortality Ssyndrome (EMS)

The novel emerging bacterial infection disease found in shrimp farming is called Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS). EMS is an evolving disease caused by bacteria with severes mortalities 100% of *P. monodon*, *L. vannamei*, *P. chinesis* in 20-30 days. The EMS/AHPNS pathogen as a unique strain of a relatively common bacterium, *Vibrio parahaemolyticus*. EMS is caused by a bacterial agent, which is transmitted orally, colonizes the shrimp gastrointestinal tract and produces a toxin that causes tissue destruction and dysfunction of the shrimp digestive organ known as the

hepatopancreas. The disease does not affect humans but is often fatal to shrimp. (GOAL, 2013).

The symptom of EMS is erratic swimming or swimming near the bottom of the pond, being reduced growth, whitening of the hepatopancreas, being reduced in size of hepatopancreas, soft texture of the exoskeleton, dark spots or streaks on the hepatopancreas, hardening of hepatopancreas.

EMS was first reported in 2009 in Asia. It caused large-scale die-offs of cultivated shrimp in such countries as China in 2009 (Panakorn 2012), Vietnam in 2010 (Mooney 2012), Malaysia in mid-2010 and Thailand in 2012 (Flegel 2012). *Vibrio parahaemolyticus* was consistently isolated from EMS/AHPNS-infected shrimp (Tran et al. 2013).

Figure 1.11 The infected shrimp caused by early mortality syndrome. Juvenile *Penaeus vannamei* from Viet Nam showing gross signs of EMS/AHPNS, specifically a pale atrophied hepatopancreas and an empty stomach and midgut (Tran et al. 2013).

1.4 Shrimp immunity

Shrimp lack immunoglobulin in adaptive immunity but they have a non-specific immune called "innate immunity". Innate immunity is the first line of defense against microbial, bacterial, fungal and viral infections. The cells and soluble molecules of innate immunity either exist in a fully functional state before encounter with microbes or are rapidly activated by microbes, faster than the development of adaptive immune responses. The innate immunity is relying on the cellular and humoral immune responses.

The cellular immune reactions include phagocytosis, nodulation and encapsulation, whereas the humoral responses involve the synthesis and release of several immune proteins such as antimicrobial peptides (AMPs), prophenoloxidase (proPO) system-mediated melanization, proteinase inhibitors, cytokine-like factors, clotting system, etc.

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The circulatory system of shrimp consists of cellular and humoral immune responses. Several immune molecules are produced and stored in the granules of hemocytes before being released into the hemolymph upon activation by bacterial and/or fungal cellwall components (Tassanakajon et al. 2013).

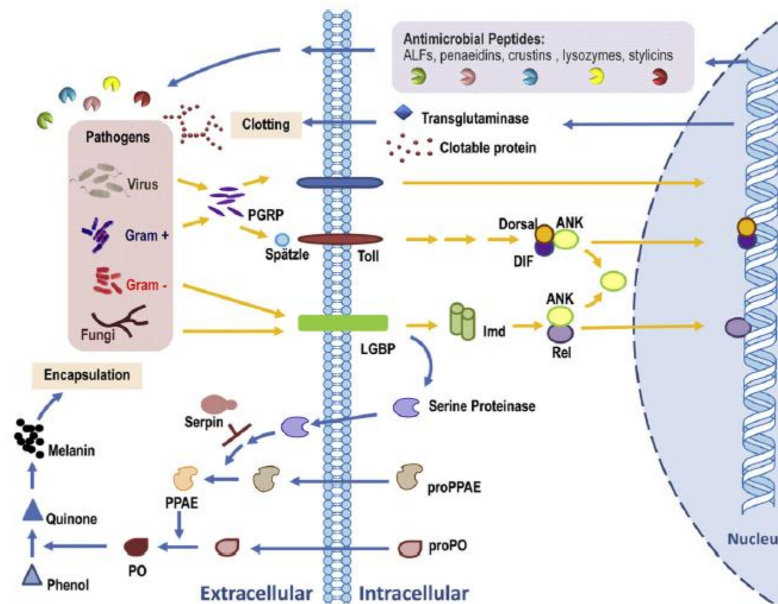

Figure 1.12 The model of shrimp *P. monodon* immune system

(Tassanakajon et al. 2013)

1.4.1 The prophenoloxidase system

The prophenoloxidase (proPO)-activating system is considered to be an important innate defense mechanism in invertebrate immunity. The proPO activation can be triggered by specific pattern recognition proteins (PRPs) which recognize microbial cell wall components such as lipopolysaccharide (LPS) from gram-negative bacteria, peptidoglycan (PGN) from gram-positive bacteria and β-1,3glucan from fungi, leading to activation of a serine proteinase cascade that results in the activation of proPO-activating enzymes (PPAEs). Then, the activated PPAE(s) converts the zymogen proPO to the functionally active phenoloxidase (PO) by specific proteolytic cleavage. POs or tyrosinase-type POs consists of two catalytic activities: the oxygenase activity hydroxylates monophenols to o-diphenolsand the oxidase activity converts o-

diphenols to quinones (Sugumaran 2002; Nappi and Christensen 2005). Subsequently, PO catalyzes the formation of quinone reactive intermediates for melanin synthesis (melanization) at the injury site or around the invading microorganisms (Cerenius and Soderhall 2004; Nappi and Christensen 2005; Amparyup et al. 2012)

The proPO system has been reported in many species such as crayfish *Pacifastacus leniculus*, insect *Manduca sexta*, *Bombyx mori* and *Drosophila melanogaster*. In penaeid shrimp, the enzymes that involving in proPO system are stored in semigranular and granular cell types (Perazzolo and Barracco 1997). *P. monodon* POs (*Pm*proPO1 and *Pm*proPO2) identified and characterized by Amparyup et al., 2009 are the key enzyme in this system.

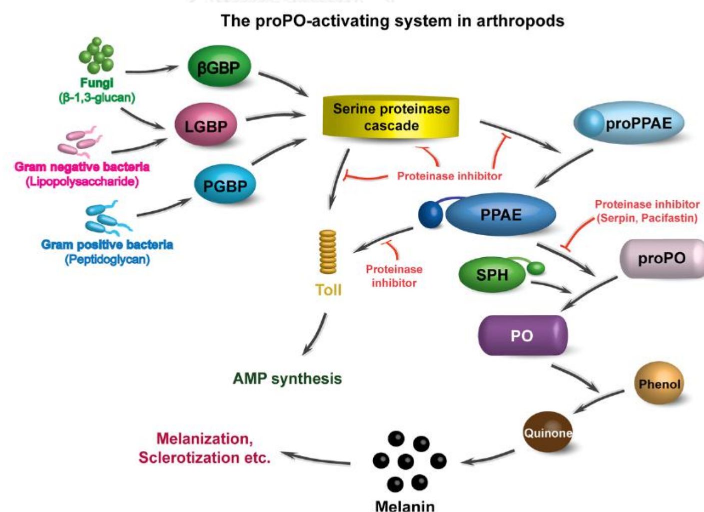

Figure 1.13 The prophenoloxidase-activating system in *P. monodon*

(Amparyup et al. 2013b)

1.4.1.1 Pattern Recognition Proteins (PRPs)

The PRPs recognizes the cell wall of pathogens and act as a crucial step for the activation of the proPO cascade such as LPS, PGN and β-1,3glucan. The various types of PRPs in the proPO system have been reported such as peptidoglycan recognition proteins (PGRPs) (Yoshida et al. 1996; Kanost et al. 2004; Charroux et al. 2009; Sumathipala and Jiang 2010), C-type lectins were found to activate proPO in the cockroach hemolymph and to enhance the laminarin-stimulated proPO system activation. (Chen C 1995; Yu and Kanost 2004; Yu et al. 2006), β-glucan-binding proteins (bGBPs) first discovers in crayfish, enhances the activation of the proPO system and induces hemocyte degranulation and opsonization (Cerenius et al. 1994; Romo-Figueroa et al. 2004) and LPS and-1,3-glucan binding proteins (LGBPs) could bind to LPS or β-1,3-glucan has been documented to activate the proPO system (Beschin et al. 1998; Lee et al. 2000; Amparyup et al. 2012). In *P. monodon*, *Pm*LGBP, a PRP involved in the shrimp proPO system or melanization, exhibits LPS and β-1,3-glucan binding activities, and can activate the proPO cascade.

1.4.1.2 Clip domain serine proteinases (Clip-SPs)

The activation of the proPO cascade requires the proteolytic steps of Clip domain serine proteinases (Clip-SPs). Clip-SPs are synthesized as inactive zymogens which consist of one or more clip domain(s) at the N-terminus interlinked

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by three strictly conserved disulfide bonds and SP domain presence of three catalytic residues (H, D and S) that form a catalytic triad at the C-terminus. The clip domain was named by Iwanaga's group because it could be drawn in the shape of a paper clip in a schematic form to show the disulfide linkages (Jiang and Kanost 2000). Clip-SPs can be classified into catalytic SPs and non-catalytic SPs. The catalytic Clip-SPs, a group of proteolytic enzymes, and the non-catalytic SPs, referred to as Clip-SP homologues (Clip-SPHs), are similar to an amino acid sequence but the serine residue in the active catalytic triads of Clip-SPHs is replaced by glycine (Cerenius and Soderhall 2004).

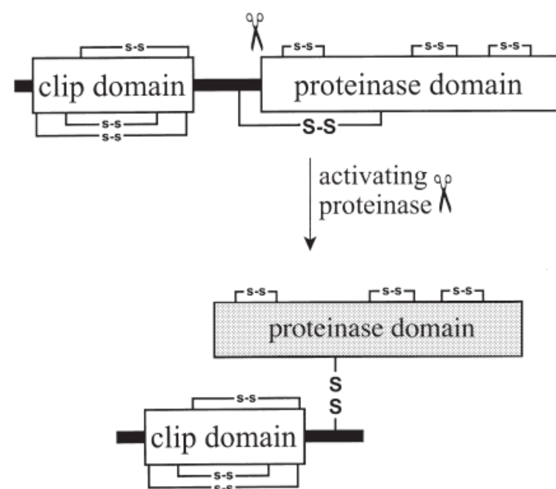

Figure 1.14 The domain organization of Clip-SPs. Clip-SPs contain a clip domain and proteinase domain linked by disulfide bridge as a zymogen or inactive form. An active form is activated by a specific proteolysis at the N-terminus of the proteinase domain, the clip domain and proteinase domain remain covalently attached (Jiang and Kanost 2000).

In arthropods, Clip-SPs play an important role in innate immunity. Several Clip-SPs have been identified and recently discovered Clip-SPs, contains key enzymes in diverse biological processes including immune responses and embryonic development (Jiang and Kanost 2000). The prototype of the clip domain was initially identified in proclotting enzyme from the horseshoe crab *Tachypleus tridentatus* (Muta et al. 1990). In *Drosophila melanogaster*, two serine proteases (snake and easter) act as the Clip-SPs that control the embryo development (Morisato and Anderson 1995; Anderson 1998). In melanization, the clip-SPs in insects (silkworm *Bombyx mori*, Korean black chafer *Holotrichia diomphalia*, tobacco hornworm *Manducasexta*) and crustaceans (crayfish *Pacifastacus lenneusculus* and black tiger shrimp *P. monodon*), named proPO-activating enzyme (PPAE) or proPO activating proteinase (PAP), which is the terminal Clip-SP of the proteolytic cascade of the proPO system that converts the proPOs to active POs, have been reported and characterized. In penaeid shrimps, clip-SPs cascade was found only in an initial and a terminal step of the proPO system. However, our research group identified several associated proteins involved in the shrimp *P. monodon* proPO system activation (Figure 1.15).

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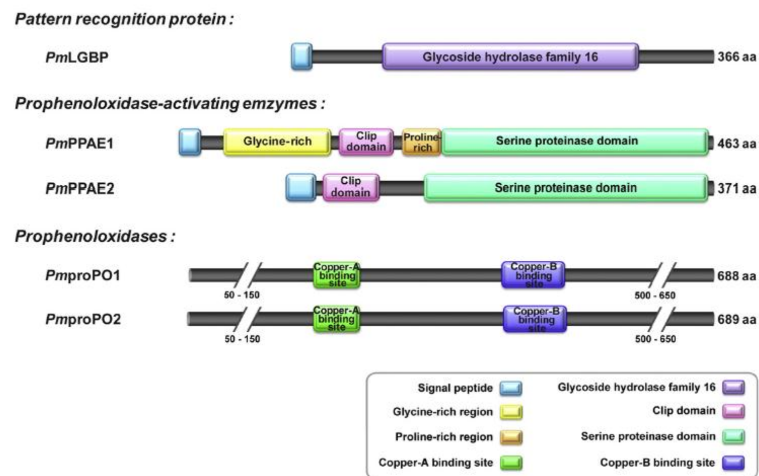

Figure 1.15 The structure of protein in proPO system of *P. monodon*

(Amparyup et al. 2013a)

The Clip-SPHs, likely to be involved in immunity, are regulated in proPO system acting as protein cofactors of PPAEs in proPO system. In insect, *D. melanogaster* is the first report of SPH that involves in muscle attachment in embryo (Murugasu-Oei et al. 1995). In *H. diomphilia*, the proPO-activating factor (PPAF)-II of the beetle is a clip-SPH functioning as a protein cofactor for PPAF-I (Kim et al. 2002) but in *B. moli*, PPAE does not require SPH (Satoh et al. 1999). In crustaceans, crayfish *P. leniusculus*, SPH has been reported to be involved in the pattern recognition of PGN-induced proPO activation, granulocyte adhesion and possibly function as protein cofactors in the PGN-binding complex with LGBP (Liu et al. 2011). In *P. monodon*, the SPH is called masquerade (Mas), *Pm*MasSPH1 and *Pm*MasSPH2, the SP-like domain mediates the hemocyte adhesion and displays bacterial-binding activity to *V. harveyi* and the LPS of *Escherichia coli* (Jitvaropas et al. 2009). The dsRNA interference

studies revealed significant decreases in the hemolymph PO activity of *Pm*MasSPH1 and *Pm*MasSPH2 knockdown shrimp, suggesting that both SPHs are involved in the shrimp proPO system (Amparyup et al. 2013a). *Pm*MasSPH1 specifically interacted with the final proteinase of the proPO cascade PmPPAE2 from yeast two-hybrid analysis and enhances PGN-induced PO activity *in vitro* (Jearaphunt et al. 2015).

Clip-SPs are identified in *P. monodon* which are involved in the proPO cascade. The Clip-SPs in this cascade are *Pm*ClipSP2 (Amparyup et al. 2013b); *Pm*PPAE1 (Charoensapsri et al. 2009); *Pm*PPAE2 (Charoensapsri et al. 2011). *Pm*ClipSP2 participates in the activation of the proPO system, leading to melanin synthesis and also acts as a PRP that exhibits LPS and β-1,3-glucan binding (Amparyup et al. 2013b). *Pm*PPAE1 and *Pm*PPAE2 have been found to be important for the proPO system in shrimp. Gene knockdown of *Pm*PPAE1 and *Pm*PPAE2 showed a significant reduction (37% and 41%, respectively) in the PO activity (Charoensapsri et al. 2009, 2011). In *M. sexta*, hemolymph proteinase 14 (HP14) is a Clip-SP that auto-activated when microbial cell wall invaded into host (Gorman et al. 2007b). Hemolymph Proteinase 21 (HP21) activates proPAP3 by limited proteolysis (Gorman et al. 2007b) and involved in the proPO system in insect.

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1.4.1.3 Serine proteinase inhibitors in the proPO cascade

Proteinase inhibitors are important factors in immune system which regulate proteinase functions. Two families of serine protease inhibitors in negative regulation of proPO system in invertebrate have been reported (Kanost et al. 2001; Cerenius et al. 2008) including serpin (Liu et al. 2009; Homvises et al. 2010) and pacifastin (Liang et al. 1997).

Serpins are high structural conserved inhibitors that act as suicide-like substrates (Huntington and Yamasaki 2011). Serpins acting as a negative regulator of proPO system have been reported in various species in invertebrates. In insect *M. sexta*, serpin-6 could inhibit prophenoloxidase activating proteinase-3 (PAP-3) (Wang Y 2004) but not PAP-1 and PAP-2 suggested that the proPO activation by PAPs is differentially inhibited by multiple serpins. *In vitro*, serpin-4 and serpin-5 formed complexes with HP6, and they inhibited the activation of proHP8 and proPAP1 to control proPO activation and antimicrobial peptides production during immune responses (An and Kanost 2010). In *Drosophila melanogaster,* Serpin27-A is required to control the phenoloxidase activity at the site of injury or infection, preventing the insect from excessive melanization (De Gregorio et al. 2002) while Serpin28-D confines PO aviability by controlling in the initial release. In *Anopheles gambiae*, serpins regulate the activation of proPO and likely toll pathway activation (Gulley et al. 2013). In shrimp *P. monodon*, *Pm*SERPIN8 was identified and has been shown to inhibit the

growth of the Gram-positive bacterium *Bacillus subtilis* and to inhibit the activation of shrimp proPO system (Somnuk et al. 2012).

Pacifastin is a specific serine proteinase inhibitor which found in crustacean. In crayfish (*P. leniusculus*), pacifastin has a molecular mass of 155 kDa and contains a light chain with nine likely proteinase inhibitor domains of pacifastin family and heavy chain containing three transferrin lobes (Liang et al. 1997). Pacifastin are the most efficient inhibitor of the prophenoloxidase activating enzyme of the prophenoloxidase activating system *Pl*PPAE1 (Hergenhahn et al. 1987; Soderhall et al. 1990). However, the function of the serine proteinase inhibitor in shrimp proPO system has not been clearly elucidated.

1.5 The objective of research

Recently, a putative clip domain serine proteinase, named *Pm*Snake, shared similarity to Hemolymph Proteinase 21 (a snake-like proteinase) that involved in proPO system in *M. sexta*, has been identified from suppression subtractive hybridization of proPO dsRNA treated hemocyte of shrimp *P. monodon*.

In the comparison of the activation mechanism of the proPO system in shrimp *P. monodon* and insect *M. sexta* (Fig, 1.15), *P. monodon Pm*clipSP2 and *M. sexta* HP14 function as pattern recognition receptors for recognition of the bacteria or fungal infection. *M. sexta* HP14 converts proHP21 to active HP21 and subsequently activates the melanization cascade, but in shrimp the downstream SP of *Pm*ClipSP2 is

still unknown. Thus, the purpose of this study is to investigate the function of snake-like serine proteinase in the shrimp proPO system. Gene expression analysis of *Pm*Snake after *Vibrio harveyi* challenge was performed. Gene silencing of *Pm*Snake was performed using RNA interference (RNAi) technique. The PO activity of the *Pm*Snake-silenced shrimp was assayed. The acquired result of this research will provide a better understanding of shrimp immunity, particularly in proPO system to draw more attention on prevention and treatment of diseases in shrimp.

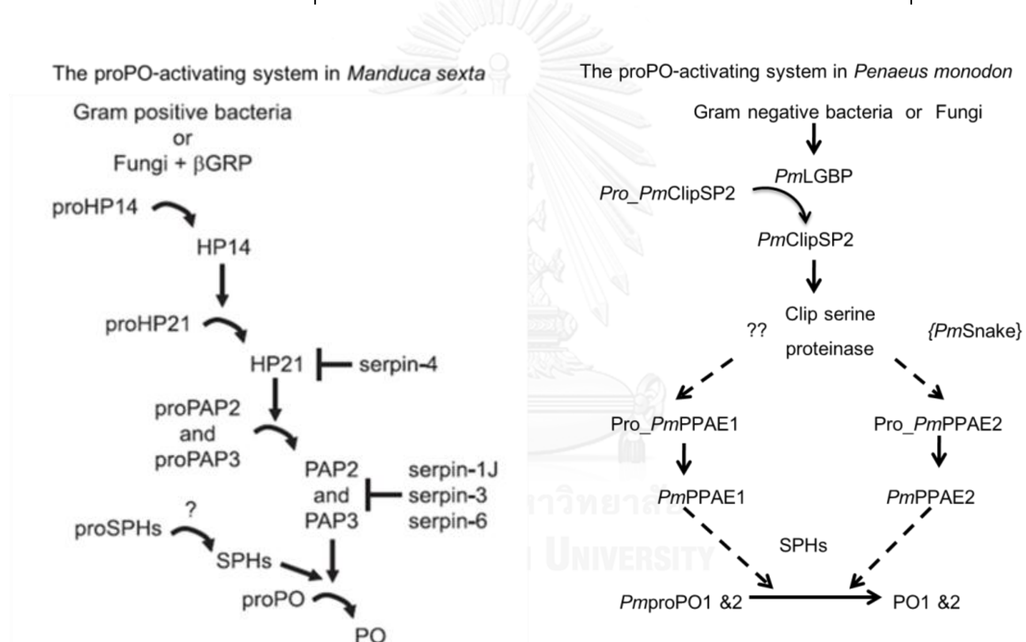

Figure 1.16 The proPO activating system in shrimp *P. monodon* compared with that in insect *M. sexta.* (modified from Gorman et al., 2007, Amparyup et al., 2013)

CHAPTER II
MATERIALS AND METHODS

2.1 Equipment and Chemicals

2.1.1 Equipment

-20˚C Refrigerator Freezer (Whirpool, SHARP), -80˚C Freezer (Thermo)

96-well plate Costar® (Corning Incorporation)

Amicon Ultra-4 concentrators (Vivaspin)

Autoclave LABO (SANYO)

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

Balance: Satorius 1702 (Scientific Promotion Co.)

Gel documentation (SYNGENE)

Gene Pulser (BIO-RAD)

Incubator (Memmert)

Incubator shaker Innova 4080 (New Brunswick Scientific)

Insulin syringes U 100 (Becton, Dickinson and Company)

Inverted confocal laser scanning microscope, FV1000

Laminar Airflow Biological Safety (NuAire, Inc.)

Microcentrifuge tube 1.5 ml, 15 ml and 50 ml (Universal 320R)

Millex syringe-driven filter unit 0.22, 0.45 μM (Milipore, MERCK)

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Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (BIO-RAD, Eppendorf AG, Germany)

PCR thin wall microcentrifuge tubes 0.2 ml (Axygen Scientific, USA)

PCR workstation Model # P-036 (Scientific Co., USA)

PCR strip tube white (BIO-RAD)

PCR cover strip (BIO-RAD)

PD-10 Column (GE Healthcare)

pH meter Model # SA720 (Orion)

Pipette tips 0.2-10, 20-200, 1000 µl (Axygen Scientific, USA)

Power supply, Power PAC 3000 (BIO-RAD)

Spectrophotometer Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Sterring hot plate (Fisher Scientific)

Semi-dry Trans-Blot® (BIO-RAD)

Touch mixer Model # 232 (Fisher Scientific)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

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2.1.2 Chemicals, Reagents and Biological substances

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

100 bpPlusGeneRuler^{TM}(Thermo Scientific)

1 kb GeneRuler^{TM} (Fermentas)

2-Mercaptoethanol (AppliChem)

5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) (Fermemtas)

5-bromo-4-chloro-indolyl phosphate (BCIP) (Fermentas)

Absolute ethanol, CH_3CH_2OH (HAYMAN)

Acrylamide page (GE Healthcare)

Agar powder (HIMEDIA)

Agarose (Research organics)

Alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, Inc.)

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Alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc.)

Ammonium persulfate, $(NH_4)_2S_2O_8$ (USB)

Ampicillin sodium salt (BIO BASIC INC.)

Anti-actin mouse Clone C4 (Milipore, MERCK)

Anti-His antiserum (GE Healthcare)

Azocaecien (SIGMA)

Boric acid, BH_3O_3 (MERCK)

Bromophenolblue sodium salt (USB)

Bovine serum albumin (SIGMA-ALDRICH)

Calcium chloride, $CaCl_2$ (MERCK)

Coomassie brilliant blue R250 (BIO BASIC INC.)

Chloroform, $CHCl_3$ (RCI Labscan)

Diethyl pyrocarbonate (DEPC), $C_6H_{10}O_5$ (SIGMA)

Dipotassium hydrogen orthophosphate (AJAX Finechem)

Dithiothereitol (DTT), $C_4H_{10}O_2S_2$ (BIO BASIC INC.)

Ethidium bromide (SIGMA)

Ethylene diaminetetraacetic acid disodium salt dehydrate, EDTA (Ajax Finechem)

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Formaldehyde, CH_2O (BDH)

Genezol reagent (Geneaid)

Gracial acetic acid, CH_3COOH (MERCK)

Glycerol, $C_3H_8O_3$ (Scharlau)

Glycine, NH_2CH_2COOH (Scharlau)

Hydrochloric acid, HCl (MERCK)

Imidazole (Fluka)

Isopropanol, C_3H_7OH (MERCK)

Isopropyl-β-D-thiogalactoside (IPTG), $C_9H_{18}O_5S$ (Thermo Scientific)

Laminarin from Laminaria (SIGMA)

Lipopolysaccharide (LPS) of *E.coli* serotype 0111:B4 (SIGMA)

Lipopolysaccharide (LPS) of *E.coli* serotype 2630 (SIGMA)

Magnesium chloride, $MgCl_2$ (MERCK)

Methanol, CH_3OH (Burdick&Jackson)

N-N dimethyl formamide (Carlo Erba)

N, N, N', N'-Tetramethylenediamine (TEMED) (USB)

N, N', methylene bisacrylamide (ACROS Organics)

N-Benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride, B2133 (SIGMA)

Nickle sulfate hexahydrate (SIGMA)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitrobluetetrazolium (NBT) (Fermentas)

Paraformaldehyde (SIGMA)

pET28b(+) vector (Novagen)

Potassium dihydrogen orthophosphate (Ajax Finechem)

Prestained protein molecular weight marker (Fermentas)

Skim milk powder (HIMEDIA)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium citrate

Sodium cacodylatetrihydrate (CAC), $(CH_3)_2AsO_2Na \cdot 3H_2O$ (SIGMA)

Sodium chloride, NaCl (Ajax Finechem)

Sodium dihydrogen orthophosphate, $NaH_2PO_4.H_2O$ (Carlo Erba)

Sodium dodecyl sulfate, $C_{12}H_{25}O_4SNa$ (Vivantis)

Sodium hydroxide, NaOH (MERCK)

Trichloroacetic acid

Tris-(hydroxyl methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (Vivantis)

Triton X 100 (Fluka)

Tryptone type I (HIMEDIA)

Tween 20 (Ajax Finechem)

Urea (SIGMA-ALDRICH)

Yeast extract powder (HIMEDIA)

2.1.3 Enzymes and Kits

Adventage® 2 Polymerase Mix (Clontech)

NucleoSpin® Extract II Kits (Reseaarch organics)

Revert Aid First Strand cDNA Synthesis kit (Thermo scientific)

Plasmid mini prep (Geneaid)

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T & A Cloning vector Kit (RBC Bioscience)

T7 RiboMAXTM Express Large Scale RNA Production System (Promega)

Taq DNA polymerase (RBC Bioscience)

Xho I (Biolabs)

Nco I (Biolabs)

RNase A (SIGMA)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase

Trypsin (SIGMA)

Chymotrypsin (SIGMA)

2.1.4 Microorganisms

Escherichia coli strain BL21

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Escherichia coli strain JM109

Vibrio harveyi 639

2.1.5 Software

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

ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/)

GENETYX 7.0.3 program (GENETYX Corporation)

GraphPad Prism 6 (GraphPad Software, Inc.)

FV10-ASW 3.0 viewer

MEGA 6.0

SMART version 4.0 (http://www.smart.emblheidelberg.de/)

2.2 Sequence analysis

Nucleotide and amino acid sequences of *Pm*Snake obtained from Amparyup (unpublished data) were analyzed with the GENETYX 7.0.3 program (GENETYX Corporation) that was used to edit and translate amino acid of the full length cDNA sequence and the BLASTX programs of GeneBank database were used to analyze the sequence compare with sequence database. The deduced amino acid sequence and the putative signal peptide were predicted by the simple modular architecture research tool SMART version 4.0. The ClustalW multiple sequence alignment program was used to create the sequence alignments of *Pm*Snake with *Manduca sexta* hemolymph proteinase 21 (*Ms*HP21) and other serine proteinases of shrimp. The MEGA 6.0 program was used to analyze phylogenetic tree.

2.3 Primer design

All primers were designed base on nucleotide sequence of *Pm*Snake cDNA by Primer Premier 5 Software (Premier Biosoft) and SECentral program (Scientific & Education Software). The primer-dimer formation, GC content and melting temperature were carefully designed. The primers of control gene were the same as the previous research (Amparyup et al., 2013) (Table 2.1)

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Table 2.1 Nucleotide sequence of the primers

Primer	Sequence (5'-3')	Purpose
T7Snake-F	GGATCCTAATACGACTCACTATAGGTTTCTATCGCAACGCTCCAC	RANi
T7Snake-R	GGATCCTAAGACTCACTATAGGTCGATGCAATGGGCGGCTGT	RNAi
Snake-F	TTTCTATCGCAACGCTCCAC	RNAi
Snake-R	TCGATGCAATGGGCGGCTGT	RNAi
*Pm*Snake-F	GAGCTCTCCATGGTCCAGGGGACGGGCGAGCAGTG	Recombinant Protein
*Pm*Snake-R	CTCGAGCTAATGATGATGATGATGATGCACATTTTCTTTGAGCCAAAC	Recombinant Protein
RTSnake-F	TCGCAAACCTTCCAAGTTCTGA	RT-PCR
RTSnake-R	GACAGGCAGGCAACACGCTC	RT-PCR
GFPT7-F	TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA	RNAi
GFPT7-R	TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA	RNAi
GFP-F	ATGGTGAGCAAGGGCGAGGA	RNAi
GFP-R	TTACTTGTACAGCTCGTCCA	RNAi
PPAE1-F	TGGGGCGAAGGCAGGGCACAAGGCGCAG	RT-PCR
PPAE1-R	CTCTTCTTCAAGCTCACCACTTCTATCT	RT-PCR
PPAE2-F	GCGGCGGTCACGCTCCTTGTTC	RT-PCR
PPAE2-R	ACTCTCGGGGGCACGCTTGTTG	RT-PCR
SP1-F	TGAGAGCACAAATAGTGGAGGGGTA	RT-PCR
SP1-R	TGGAGGCAGGCACACAGGCAAC	RT-PCR
SP2-F	GGCGTTGGTCTTCACTGCTCTC	RT-PCR
SP2-R	CAGAACTGCCTTCCAAGGATAG	RT-PCR
PO1-F	GGTCTTCCCCTCCCGCTTCG	RT-PCR
PO1-R	GCCGCAGGTCCTTTGGCAGC	RT-PCR
PO2-F	GCCAAGGGGAACGGGTGATG	RT-PCR
PO2-R	TCCCTCATGGCGGTCGAGGT	RT-PCR
Pen3-F	GGTCTTCCTGGCCTCCTTCG	RT-PCR
Pen3-R	TTTGCATCACAACAACGTCCTA	RT-PCR
EF1-α-F	GGTGCTGGACAAGCTGAAGGC	RT-PCR
EF1-α-R	CGTTCCGGTGATCATGTTCTTGATG	RT-PCR

2.4 Gene expression analysis of *Pm*Snake

2.4.1 Tissue distribution analysis

2.4.1.1 Tissue collection

To investigate the tissue-specific expression of *Pm*Snake transcripts, various tissues of healthy shrimp (hemocytes, hepatopancreas, intestine, hematopoietic tissue, gills, heart, muscle, foregut, midgut and lymphoid organ) were collected and stored immediately in liquid nitrogen. Hemocytes were prepared by collecting hemolymph under 10% sodium citrate and centrifugation at 800 ×g for 10 min 4°C. Hemocyte pellet was stored in liquid nitrogen.

2.4.1.2 Total RNA preparation and first-strand cDNA synthesis

Total RNA was extracted from samples homogenizing by GeneZol (Geneaid) reagent according to the manufacturer's protocol. Briefly, 200 ul of chloroform was added and incubated on ice 30 min before centrifugation (12,000 rpm) at 4°C for 15 min. The upper face of solutions were transferred to new tube and precipitated with 1 volume of isopropanol. Then, the sample was centrifuged at 12,000 rpm for 15 min at 4°C. Pellets were collected and washed with 75% ethanol. RNA pellets was air-dried and dissolved with Diethyl pyrocarbonate (DEPC) water. Then, total RNA was measured by using nanodrop (Thermo scientific) at A_{260}/A_{280}. After that, total RNA was treated with DNase I (RBC) ratio 1:10 to remove any contaminating DNA. Equal amount of each obtained from the pooled RNA from the

respective tissue types from three healthy shrimps. First-strand cDNA was synthesized using Revert Aid First Strand cDNA Synthesis kit (Thermo scientific) and stored at -20°C refrigerator for further investigation.

2.4.1.3 Semi-quantitative Reverse transcriptase-PCR (RT-PCR) analysis

Semi-quantitative RT-PCR was analyzed by using gene-specific primers for *Pm*SnakeRTSnake-F/R (Table 2.1). The tissue distribution expression levels were normalized relative to that of the elongation factor 1-α (EF1-α) gene (Table 2.1). A fragment of the EF1-α gene was amplified in a separate tube and served as an internal control. PCR reactions (25 μl) consist of 1 μl of 10-fold diluted cDNA as a template, 5 μM of each forward and reverse primers, 2.5mM dNTP and 1 U Taq polymerase (RBC Bioscience). The PCR thermal cycling conditions were 94°C for 1 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and then a final extension at 72°C 5 min. PCR product was analyzed by 1.8% agarose gel electrophoresis and visualized by UV-transillumination.

2.5 Gene expression analysis in response to pathogenic bacterial challenge

2.5.1 *Vibrio harveyi* challenge

The pathogenic *V. harveyi* strain 639 was grown in a Tryptic soy broth (TSB) containing 2% NaCl and incubated at 30°C with shaking at 250 rpm for overnight. The starter cultured was diluted 1:200 TSB 2% NaCl and measured Absorbance A_{600} reached 0.6. The cultured was diluted to 2×10^5 colony forming unit (CFU) of *V.*

harveyi strain 639 in 150mM NaCl for shrimp injection and control shrimp were injected with 150mM NaCl.

2.5.2 Shrimp preparation and total RNA extraction

Healthy shrimp were obtained from local farm in Thailand. Shrimp were maintained in 20 ppt seawater at least 7 days before proceeding in experiments. For the expression analysis of bacterial challenge, shrimps were divided in to 2 groups; *V. harveyi*–injected shrimp and NaCl-injected shrimp (control). Thereafter, the hemolymph of three individual shrimp was collected at 0, 6, 24, 48 and 72 hours after injection. Total RNA was extracted from hemocytes using Genezol REAGENT® following to manufacturer's protocol.

2.5.3 Quantitative Real-time PCR analysis

First-strand cDNA was synthesized using Revert Aid First Strand cDNA Synthesis kit (Thermo scientific). A real-time RT-PCR analysis was performed by SsoFast™ Evagreen® Supermix (Bio-RAD) detection. The amplification and thermal profile was performed using RTSnake-F/R primer (Table 2.1). The amplification was performed in PCR white strip tube in a 10 μl reaction volume containing 5 μl of SsoFast™ Evagreen® Supermix (Bio-RAD), 0.4 μl of RTSnake-F and RTSnake-R primers (10 mM), and 0.5 μl of 1:10 diluted cDNA template. The thermal profile for SsoFast™ Evagreen® Supermix (Bio-RAD) real-time PCR was 95°C for 8 min followed by 40 cycles of denaturation (95°C for 30 s), annealing (58°C for 30 s) and extension (72°C

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for 30 s). The specificity of PCR was verified by measuring the melting curve of the PCR product at the end of the reaction. The reaction was incubated at 95°C for 5 min and subsequently 55°C for 5 min, followed by heating for 10 s starting at 55°C with 0.5°C increments. The relative quantification was analyzed the amount of target transcript relative to an internal standard, elongation factor 1-alpha gene (EF1-α) in the same sample of *V. harveyi*-injected shrimp hemocytes. The Ct values of *V. harveyi*-injected sample at each time point were normalized with saline-injected samples. A mathematical model was used to determine the relative expression ratio (Pfaffl, 2001).

$$\text{Real expression ratio (R)} = (E_{target})^{\Delta Ct_{target}\,(control-sample)} / (E_{ref})^{\Delta Ct_{ref}\,(control-sample)}$$

2.6 *In vivo* Gene silencing

2.6.1 Preparation double-stranded RNAs (dsRNAs)

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Double-stranded RNAs (dsRNA) of *Pm*Snake was generated *in vitro* using T7 RiboMAX™ Express Large Scale RNA Production System. DNA fragment for preparation dsRNA of *Pm*Snake was amplified by PCR from a full-length *Pm*Snake containing plasmid using gene specific primers (Table 2.1). The primers for the dsRNA synthesis consist of the same primer sequences but flanked at the 5’ end by a T7 promoter sites. Two separate PCR were set up, one with T7Snake-F and Snake-R (Table 2.1) for the sense strand template, the other with Snake-F and T7Snake-R (Table 2.1) for the anti-sense strand template. In addition, the exogenous gene (GFP

gene) was amplified as a negative control with pEGFP-1 vector by using GFPT7-F and GFP-R (Table 2.1) for the sense strand template, and GFP-F andGFPT7-R (Table 2.1) for the anti-sense strand template. T7 RiboMAX™ Express Large Scale RNA Production System was used to generate single stranded RNAs. Equal amount of single stranded RNAs as annealed to produce dsRNA. The quality of dsRNAs are verified by 1.5% agarose gel electrophoresis, quantified by using UV visualization following ethidium bromide staining, and UV spectrophotometer. The dsRNAs were stored at -20°C for further *in vivo* experiment.

2.6.2 Shrimp preparation and injection

Shrimp were divided in to 3 groups with an average wet weight 3 g. Shrimp were injected with either *Pm*Snake or GFP dsRNA or 150 mM NaCl, approximately 25µl volume containing 2.5 µg of dsRNA in 150 mM NaCl per 1 g of a shrimp was injected through the lateral area of the fourth abdominal segment using a 0.5ml insulin syringe. For the control groups, GFP dsRNA and 150mM NaCl were injected into the shrimps. After 24 hour first injection, repeated injection of dsRNA was carried out together with 1 mg of each of *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) (Sigma) and laminarin (b-1,3-glucan) (Sigma) to stimulate the immune response.

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2.6.3 Extraction of total RNA and synthesis of cDNA

At the end of the experiment (48 h after the second RNAi treatment) shrimp hemolymph was collected for total RNA extraction using TRI Reagent® (Molecular Research Center, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (180 ng) using Revert Aid First Strand cDNA Synthesis kit (Thermo scientific) and oligo (dT) primer. First-strand cDNA were stored at -20°C for further investigated.

2.6.4 Semi-quantitative RT-PCR analysis of gene expression in silence shrimp

The efficiency of the *Pm*Snake knockdown was analyzed by semi-quantitative RT-PCR analysis using gene-specific primers for *Pm*Snake RTSnake-F/R (Table 2.1). The EF1-α gene was amplified in a separate tube and used as an internal control for normalization by using EF1-α-F/R (Table 2.1). The PCR thermal cycling conditions were 94°C for 1 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and then a final extension at 72°C 5 min. PCR product was analyzed by 1.8% agarose gel electrophoresis and visualized by UV-transillumination.

The specificity of the *Pm*Snake knockdown was checked by the individual amplification with the set of gene specific primers of the other shrimp clip-domain serine proteinase of *P. monodon* such as *Pm*ClipSP1 (*Pm*ClipSP1-f/R), *Pm*ClipSP2 (*Pm*ClipSP2-F/R), *Pm*PPAE1 (*Pm*PPAE1-F/R) and *Pm*PPAE2 (*Pm*PPAE2-F/R) (Table 2.1);

the other set of gene in proPO system and antimicrobial peptide such as *Pm*proPO1 (*Pm*proPO1-F/R), *Pm*proPO2 (*Pm*proPO2-F/R) and *Pm*Penedin3 (*Pm*Pen3-F/R) respectively. The GFP dsRNA and 150mM NaCl were used as a control. The PCR thermal cycling conditions were 94°C for 1 min, 25-35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1min, and then a final extension at 72°C 5 min. PCR product was analyzed by 1.2% agarose gel electrophoresis and visualized by UV-transillumination.

2.6.5 Hemolymph PO activity of silenced shrimp

The hemolymph, collected 48 h after the second dsRNA injection without anti-coagulant, was subjected to a phenoloxidase (PO) assay. Total protein concentration was measured using a Bradford assay kit (Bio-RAD). Briefly, shrimp hemolymph protein (2 mg) in 435 ml of Tris–HCl (10 mM, pH 8.0) was mixed with 65 ml of Dopamine (3 mg/ml in water) (Fluka). The reaction mixture was incubated at room temperature for 30 min and the reaction was stopped by adding 500 ml of 10% (v/v) acetic acid. The remaining PO activity was monitored by spectrophotometry at 470 nm. To follow enzyme reaction, dopamine quinone to melanochrome was measured the absorbance. PO activity was recorded as A470/mg total protein/min against control. Each experimental group (6 shrimp/group), including the *Pm*Snake dsRNA, GFP dsRNA and 150mM NaCl injected shrimp was repeated at least three times.

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2.7 Production and purification of recombinant *Pm*Snake protein

2.7.1 Amplification of mature *Pm*Snake gene

Gene specific primers (name *Pm*Snake-F and *Pm*Snake-R) (Table 2.1) were designed from nucleotide sequence of *P.monodon* to amplify the mature *Pm*Snake gene. The PCR conditions of 25 µl amplification reaction were consisted of 1X $MgCl_2$ reaction buffer, 2.5 mM dNTP, 5 µM each primer, 1 µl cDNA normal shrimp sample and 1 U Advantage 2 Taq DNA Polymerase (Clontech). The PCR thermal cycling conditions were 94˚C for 10 min, 30 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min, and then a final extension at 72˚C 10 min.

2.7.2 Agarose gel electrophoresis

The PCR products were analyzed by using 1.2% agarose gel electrophoresis. Agarose powder was dissolved in 1X TBE buffer (Tris-HCl, Boric acid, EDTA), boiled the solution. The gel was poured into tray and the plastic comb was placed in the gel after the solution cool down. Then, the gel was completely set PCR products were mixed with 6X dye DNA and loaded each well. The size was determined by comparing with DNA Marker (100 bp or 1 kb). Electrophoresis was performed at 100 mV 30 min and stained with ethidium bromide solution 1 min. After destained in water for 15 min, DNA fragment was detected by UV transilluminator and photographed.

2.7.3 Purification PCR product from agarose gel

The expected bands were cut from agarose gel. NucleoSpin® Extract II kits (MACHEREY-NAGEL) was used for DNA purification from gel. Briefly, the 700 µl of NT buffer was added to dissolved gel and melted at 55-60 °C for 10 min or until gel completely dissolved. The solution was transfer into NucleoSpin® column and then centrifuged at 12,000 rpm for 1 min room temperature. The supernatant was removed, washed by adding 600 µl of NT3 buffer and centrifuged at 12,000 rpm for 1 min 2 times to remove ethanol from NT3 buffer. The ultrapure water 25 µl was used as elution buffer and stored at -20°C.

2.7.4 Construction of *Pm*Snake to T&A vector

The purified PCR was ligated into T&A cloning vector (RBCBioscience) (Figure2.1). The ligation product was transformed into *E. coli* strain JM109, the positive colonies were confirmed by colony PCR and digested with NcoI/ XhoI restriction enzyme. Recombinant plasmid was extracted by Presto® plasmid mini prep (Geneaid) and sequenced by Macrogen Inc (Korea).

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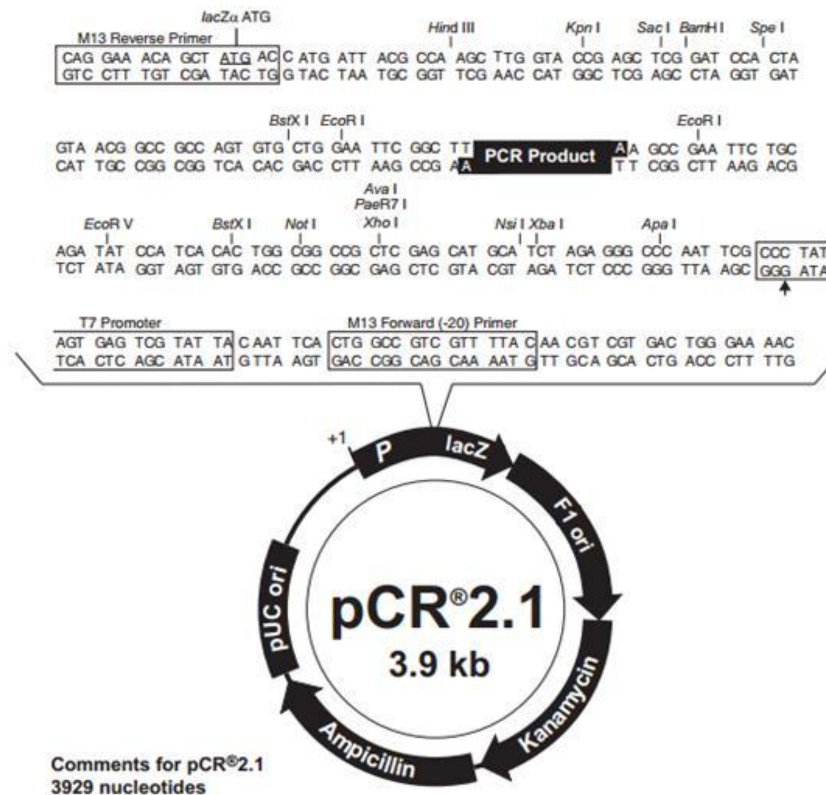

Figure 2.1 The map of TA cloning vector (www.invitrogen.com)

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2.7.5 Expression, purification and antibody production of recombinant protein

After the correct sequence was confirmed, the mature *Pm*Snake gene was amplified using a pair of primers contained 5' flanking *NcoI* restriction sites and 3' flanking *XhoI* restinction site and 6X histidine tag. Then, PCR product was digested with *NcoI* and *XhoI*, and clone into the pET-28b(+) vector (Figure 2.2). The fragment was ligated with pET-28b(+) vector and was cut with the same restriction enzymes by using T4 ligase (BioLabs) and incubation at 16°C overnight. The ligation mixture was transformed into *E. coli* JM109 and confirmed by nucleotide sequence

(Macrogen, Korea). The recombinant plasmid pET28b-*Pm*Snake was transformed into *E. coli* strain BL21 (Heat shock method). The recombinant clone was grown in LB medium containing 50 µg/µl kanamycin. The protein expression was induced with 1 mM Isopropyl-β-D-thiogalactoside (IPTG) and harvested cell at 0, 1, 2, 3, 4, 5 and 6 hours post IPTG-induction. Centrifugation was performed at 8,000 rpm 4 °C for 5 min. Sonication was used to break cell at 35% amplitude 2 min pulse on 1 sec and centrifuged for separate inclusion bodies and soluble fractions. The expression of recombinant (r)*Pm*Snake protein was analyzed by 12.5% SDS-PAGE and strained with coomassie brilliant blue.

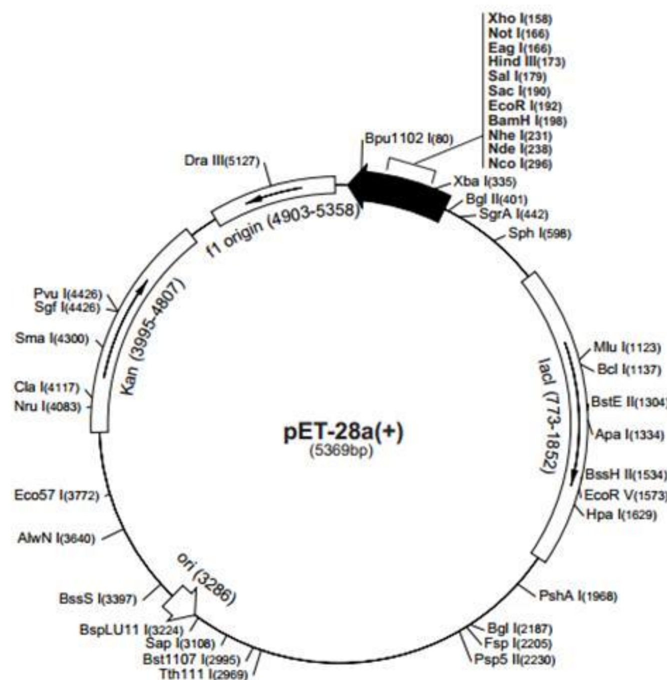

Figure 2.2 The map of pET-28 b(+) expression vector. (Novagen)

The inclusion bodies of r*Pm*Snake protein were solubilized with 8M Urea for completely dissolved. The r*Pm*Snake was purified by using Ni-NTA affinity chromatography column (GE Healthcare). The crude r*Pm*Snake was pre-incubated with Ni-NTA bead and collected flow through. After that, Ni-NTA bead was washed with lysis buffer (20 mM Tris-HCl pH8.0, 10 mM Immidazole, 8M Urea and 0.3 M NaCl) 2 ml and wash buffer (wash 20; 20 mM Immidazole, wash 50; 50mM Immidazole, wash 100; 100 mM Immidazole). The elution fraction was carried out by using 20 mM Tris-HCl pH8.0, 250 mM Immidazole, 8M Urea and 0.3 M NaCl. The purification of r*Pm*Snake was analyzed by 12.5% SDS-PAGE and coomassie brilliant blue for staining. The fraction purified r*Pm*Snake were dialyzed with 20 mM Tris-HCl pH 8.0 and concentrated with VivaSpin® (GE Healthcare) molecular weight cut off 10 kDa Column. The concentration of r*Pm*Snake was measured by Bradford assay.

The purified r*Pm*Snake was used to synthesize the rabbit antibodies in order to generate anti-*Pm*Snake polyclonal antiserum at the Biomedical Technology Research Unit, Chaingmai University, Chaingmai Thailand.

2.7.6 Purification of anti-r*Pm*Snake polyclonal antibody

The rabbit polyclonal antiserum specific r*Pm*Snake was purified by using protein A column for incubated with protein A bead (GE Healthcare). Then, flow through was collected and washed 10 column volumes with 1X PBS (Phosphate

buffer saline) pH 7.4. The elution step was carried out by using 100mM glycine pH 2.5 200 µl and mixed with 50 µl of 2 M Tris-HCl pH 9.5.

2.7.7 Analysis of *Pm*Snake protein in hemolymph of shrimp by using Immunoblotting

Hemolymph was collected from the segment of ventral by 1 ml of syringe from healthy shrimp and mixed with anticoagulant solution (10% sodium citrate). Then, hemolymph was separate by centrifugation at 800×g for 10 min at 4°C. After that, hemolymph was separated the plasma solution (supernatant) and hemocyte cells (pellets). Hemocyte cells were washed with CAC buffer (10mM Sodium cacodylate) 2 times, homogenized with the same buffer and centrifuged at 25,000×g at 4°C for 20 min. The protein in hemocyte contain hemocyte lysate supernatant (HLS) was measured the concentration by Bradford assay.

For Western blot analysis, 12.5% SDS-PAGE was performed to analyze by loading 20 µg HLS and 100 µg plasma solution. The gel, nitrocellulose membrane and filter papers were incubated with transfer buffer (25 mM Tris, 150 mM glycine and 20% methanol) and placed on Trans-Blot® Semi-Dry (Bio-RAD) at a constant 110 mM for 3 hours. After that 5% of skim milk was blocked in 1X Phosphate buffer saline and 0.05 % (v/v) tween20 pH 7.4 (PBST) at room temperature with shaking 1 hour before stored at 4 °C refrigerator. After washing out the blocking solution by using PBST 3 times for each 10 min, nitrocellulose membrane was incubated with primary antibody (rabbit polyclonal anti-*Pm*Snake) 1:15,000 dilution in PBST for 1

hour at room temperature. Then, the membrane was washed 3 times and incubated with secondary antibody (goat anti-rabbit alkaline phosphstase conjugated antibodies) 1:20,000 dilution in PBST for 1 hour at room temperature. After that, membrane was washed 3 times with PBST and 2 times with DI water before the protein detection by color development using NBT and BCIP as substrate.

2.8 PO Activation assay of the r*Pm*Snake protein

Hemolymph was collected from healthy shrimp. The 10 µM r*Pm*Snake (25 µl) and activators (LPS and β 1,3-glucan, 25 µl) were added into 250 µg of hemolymph (25 µl) in 96 well plate Costar® Clear and incubated at room temperature for 10 min. Then, 3 mg/ml of L-DOPA (25 µl) was added in each reaction and the PO activity was measured at 490 nm by using spectrophotometry microplate reader SpectraMaxM5 (Molecular Devices) every 5 min for 30 min. The reactions were stopped by 10% (v/v) acetic acid and BSA (Bovine serum albumin) used as negative control. The experiment was repeated three times. PO activity was reported as A490/mg total protein/min against control.

2.9 Proteinase activity

Hemolymph was collected from healthy shrimp. The 10 µM of r*Pm*Snake (25 µl) and activators (LPS 50 µg) were added into 250 µg of hemolymph (25 µl) in 96 well plate Costar® Clear and incubated at room temperature for 10 min. Then, 100

μM of B2133 (N-Benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride) 25 μl was added in each reaction. Then, the reactions were measured at 405 nm by using spectrophotometry. The experiment was repeated three times.

2.10 Immunofluorescence of *Pm*Snake protein in shrimp hemocyte

The expression of *Pm*Snake in hemocyte was analyzed by the purified polyclonal antibody specific to *Pm*Snake. The hemolymph was collected and fixed in 4% paraformaldehyde (ratio 1:1) for 10 min at room temperature. Then, Hemocytes were separated by using centrifuge at 800×g at 4°C for 10 min. The hemocytes were resuspended with 1 X PBS pH 7.4, counted by hemocytometer and centrifuged at 1,000 × g at 4°C for 10 min onto poly-L-lysine slide (Thermo Scientific) (1×10^6 cells/slide). The cells were washed with 1 X PBS pH 7.4 three times, permibilized by 1% triton X-100 in 1 X PBS pH 7.4 for 5 min at room temperature and washed three times with 1X PBS pH 7.4 for 5min. Then, the cells were blocked with 10% fetal bovine serum (FBS) in 1X PBs pH7.4 at room temperature for 1 hour. The cells were probed with 1:1000 dilution the purified rabbit polyclonal antibody specific *Pm*Snake at room temperature for 3 hours and the negative control were incubated with 1% FBS in 1X PBS pH7.4. The slides were washed and probed with secondary antibody, 1:1000 dilution of goat anti-rabbit antibody conjugated with Alexa Fluor 488 in 1% FBS in 1X PBS pH 7.4 at room temperature for 1 hour. To stain the nuclear DNA, The cells were incubated with

4´,6-diamidino-2-phenylindole (DAPI) before mounting with medium Prolong® Gold antifade reagent. The fluorescent staining was observed under FV1000 confocal laser scanning microscope (Olympus).

2.11 Detection of *Pm*Snake protein in shrimp hemocyte

2.11.1 Hemocyte lysate and cell-free plasma preparation

Hemolymph was collected from *Pm*Snake silencing shrimp with 10% (v/v) sodium citrate as a shrimp anticoagulant. The shrimp hemocyte was separated by centrifugation at 800×g 4°C for 10 min. The supernatant was collected as cell-free plasma and hemocyte pellets were washed 2 times with 10mM sodium cacodylate buffer (CAC buffer). Then, the solutions were homogenized in 10 mM CAC buffer and separated by centrifugation at 13,000 rpm for 10 min at 4°C. The HLS and cell-free plasma were measured the concentration by using Bradford assay, approximately 20 µg of HLS and 100 µg of cell-free plasma were used in this experiment.

2.11.2 SDS-PAGE and Western blot analysis

To detect *Pm*Snake protein in shrimp hemocyte of healthy shrimp using SDS-PAGE and Western blot analysis. The samples were loaded on a reducing SDS-PAGE gel (12.5% (w/v) acrylamide resolving gel), and blotted onto a nitrocellulose membrane (GE Healthcare). Membranes were then blocked in 5% (w/v) skim milk in 1XPBS and 0.05% (v/v) Tween 20. The membrane was detected with 1:10,000

dilution rabbit polyclonal antibodies *Pm*Snake as the primary antibody and the mouse anti β-actin as internal control, washed 3 times with PBST. Then, the membrane was incubated with 1:20,000 dilution goat anti-rabbit alkaline phosphatase conjugated antibodies as a secondary antibody (Jackson & Burdich, USA) and was detected with 5-Bromo-4-chloro-3-indolyl Phosphate/ NitroblueTetrazolium (BCIP/NBT) as a substrate.

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CHAPTER III
RESULTS

3.1 Sequence analysis of *Pm*Snake

Previously, a cDNA encoding an open reading frame (ORE) of Snake-like serine proteinase (*Pm*Snake) gene was identified from the suppression subtractive hybridization cDNA library of proPO dsRNA treated hemocyte (unpublished data). An open reading frame of *Pm*Snake contains 1,068 bp encoding a predicted protein of 355 amino acid residues (Figure 3.1). Using SMART analysis revealed a putative signal peptide of 22 amino acid residues with the six cysteine residues forming three disulfide bridges of clip-domain at the N-terminal region and a conserved catalytic triad (Histidine, Aspartate and Serine) at the C-terminal region of serine proteinase domain (Figure 3.2)

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```
        10        20        30        40        50        60
ATGTTTGTGAGGTCTTTATGTATTTCTATCGCAACGCTCCACCTCCTCCTCCCATGTGGC
 M  F  V  R  S  L  C  I  S  I  A  T  L  H  L  L  L  P  C  G
        70        80        90       100       110       120
GCGGCGCAGGGGACGGGCGAGCAGTGCGAACGCGTCGACGGGACGATCGGAACCTGTATG
 A  A  Q  G  T  G  E  Q  C  E  R  V  D  G  T  I  G  T  C  M
       130       140       150       160       170       180
GAGTTCAGCCGCTGCTTGCAATCAGACGGAAACGCGCAGAGTGTCGTCAACCTGAGATCT
 E  F  S  R  C  L  Q  S  D  G  N  A  Q  S  V  V  N  L  R  S
       190       200       210       220       230       240
TGCGCGACTACCTGGGGAGACATTAGGCAGACACCCGTGTGTTGCAGAAGCAATCCTAGG
 C  A  T  T  W  G  D  I  R  Q  T  P  V  C  C  R  S  N  P  R
       250       260       270       280       290       300
AATCTGGCAAGAGCGATGTGTTCCAAGTGGAACAGGATTGCCAGTAACTTCGGCGTCAGA
 N  L  A  R  A  M  C  S  K  W  N  R  I  A  S  N  F  G  V  R
       310       320       330       340       350       360
TGCACATCCACAGAAACGCGAATCCAAGGTGGGACCTTCGCTCAGGTTAACGAATTCCCT
 C  T  S  T  E  T  R  I  Q  G  G  T  F  A  Q  V  N  E  F  P
       370       380       390       400       410       420
CATATGGCCGCACTCGTAGACCGTCTCAAAGGCCAAAACGCCTTCTGTGGGGGAACTCTC
 H  M  A  A  L  V  D  R  L  K  G  Q  N  A  F  C  G  G  T  L
       430       440       450       460       470       480
ATTTCAGAAAATTTCGTCTTAACAGCCGCCCATTGCATCGACGGAATAAAAACCGCGTTT
 I  S  E  N  F  V  L  T  A  A  H  C  I  D  G  I  K  T  A  F
       490       500       510       520       530       540
GAATTATCCGTCCGCGTCGGTGTCATCAACCTGCAGGAGCAGGATTCGCAAACCTTCCAA
 E  L  S  V  R  V  G  V  I  N  L  Q  E  Q  D  S  Q  T  F  Q
       550       560       570       580       590       600
GTTCTGAAGGTCATCAAGCACCCGCTGTATAGGCCGCCTTCTTCGTACCATGACATCGCT
 V  L  K  V  I  K  H  P  L  Y  R  P  P  S  S  Y  H  D  I  A
       610       620       630       640       650       660
TTGCTGCAGTTGGCGACCAAGGTTCCACTGTCGAAGAGCGTGTTGCCTGCCTGTCTGCCA
 L  L  Q  L  A  T  K  V  P  L  S  K  S  V  L  P  A  C  L  P
       670       680       690       700       710       720
ACCCAGAACAGACGCCTGCAAGAGGGAAAAATTCTCACTGTGGCTGGCTGGGGGTCGACT
 T  Q  N  R  R  L  Q  E  G  K  I  L  T  V  A  G  W  G  S  T
       730       740       750       760       770       780
GAAGTAGCTAAATTCTCCAACGTGTTACTGAAAGGCTTCGGAAGAACAATTTCTCGCTTT
 E  V  A  K  F  S  N  V  L  L  K  G  F  G  R  T  I  S  R  F
       790       800       810       820       830       840
GGGTGTGACGATGCACTAGACACAGGTGCTCTCAATAAAGACTTTTATAGAGCAGGGATT
 G  C  D  D  A  L  D  T  G  A  L  N  K  D  F  Y  R  A  G  I
       850       860       870       880       890       900
ACCGACTCCATCATCTGTTTGGATGAAAGCAGTTCTGGCCCCTGCAAGGGTGACAGCGGC
 T  D  S  I  I  C  L  D  E  S  S  S  G  P  C  K  G  D  S  G
       910       920       930       940       950       960
GGTCCTCTCACTGACGAAAATGAGCCCACTTGTCAACATATTGTCTTCGGCGTAGTGGCT
 G  P  L  T  D  E  N  E  P  T  C  Q  H  I  V  F  G  V  V  A
       970       980       990      1000      1010      1020
AAGGGCTCGCCACAATGCCAGGGAACATCTGTGCCTGGGATTTACACCAATGTCGAGCAC
 K  G  S  P  Q  C  Q  G  T  S  V  P  G  I  Y  T  N  V  E  H
      1030      1040      1050      1060      1070
TACATGCAATGGATTGTTGATATCGTTTGGCTCAAAGAAAATGTGTAA
 Y  M  Q  W  I  V  D  I  V  W  L  K  E  N  V  *
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Figure 3.1 The nucleotide and deduced amino acid sequences of *Pm*Snake. The signal peptide predicted by signal 4.0 server is highlighted in grey. The ORF contains 1,068 bp encoding a predicted protein of 366 amino acid residues.

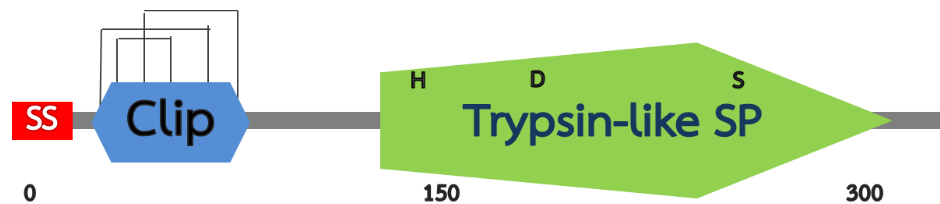

Figure 3.2 The domain organization of *Pm*Snake. Signal peptide sequence, Clip-domain and serine proteinase domain are shown as a rectangle, hexagon and pentagon, respectively. The disulfide linkages are indicated by solid lines. The conserved amino acid residues represented catalytic traid (H, D and S).

The sequence analysis by BlastX showed that the deduced amino acids of *Pm*Snake shared highest similarities to snake-like Hemolymph Proteinase 21 (49% sequence similarity) of silkworm *Manduca sexta* that involved in the proPO system. Multiple sequence alignment of deduced amino acid sequence of *Pm*Snake with other clip-domain serine proteinases in *P. monodon* and Hemolymph proteinase 21 in *M. sexta* from ClustalW2 performed the six conserved cysteine residues in the clip-domain region. In addition, the conserved catalytic traid and cysteine residues in the SP domain were found in *Pm*Snake, *Pm*ClipSP1, *Pm*ClipSP2, *Pm*PPAE2 and *Ms*HP21. (Figure 3.3)

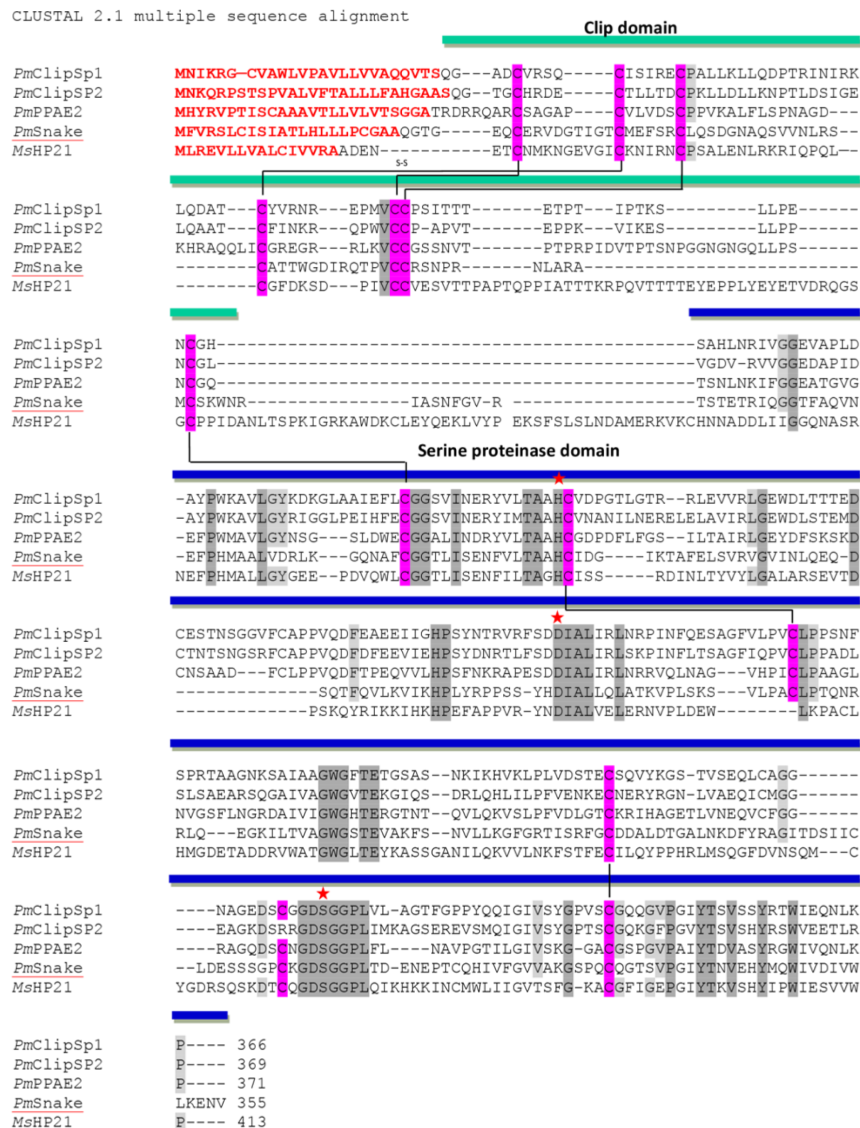

Figure 3.3 Predicted amino acid sequence alignment using ClustalW2 of *Pm*Snake with other Clip-SPs in *P. monodon* and hemolymph proteinase 21 from *M. sexta* (*Ms*HP21). The signal peptide is shown in red. Grey indicates conserved residues. Pink highlights indicate conserved cysteine residues. The disulfide linkages are shown by black solid lines. The amino acid residues corresponding to the catalytic triad of serine proteinases are marked by red stars.

3.2 Phylogenetic analysis

To analyze the relationship of *Pm*Snake protein with Clip-SPs and ClipSPHs of arthropods, the NJ distance based method was used to construct phylogenetic tree by comparison of amino acids sequences. NJ analysis categorized arthropod clip-SPs and clip-SPHs into two distinct and separate major groups. *Pm*Snake clusters in the same clade with those of the insect clip-SPs and the closest to *Ae*Snake from the ant *Acromyrmex echinatior* Serine protease snake. The results suggested *Pm*Snake is closely related to insect snake-like serine proteinase. (Figure 3.4)

Figure 3.4 Bootstrapped unrooted neighbor-joining tree of the serine proteinase domain of clip-SPs, clip-SPHs and snake from arthropods: *Penaeus monodon* snake-like serine proteinase (*Pm*Snake), prophenoloxidase-activating enzyme 1 (*Pm*PPAE1; ACP19558.1), prophenoloxidase-activating enzyme 2 (*Pm*PPAE2; ACP19559.1), clip domain serine proteinase 1 (*Pm*ClipSP1; ACP19562.1), clip domain serine proteinase 2 (*Pm*ClipSP2; ACP19561.1), prophenoloxidase activating factor Mas-like SPH1 (*Pm*MasSPH1; ABE03741.1), masquerade-like serine proteinase-like protein 2 Mas-like SPH2 (*Pm*MasSPH2; ACP19560.1), masquerade-like serine proteinase-like protein 3 Mas-like SPH3 (*Pm*MasSPH3; ACP19563.1), Mas-like protein (*Pm*CSPH; AAT42131.1); *Fenneropenaeus chinensis* serine proteinase SPH (*Fc*SPH1; ABC33918.1); *Pacifastacus leniusculus* serine protease PPA (*Pl*PPA; CAB63112.1), masquerade-like protein Mas-like protein (*Pl*Mas; CAA72032.2), serine proteinase-like protein 1 SPH1 (*Pl*SPH1; AAX55746.1), serine proteinase-like 2a SPH2a (*Pl*SPH2a; ACB41379.1); *Callinectes sapidus* phenoloxidase activating factor PPAF (*Cs*PPAF; AAS60227.1); *Anopheles gambiae* serine protease 14D (*Ag*SP14D; ACN38198.1), serine protease 14D2 (*Ag*Sp14D2; AAD38335.1); *Drosophila melanogaster* melanization protease 1 (*Dm*MP1; NP_649450.3), Spatzle-processing enzyme (*Dm*SPE; NP_651168.1), snake (*Dm*Snk; NP_524338.2), easter (*Dm*Ea; NP_524362.2), serine protease 7 (*Dm*SP7; NP_649734.2); *Bombyx mori* prophenoloxidase activating enzyme precursor PPAE (*Bm*PPAE; NP_001036832.1), BzArgOEtase precursor SP zymogen (*Bm*proBAEEase; NP_001036844.1); *Holotrichia diomphalia* pro-phenoloxidase activating enzyme-I

precursor PPAF-I (HdPPAFI; BAA34642.1), prophenoloxidase activating factor PPAF-II (*Hd*PPAFII; CAC12665.1), prophenoloxidase activating factor-III PPAF-III (*Hd*PPAFIII; BAC15604.1); *Manduca sexta* prophenoloxidase-activating proteinase-1 PAP1 (*Ms*PAP1; AAX18636.1), prophenoloxidase-activating proteinase-2 PAP2 (*Ms*PAP2; AAL76085.1), prophenoloxidase-activating proteinase-3 precursor PAP3 (*Ms*PAP3; AAO74570.1), serine proteinase-like protein 1 SPH1 (*Ms*SPH1; AAM69352.2), serine proteinase-like protein 2 SPH2 (*Ms*SPH2; AF518768_1), hemolymph proteinase 6 (*Ms*HP6; AAV91004.1), hemolymph proteinase 21 (*Ms*HP21; AAV91019.1) ; *Tenebrio molitor* prophenoloxidase activating factor PPAF (*Tm*PPAF; CAC12696.1), masquerade-like serine proteinase homologue Mas-like SPH (*Tm*MasSPH; BAC15605.1), 41 kDa zymogen (*Tm*41kDa; BAG14261.1), 44 kDa zymogen (*Tm*44kDa; BAG14262.2); *Tachypleus tridentatus* proclotting enzyme (*Tt*PCE; AAA30094.1) and coagulation factor B precursor (*Tt*CFB; BAA03528.1); *Acromyrmex echinatior* Serine protease snake (*Ae*Snake; EGI60137.1); *Bombyx mori* serine protease HP21 precursor (*Bm*HP21; NP_001243984.1), *Danaus plexippus* hemolymph proteinase 6 (*Dp*HP6; EHJ76340.1); *Aedes aegypti* serine protease snake (*Aa*SP; XP_001649319.1); *Tribolium castaneum* serine protease P56 (*Tc*SPP56; EEZ99345.1), serine protease H17 (TcHP17; EEZ99231.1); *Ctenocephalides felis* trypsin-like serine protease (*Cf*SP; AAD21841.1); *Camponotus floridanus* Serine protease snake (*Cf*Snake; EFN63907.1); *Acyrthosiphon pisum* serine protease-like precursor (*Ap*SP-like; NP_001155379.1); *Tribolium castaneum* serine protease P138 (*Tc*SP138; EFA07560.1); *Harpegnathos saltator*

Serine protease snake (*Hs*Snake; EFN87035.1); *Culex quinquefasciatus* serine protease (CqSP; XP_001845292.1). Bootstrap values indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

3.3 Tissue distribution of *Pm*Snake

Semi-quantitative RT-PCR analysis was employed to determine the transcript expression of *Pm*Snake mRNA in different tissues, using EF1- α as an internal control gene. The RT-PCR revealed that *Pm*Snake was expressed in many shrimp tissues such as hemocytes, gill, forgut, midgut/hindgut, hematopoietic tissue, hepatopancrease and lymphoid organ. However, the *Pm*Snake gene showed the highest expression level in hemocyte and the relative gene expression was confirmed by Gel pro analyzer 31 program. (Figure 3.5)

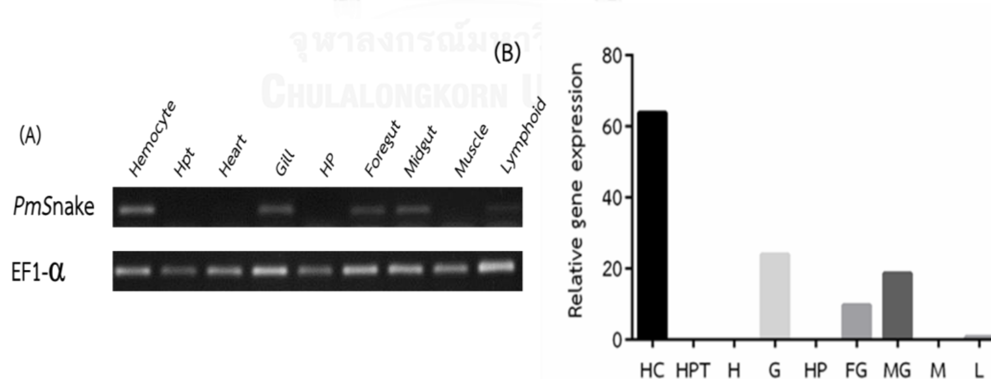

Figure 3.5 The semi-quantitative RT-PCR analysis of *Pm*Snake gene expression in shrimp tissues. (A) Analysis of *Pm*Snake transcript of healthy shrimp in hemocyte (HC), hematopoietic tissue (HPT), heart (H), gill (G), hepatopancrease (HP),

foregut (FG), midgut/ hindgut (MG), muscle (M) and lymphoid organ (L). The EF1-α used as an internal control. (B) The relative gene expression of *Pm*Snake from shrimp tissues analyzed by Gel pro 31 analyzer.

3.4 Gene expression analysis in response to bacterial challenge

To observe the response of *Pm*Snake after bacterial challenge, the transcript levels of *Pm*Snake were determined after systemic *V. harveyi* challenge and normal saline injection by quantitative real-time RT-PCR analysis. Total RNA was extracted from the hemocytes of shrimp at various time points (0, 6, 24, 48 and 72 h.) after injection of the pathogenic bacterium *V. harveyi* and normal saline. The first-strand cDNA was synthesized and the *Pm*Snake transcript level was determined by real-time RT-PCR, the EF1-α housekeeping gene as an internal control. The relative gene expression was analyzed by using data from real-time RT-PCR CFX 96 (Bio-RAD) and calculated from Ct value of bacteria injected shrimp compared with normal shrimp. The transcription level of *Pm*Snake gene was increased 1.9-fold at 6 h and gradually increased at 24 h by 3.1-fold and reached the highest expression level 8.0-fold at 48 h post-challenge. At 72 h the expression level of the *Pm*Snake gene was drastically decreased to 0.4-fold. (Figure 3.6)

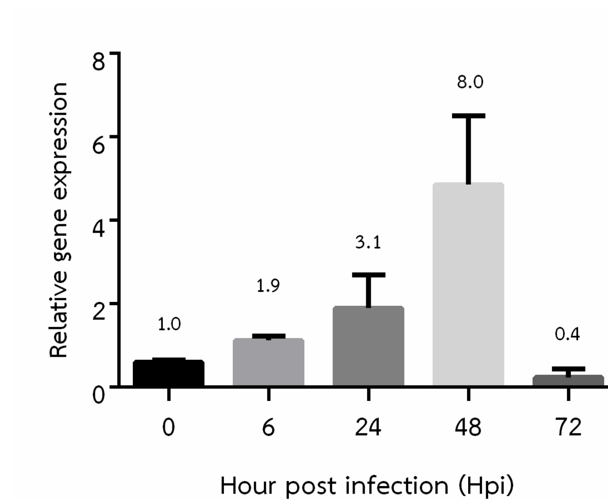

Figure 3.6 Relative expression levels of *Pm*Snake transcript in the shrimp hemocytes post-*V.harveyi* injection by real-time PCR at the indicated times post-injection. Relative expression levels of mRNA were calculated according to Pfaffl (Real expression ratio (R) = $(E_{target})^{\Delta Ct_{target}(control\text{-}sample)} / (E_{ref})^{\Delta Ct_{ref}(control\text{-}sample)}$), using EF1-$\alpha$ as the internal reference gene. Data are shown as the mean ± 1 SEM, derived from triplicate samples per assay and three independent assays.

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3.5 Functional characterization of *Pm*Snake silence shrimp by RNA interference (RNAi)

To investigate the role of *Pm*Snake in shrimp innate immunity, RNA interference was used to suppress the *Pm*Snake mRNA. A semi-quantitative RT-PCR was used to determine the gene transcription of the targeted gene after systemic dsRNA treatment. To study the effect and specificity of *Pm*Snake RNAi-mediated insufficiency on the proPO-activating system, the total PO activity in the *Pm*Snake-knockdown shrimp was determined.

3.5.1 Preparation of dsRNA

The recombinant plasmid of full length *Pm*Snake cDNA was used to amplify the DNA fragment of *Pm*Snake by using gene specific primers (Table 2.1). The primers for the dsRNA synthesis consist of the same primer sequences but flanked at the 5' end by a T7 promoter sites. The sense strand template and the anti-sense strand template were synthesized by using T7Snake-F/Snake-R, the other with Snake-F/T7Snake-R. The exogenous gene (GFP gene) was amplified as a negative control with pEGFP-1 vector by using GFPT7-F and GFP-R for the sense strand template, and GFP-F and GFPT7-R for the anti-sense strand template. The expected band was cut, purified and used for synthesis dsRNA with a T7 RNA polymerase by using a T7 RiboMAX^{TM} Express Large Scale RNA Production System (Figure 3.7). The concentration of single-stranded RNA was measured before annealing step. The major band of *Pm*Snake and GFP dsRNA were observed on 1.5% agarose gel electrophoresis after the purification of dsRNA (Figure3.8).

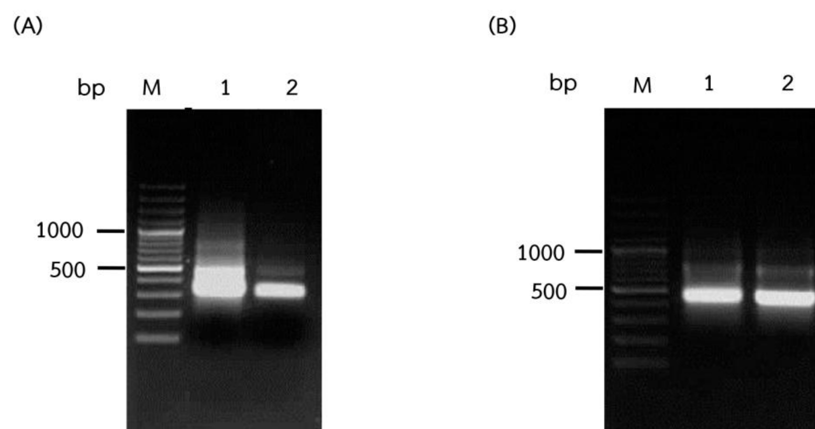

Figure 3.7 The agarose gel electrophoresis of single strand RNA of *Pm*Snake and GFP (A) The sense and anti-sense strand of *Pm*Snake (B) The sense and anti-sense strand of GFP. Products were analyzed by 1.5 % agarose electrophoresis. Lane M is GeneRuler^{TM} 100 bp DNA ladder, Lane1 is sense strand, Lane 2 is anti-sense strand

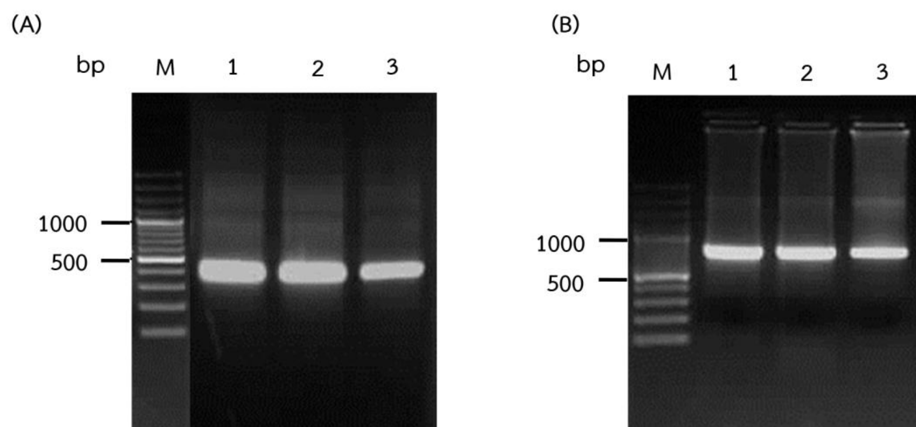

Figure 3.8 The purification of dsRNA (A) Purified *Pm*Snake dsRNA (B) Purified GFP dsRNA. Products were analyzed by 1.5 % agarose electrophoresis. Lane M is GeneRuler^{TM} 100 bp DNA ladder, Lane 1 is dsRNA after annealing, Lane 2 is dsRNA after DNase1 Treatment, Lane 3 is purified dsRNA

3.5.2 Gene silencing of PmSnake

To evaluate the effect of *Pm*Snake dsRNA knockdown, shrimp were injected with 2.5 μg of *Pm*Snake dsRNA and double injection after 24 h in the same concentration of dsRNA. The control groups, GFP dsRNA and 150 mM NaCl were injected into shrimp. At 48 h after double injection the hemolymph were collected. Total RNA were extracted, first-strand cDNA was synthesized and analyzed by semi-quantitative RT-PCR technique, EF1-α as an internal control. The result showed that the expression of *Pm*Snake gene was suppressed by *Pm*Snake dsRNA but not shown in control group. (Figure 3.9)

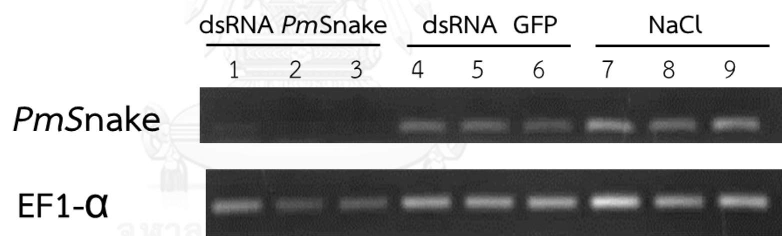

Figure 3.9 Gene-silencing of *Pm*Snake transcript levels in *P. monodon* hemocytes. Lanes 1-3 shrimp were injected with *Pm*Snake dsRNA, Lanes 4-6 shrimp were injected with GFP dsRNA and Lanes 7-9 shrimp were injected with 150 mM NaCl. EF1-α was used as internal control.

To specificity and effect of gene knockdown by *Pm*Snake dsRNA was determined by semi-quantitative RT-PCR analysis using gene specific primers for the other Clip-SPs of *P. monodon* such as *Pm*ClipSP1, *Pm*ClipSP2, *Pm*PPAE1 and

*Pm*PPAE2, *Pm*proPO1, *Pm*proPO2 and *Pm*Penedin 3 genes (Figures 3.10, 3.11). The result showed a significant up-regulation of *Pm*PPAE2 transcript, *Pm*ClipSP2, *Pm*PPAE1 and *Pm*proPOs no significant change. No effect of dsRNA injection was observed in *Pm*Penedin3 gene expression as compared to the control GFP dsRNA-injected shrimp.

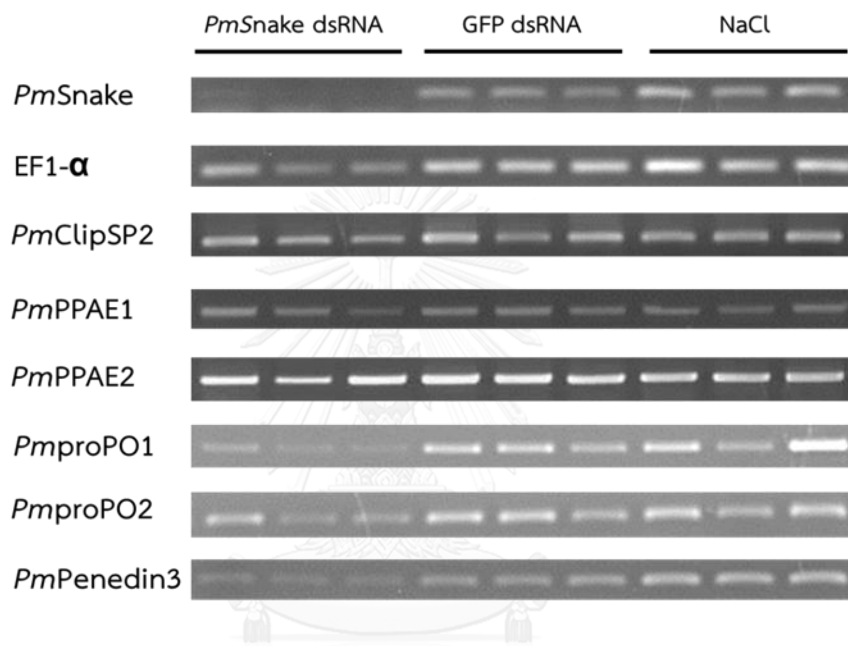

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Figure 3.10 Specificity and effect of gene silencing of the *Pm*Snake transcripts in the hemocytes of *P. monodon*. The transcription levels of the other shrimp Clip-SPs (*Pm*ClipSP2, *Pm*ClipSP1, *Pm*PPAE1 and *Pm*PPAE2), shrimp proPO gene (*Pm*proPO1 and *Pm*proPO2) and shrimp AMP (*Pm*Penedin 3) in *Pm*Snake dsRNA, GFP dsRNA and NaCl injected-shrimp were determined by RT-PCR and observed 1.5% agarose gel electrophoresis.

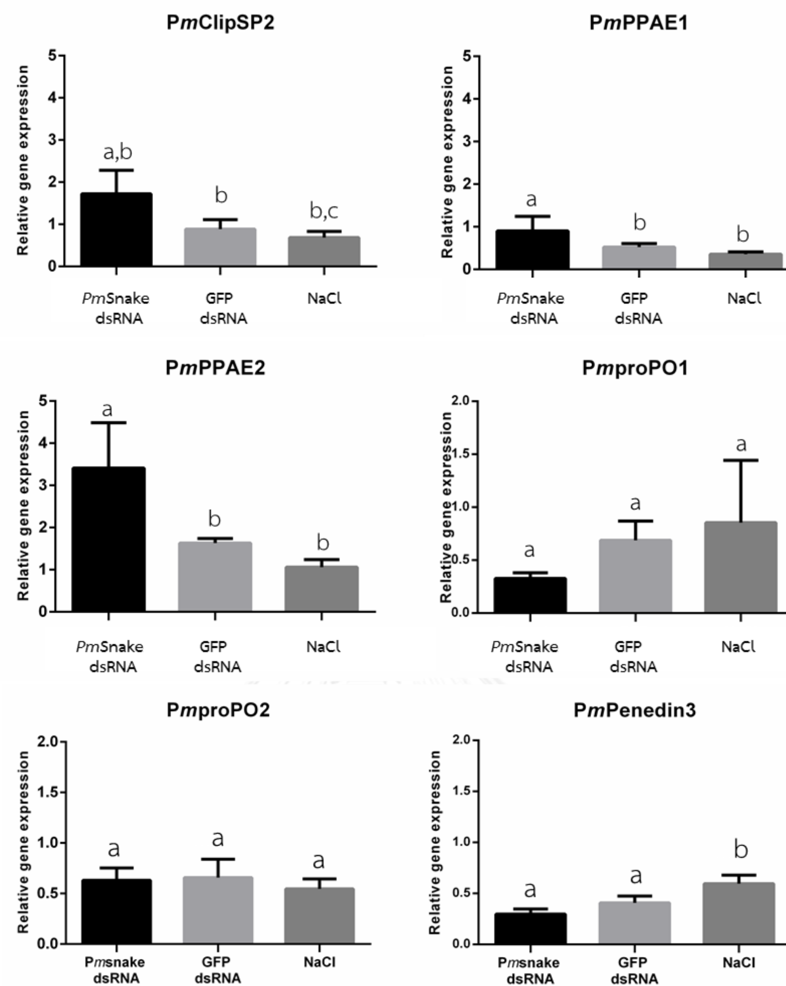

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Figure 3.11 The relative gene expression ratio of specific dsRNA-mediated in *Pm*Snake knockdown shrimp. The transcription level was determined by individual amplification with the set of gene specific primers of Clip-SPs, proPOs and Penaedin. The relative gene expression ration was analyzed by using program gel pro 31 (A) *Pm*ClipSp2 (B) *Pm*PPAE1 (C) *Pm*PPAE2 (D) *Pm*proPO1 (E) *Pm*proPO2 (F) *Pm*Pen3. Data are shown as mean ± 1 SD (error bars) and represent three replicates. The statistical analysis was performed using one-way ANOVA. Means with different Lower case letter (above each bar) are significantly different at $P<0.05$ level.

3.5.3 The efficiency of dsRNA-mediated suppression of *Pm*Snake protein.

To determine whether the *Pm*Snake dsRNA could mediate suppression of *Pm*Snake at the protein level, the Western blot analysis was conducted. The hemocyte lysates were prepared from each group of knockdown shrimp and control shrimp. The rabbit polyclonal antibody *Pm*Snake (1:15,000 dilution) and mouse anti-actin (1:5,000 dilution) were used to detect the protein levels. The immunoblotting results suggested that *Pm*Snake dsRNA could also suppress *Pm*Snake at the protein level when compared with β-actin as an internal control. (Figure 3.12)

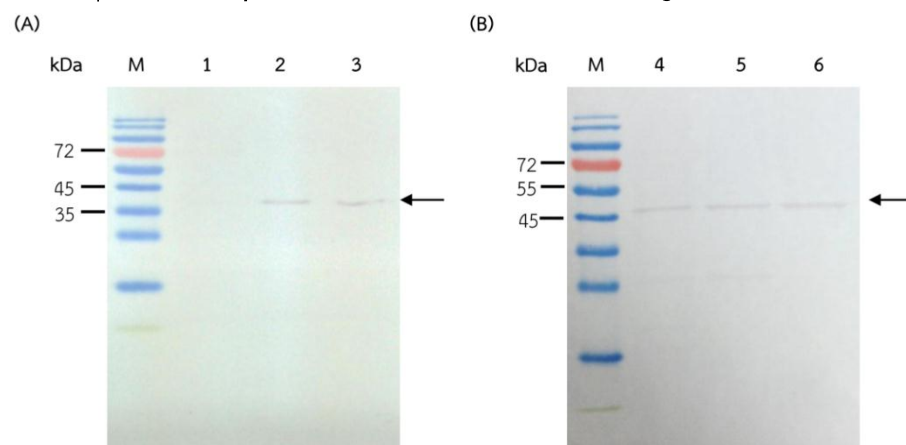

Figure 3.12 RNAi-mediated suppression of *Pm*Snake in *P. monodon*. (A) The efficiency of dsRNA-mediated gene silencing of *Pm*Snake proteins determined by using Western blot analysis. Shrimp injected with GFP dsRNA in 150mM NaCl or with 150mM NaCl alone served as controls. (B) The β-actin was used as a loading control for Western blot analysis. In the shown gel, the lane for each condition represents the result from individual shrimp, arrows indicate size of *Pm*Snake protein. Lanes 1 and 4 are *Pm*Snake dsRNA knockdown shrimp. Lanes 2 and 5 are GFP dsRNA knockdown shrimp. Lanes 3 and 6 are NaCl.

3.5.4 Hemolymph PO activity of PmSnake silencing shrimp

The hemolymph of *Pm*Snake silencing and control were collected 48 h after the second dsRNA injection. The PO activity was monitored by spectrophotometry at 470 nm by spectrophotometry. To follow enzyme reaction, dopamine quinone to melanochrome was measured at the absorbance of A470. PO activity was recorded as A470/mg total protein/min against control. The result showed significant decrease in the total PO activity (36%) in *Pm*Snake dsRNA injected-shrimp when compared to control groups with GFP dsRNA injected shrimp and NaCl. This result suggested that *Pm*Snake is involved in the regulation of the proPO system in shrimp. (Figure 3.13)

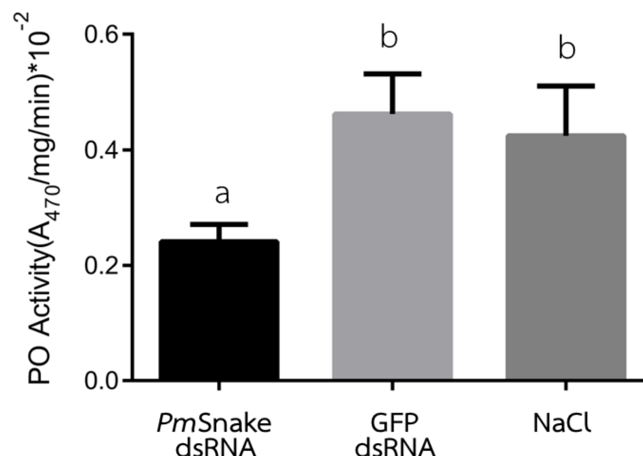

Figure 3.13 Total hemolymph phenoloxidase (PO) activity in *Pm*Snake silenced shrimp, GFP dsRNA and NaCl. The PO activity was measured by spectrophotometry at 470 nm and recorded as A470/mg total protein/min. Experiments were repeated three times and the data is shown as the mean ± standard deviation and are derived from three independently replicated experiments. Means with a different lower case letter are significantly at the $p<0.05$ level.

3.6 Construction, expression and purification of recombinant *Pm*Snake protein

To further characterize the function of *Pm*Snake, the gene coding for mature *Pm*Snake protein was synthesized from cDNA of normal shrimp by using gene specific primers (*Pm*Snake-F/ *Pm*Snake-R). The *Pm*Snake gene was cloned and expressed in pET-28b(+) as an expression vector.

The mature *Pm*Snake contained six histidine tag at C-terminus to facilitate purification step. Then, the *Pm*Snake was amplified by using *Pm*Snake gene specific primers (*Pm*Snake-F/ *Pm*Snake-R). A single band of *Pm*Snake was detected on UV transilluminator by agarose gel electrophoresis and size approximately 1068 bp. Then, the product was cloned and sequenced (Figure 3.14).

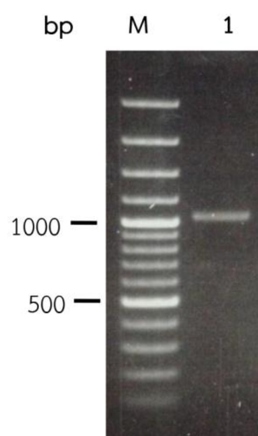

Figure 3.14 The amplification of gene coding for mature *Pm*Snake by RT-PCR and agarose gel electrophoresis. Lane 1 is the PCR product of *Pm*Snake gene with size ∼1068 bp detected on 1.5% agarose gel electrophoresis, Lane M is 100 bp DNA marker.

The *Pm*Snake gene was cloned in expression vector pET-28b(+) at *Nco* I and *Xho* I sites. After ligation, the recombinant plasmid of *Pm*Snake was transformed into *E.coli* JM109 (Figure 3.15). Then, the recombinant plasmid was extracted, confirmed by colony PCR and sequenced.

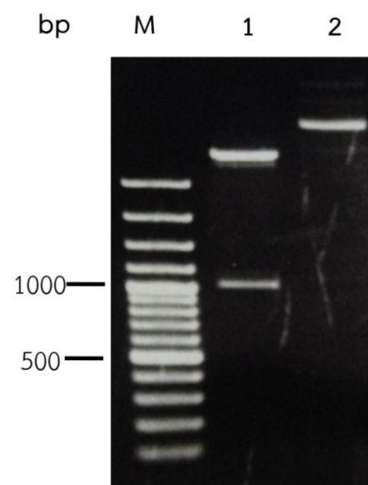

Figure 3.15 Screening of the recombinant plasmid. The *Pm*Snake gene was cloned into pET28b(+), digested with *Nco* I and *Xho* I and analyzed by 1.5% agarose electrophoresis. Lane M is 100 bp DNA ladder, Lane 1 is the digestion with *Nco* I and *Xho* I, Lane 2 is pEt 28b(+)-*Pm*Snake.

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The corrected plasmid was transformed into *E. coli* BL21 cells for protein expression. The single colony was selected to grow in LB broth containing kanamycin resistance drug. The culture medium was grown until OD 600 reached approximately 0.6. After the induction with 10mM IPTG, the cells were harvested at 0, 1, 2, 3, 4, 5 and 6 hours by centrifugation. The expressed proteins were detected by using 12.5% SDS-PAGE gel and the coomassie brilliant blue staining (Figure 3.16).

The total cells were broken by sonication in 20 mM Tris-HCl pH 8.0 as a buffer after washing with 1% triton X. The soluble fraction and inclusion body were separated and analyzed with 12.5% SDS-PAGE gel. The r*Pm*Snake was mainly expressed in inclusion body fraction and showed the major band about 37 kDa (Figure 3.16).

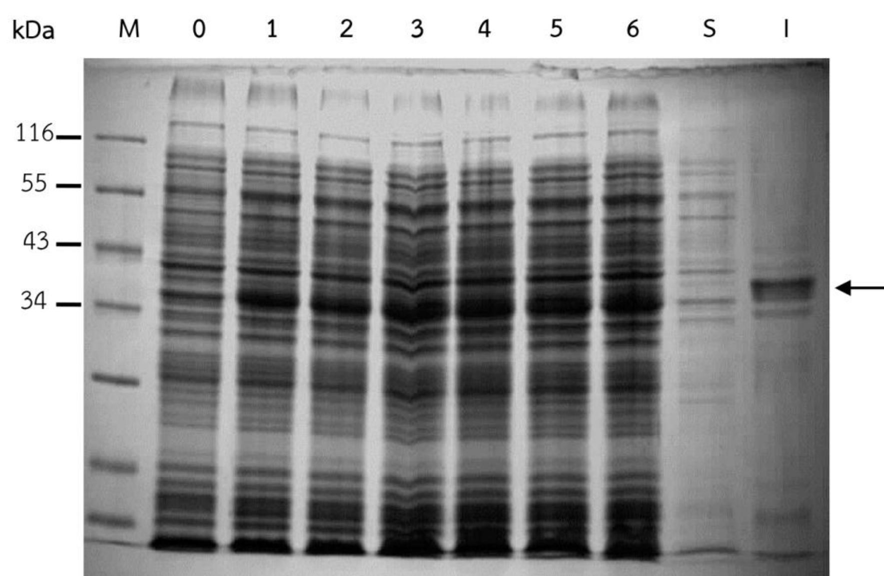

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Figure 3.16 The expression of r*Pm*Snake in *E. coli* BL21. The cells were harvested and induced with IPTG at 0, 1, 2, 3, 4, 5 and 6 hours. Lane M : Unstained protein marker, Lanes 0-6 : The expressed proteins after IPTG induction at 0 to 6 hours. Lane S is the soluble fraction. Lane I is the inclusion body fraction, arrow indicates r*Pm*Snake.

3.5.1 Purification of *Pm*Snake by Ni-NTA affinity chromatography

The expressed r*Pm*Snake protein was purified from inclusion body by Ni-NTA affinity chromatography. The pellets were dissolved in 8M urea before purification. The differential concentration of imidazole was used to wash and elute the r*Pm*Snake. The expected r*Pm*Snake was successfully purified with Ni-NTA chromatography. The purified protein was dialyzed in 20 mM Tris-HCl pH 8.0 buffer the concentration of r*Pm*Snake was measured by Bradford assay and determined the purity by using 12.5% SDS-PAGE (Figure 3.17). The major band of r*Pm*Snake had predicted molecular weight at 36.7 kDa. (Figure 3.18)

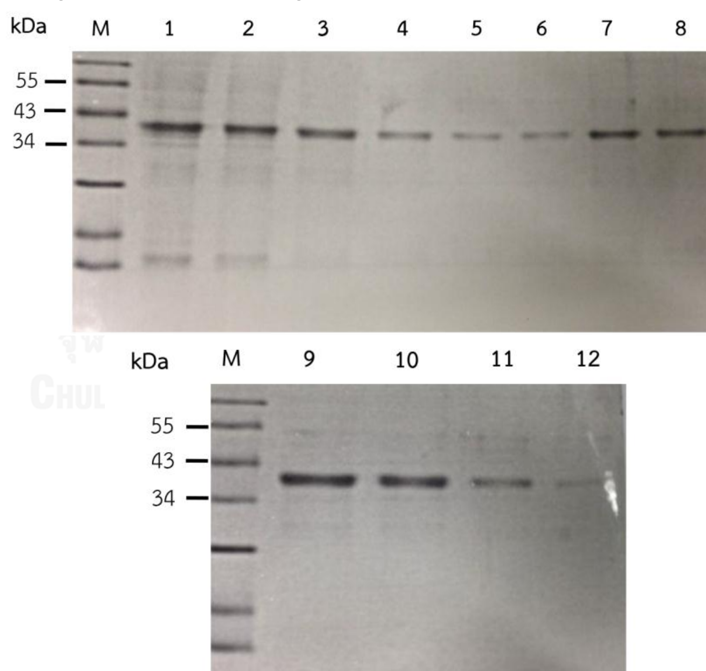

Figure 3.17 Purification of r*Pm*Snake using Ni-NTA chromatography. Lane M is Unstained protein marker, lane 1 is Flow through, lanes 2-6 are washed fraction containing 10 mM, 20 mM, 50 mM imidazole, lanes 7-12 are eluted fraction containing 100 mM and 250 mM imidazole

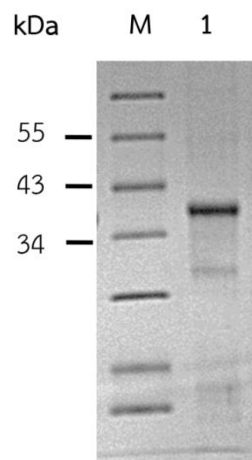

Figure 3.18 The 12.5% SDS-PAGE analysis of purified r*Pm*Snake by using Ni-NTA chromatography. Lane M is Unstained protein marker, Lane 1 is Purified r*Pm*Snake protein

3.6 The specificity of antibody *Pm*Snake

To substantiate the polyclonal rabbit anti-*Pm*Snake specific to *Pm*Snake protein, the purified r*Pm*Snake was used to synthesize the rabbit antibodies in order to generate anti-*Pm*Snake polyclonal antiserum at the Biomedical Technology Research Unit, Chiangmai University, Chiangmai Thailand. The major band was observed by using western blot analysis. The purified polyclonal anti-*Pm*Snake was used as a primary antibody and detected with substrate link secondary antibody. The result specified that, the rabbit polyclonal anti-*Pm*Snake is extremely specific to r*Pm*Snake. (Figure 3.19)

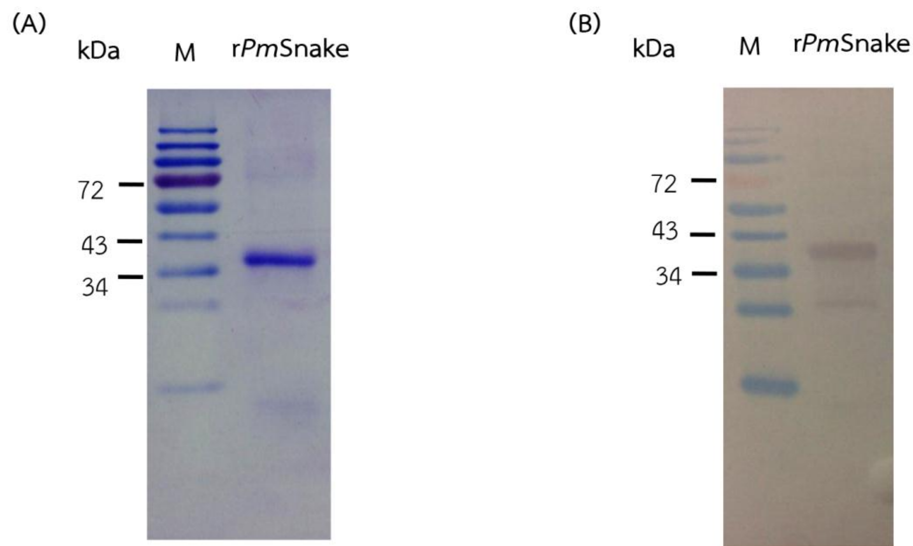

Figure 3.19 The SDS-PAGE and western blot analysis showed the specificity of rabbit polyclonal anti-*Pm*Snake. (A) 2 µg of r*Pm*Snake were run on 12.5% SDS-PAGE and staining with coomassie brilliant blue (B) 2 µg of r*Pm*Snake were run on 12.5% SDS-PAGE, transferred into nitrocellulose membrane and detected by rabbit polyclonal anti-*Pm*Snake.

3.7 Analysis of *Pm*Snake protein in hemolymph of shrimp by using Immunoblotting

To verify the *Pm*Snake protein in shrimp hemocyte, 20 µg of *P. monodon* hemocyte lysate supernatant and 100 µg of cell free plasma were run on 12.5% SDS-PAGE. Then, the proteins were transferred from the gel to nitrocellulose membrane and detected by purified anti-*Pm*Snake as a primary antibody. The expected band was observed as a major band, which corresponding to the predicted molecular mass 36.7 kDa of the mature r*Pm*Snake protein. The result showed that the *Pm*Snake

protein was detected in hemocyte of normal shrimp but not in cell free plasma (Figure 3.20).

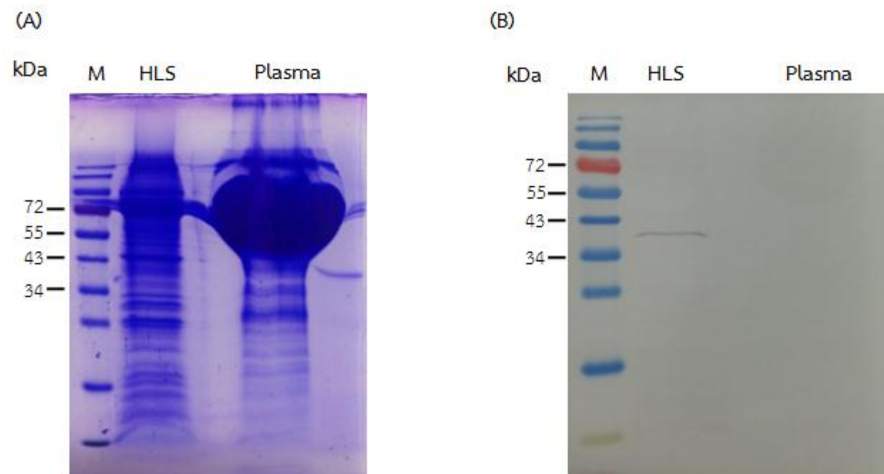

Figure 3.20 The SDS-PAGE and western blot analysis of the *Pm*Snake protein in shrimp hemocyte (A) The 12.5% SDS-PAGE staining with coomassie brilliant blue (B) The immunoblotting using anti-*Pm*Snake as primary antibody. Lane M is Prestained protein marker

3.8 Immunofluorescence of *Pm*Snake protein in shrimp hemocyte

Previously, the *Pm*Snake protein was found to be mainly expressed in hemocyte. To observe the expression of *Pm*Snake protein in different type of hemocytes, the hemocytes were collected and fixed by using 4% paraformadehyde into the slide. Then, the purified anti-*Pm*Snake was incubated and probed with Alexa 488 conjugated secondary antibody. The TOPO III was used to stain nucleus and observed under confocal FV1000. The results revealed that *Pm*Snake was expressed

in all three types of shrimp hemocytes such as hyaline, granular and semi-granular (Figure 3.21).

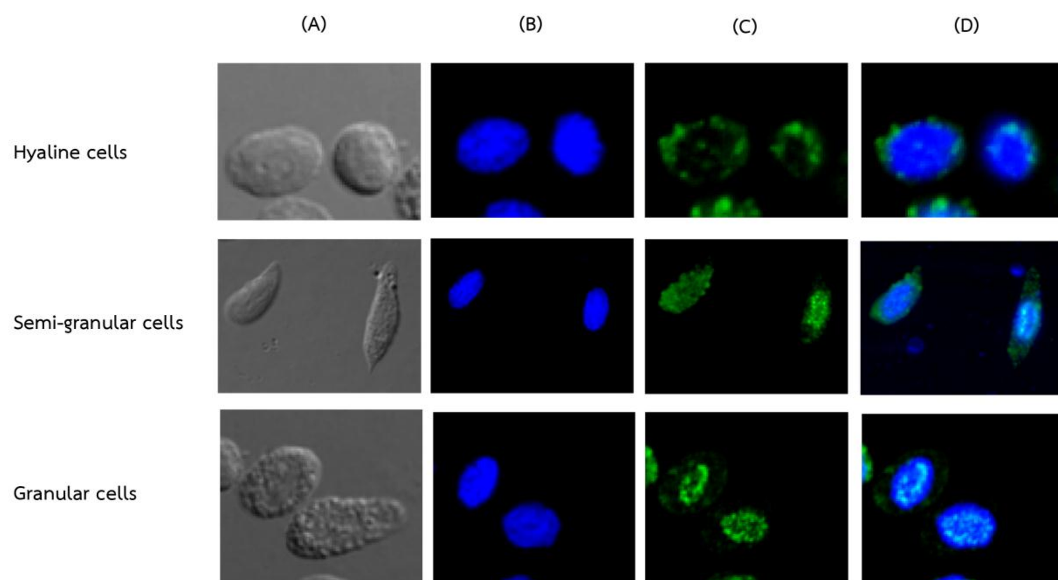

Figure 3.21 Fluorescence detection of *Pm*Snake-producing hemocytes. *Pm*Snake protein expression in different type of hemocytes (hyaline, granular and semi-granular) were observed by confocal microscope. The *Pm*Snake was probed in green color and nucleus hemocyte in blue color. Images were represented 4 fields of samples. (A) Bright field (B) TOPO-3 (C) Anti-*Pm*Snake antibody (D) Merge

3.9 PO Activation assay of the r*Pm*Snake protein

To study the biological function of r*Pm*Snake in PO activating, hemolymph was collected, incubated with r*Pm*Snake and L-DOPA was used as a substrate. The spectrophotometer was used to detect the absorbance at A_{490} nm which was calculated to PO activity unit. The results showed that adding r*Pm*Snake could

increase the total hemolymph phenoloxidase (PO) activity by 35% when compared with control. (Figure 3.22)

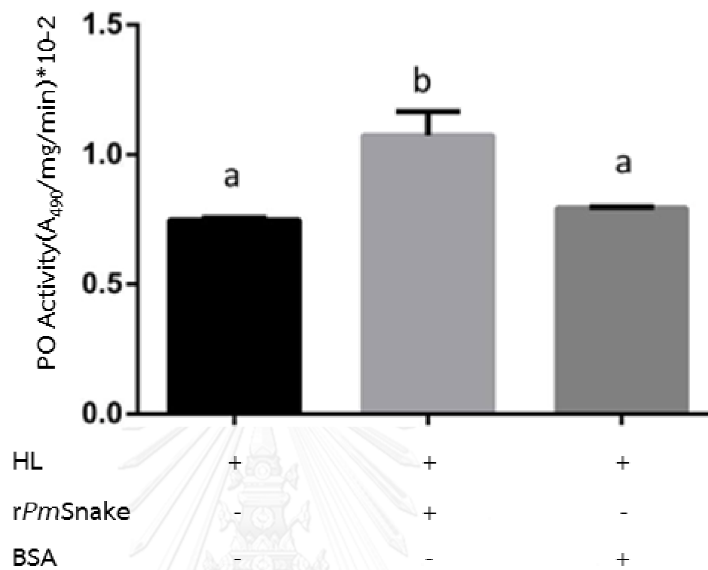

Figure 3.22 The *in vitro* PO activation of r*Pm*Snake protein. The L-dopa as substrate was used to detect PO activity at A490 nm and reported as PO activity (A490/mg/min). Experiments were repeated three times and the data is shown as the mean ± standard deviation and are derived from three independently replicated experiments. Means with a different lower case letter are significant at the $p<0.05$ level.

3.10 Proteinase activity

To determine the proteinase activity of r*Pm*Snake protein, the normal shrimp hemolymph was collected and incubated with r*Pm*Snake protein and adding activator and B2133 N-Benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride as a

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substrate. The colorimetric of enzyme activity was adding measured under the Spectra max spectrophotometer at A405 nm. The results revealed that the r*Pm*Snake significantly increased the proteinase activity as compared to that of BSA protein and trypsin as a negative and positive control respectively. (Figure3.23)

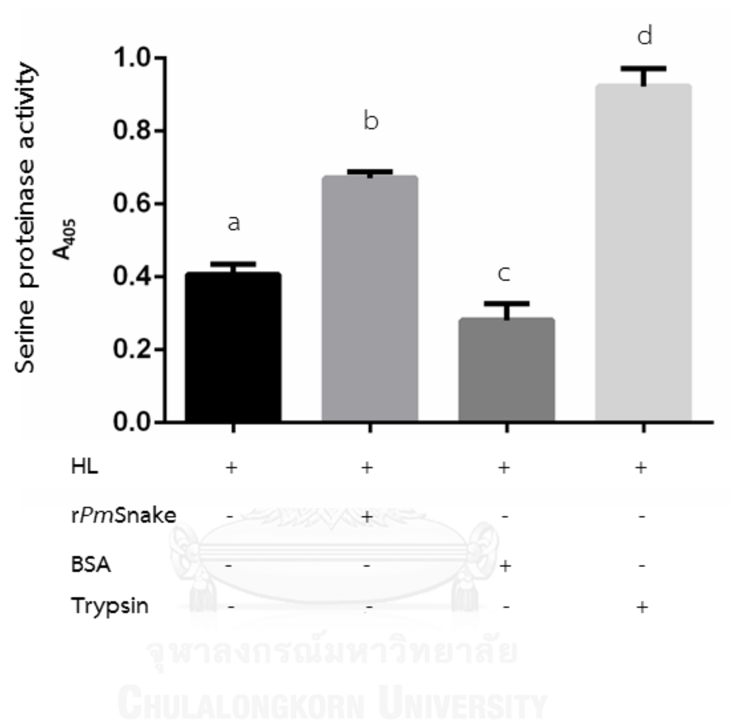

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Figure 3.23 The proteinase activity assay of r*Pm*Snake protein. Hemolymph were incubated with r*Pm*Snake (10 μM) and LPS as an activator. The BSA protein (10 μM) and trypsin (2.5 μM) were used as negative and positive controls respectively. B2133 N-Benzoyl-Pro-Phe-Arg-p-nitroanilide was used as a substrate and the enzymatic activity was detected at A 405 nm. The data are shown as the mean ± standard deviation and are derived from three independently replicated experiments.

CHAPTER IV
DISCUSSIONS

The innate immunity in invertebrate is the first line of defense against microbial infections (Hoebe et al. 2004). Invertebrates, which lack immunoglobulin in the adaptive immunity, instead they have innate immune defenses to detect and respond to the microbial surfaces like lipopolysaccharide (LPS), β-1,3 glucan and peptidoglycan (PGN) (Iwanaga and Lee 2005). The innate immune mechanism prevents the pathogen entrance that featured cellular and humoral immune defenses. One of the most effective humoral defenses is the melanization which is activated by the prophenoloxidase system leading to the synthesis of melanin and reactive intermediate compounds to accumulate or entrap the pathogen (Amparyup et al. 2013a; Cerenius and Soderhall 2004). The system requires many steps of non-enzymatic and enzymatic processes that catalyzed or activated the proPO cascade especially the complex cascade of Clip-domain serine proteinases (Clip-SPs).

Clip-SPs play critical roles in innate immunity of arthropods (Jiang and Kanost 2000) including the proclotting enzyme from the horseshoe crab *Tachypleus tridentatus* (Muta et al. 1990), the two serine proteases, snake and easter, the Clip-SPs from the fly *Drosophila melanogaster* which are involved in the pathway establishing the dorsal–ventral axis of a developing embryo (Morisato and Anderson

1995; Anderson 1998). Clip-SPs were also reported to be involved in the proPO system in the shrimp *Penaeus monodon* (Charoensapsri et al. 2009) tobacco hornworm *Manduca sexta* (Jiang et al. 1998; Jiang and Kanost 2000; Gupta et al. 2005), Korean black chafer *Holotrichia diomphalia* (Lee et al. 1998a; Lee et al. 1998b; Kwon et al. 2000), silkworm *Bombyx mori* (Satoh et al. 1999) and fly *Drosophila melanogaster* (Jiang and Kanost 2000). Clip-SPs are composed of two domains, the clip-domain at N-terminal region and C-terminal region of serine proteinase domain with conserved catalytic sites (His, Asp, Ser) that are always found in serine proteinases (SPs) and the non-catalytic site (His, Asp, Gly) in serine proteinase homologues (SPHs).

A number of Clip-SPs and Clip-SPHs have been identified in *P. monodon* and found to be involved in the proPO cascade. The Clip-SPs in the cascade are *Pm*Clip-SP2 (Amparyup et al. 2013b), *Pm*PPAE1 (Charoensapsri et al. 2009), *Pm*PPAE2 (Charoensapsri et al. 2011) and Clip-SPHs are *Pm*MasSPH1 (Amparyup et al. 2007; Jitvaropas et al. 2009), *Pm*MasSPH2 (Jearaphunt et al. 2015), *Pm*MasSPH3 (Amparyup et al., unpublished data). Recently, snake-like serine proteinase (*Pm*Snake) gene was identified from cDNA library suppression subtractive hybridization of proPO dsRNA treated hemocyte of *P. monodon* (unpublished data). In this study, an open reading frame of *Pm*Snake was analyzed. The sequence analysis showed that *Pm*Snake contains a signal peptide, an N-terminus clip domain and a C-terminus trypsin-like SP

domain (Figure 3.2). From blastx result, *Pm*Snake is similar to Clip-SPs in arthopods, with the highest similarity to snake-like hemolymph proteinase 21 in *Manduca sexta* (49% similarity)

The multiple amino acid sequence alignments of *Pm*Snake with other Clip-SPs in *P. monodon* and *M. sexta* showed the conservation of the six cysteine residues form three disulfide linkages of clip-domain at N-terminal region and conserved catalytic site (His, Asp and Ser) at C-terminal region of serine proteinase domain suggested that *Pm*Snake is a member of the clip-SPs. The Clip domain has been reported to form secondary structure similar to antibacterial proteins (Jiang et al. 1998) and SP domain is essential for proteolytic activity.

Tissue distribution analysis revealed that *Pm*Snake mRNA expressed in various shrimp tissues and the highest expression was found in hemocyte. This expression pattern is similar to that of the other known proPO-associated transcripts in *P. monodon* such as *Pm*ClipSP2 (Amparyup et al. 2013b), *Pm*ClipSP1 (Amparyup et al. 2010), *Pm*PPAE1 (Charoensapsri et al. 2009) and *Pm*PPAE2 (Charoensapsri et al. 2011) that are primarily detected in the hemocytes and similar to the expression profile observed for PPAE mRNA in *P. leniusculus* (Wang et al. 2001). However, the *Pm*Snake mRNA was all found to be expressed in hemocyte and other tissues such as gill, lymphoid organ and digestive tissues (Figure 3.4), but genes in the proPO system (*Pm*PPAE1, *Pm*PPAE2 and *Pm*ClipSP2) were not expressed in the digestive tissues

(Charoensapsri et al. 2009, 2011; Amparyup et al. 2013b) suggesting that *Pm*Snake might have multiple functions beside an immune responsive protein.

Injection of *V. harveyi* into *P. monodon* resulted in an increase expression of *Pm*Snake at 6 and 24 hpi and the expression increased up to 8 fold at 48 hpi, but rapidly declined at 72 hpi (Figure 3.6). Comparing to other *P. monodon* clip SPs, *Pm*PPAE1 transcript levels decreased at 24 h after systemic bacterial challenge and followed by significant increase at 48 h after infection (Charoensapsri et al. 2009). In *Pm*ClipSP2 mRNA levels increased at 3 h and continuous decreased at 24 h and 48 h after infection (Amparyup et al. 2013b). In *Pm*ClipSP1 transcript levels increased at 3 h and then decreased at 6 h and remain decreased at 48 h (Amparyup et al. 2010). In the shrimp *L. vannamei*, *Lv*PPAE1 transcription levels were down-regulated in hemocytes and up-regulated in the gill after systemic *V. harveyi* challenge (Jimenez-Vega et al. 2005). In the crayfish *P. leniusculus*, the PPAE1 mRNA levels were not affected following *Aeromonas hydrophila* infection (Liu et al. 2007). In insect, the *M. sexta* PPAE (PAP-1) is expressed at high levels in the integument and less abundantly in the fat body of naive larvae. However, the transcript expression levels of PAP-1 and the two other PPAE transcripts (PAP-2 and PAP-3) are up-regulated following bacterial infection (Jiang et al. 2003a; Jiang et al. 2003b). The mRNA levels of HP21 in *M. sexta* were increased in the larval fat body and/or hemocytes after a bacterial injection, suggesting that this enzyme plays certain roles in defense responses (Zou

and Jiang 2005; Zou et al. 2005). The differences of Clip-SPs in arthropods transcript expression could be due to variations in the response in the proPO cascade or are likely from the variations in each experiment ie. dose of bacterial injection, size of the animals etc. Nevertheless, the changes in the expression levels upon bacterial injection suggested that *Pm*Snake is involved in shrimp immune response to bacteria challenge.

In this study, the RNA interference (RNAi) was performed to investigate the involvement of *Pm*Snake in the *P. monodon* proPO system. In previous research, the RNAi technique in the dipteran insects *A. gambiae* and *D. melanogaster* established the function of the three Clip-SPs, CLIPB4, CLIPB8 and CLIPB14, to be involved in the proPO pathway (Paskewitz et al. 2006; Volz et al. 2006; Volz et al. 2005). In Drosophila, suppression of Clip-SPs, MP1 and MP2/sp7 by RNAi suggested that these clipSPs are required for the insect proPO activation (Castillejo-Lopez and Hacker 2005; Tang et al. 2006). The function of Clip-SPs in *P. monodon*, *Pm*ClipSP1 (Amparyup et al. 2010); *Pm*PPAE1 (Charoensapsri et al. 2009); *Pm*PPAE2 (Charoensapsri et al. 2011); *Pm*ClipSP2 (Amparyup et al. 2013b) was investigated by using dsRNA-mediated gene silencing in shrimp hemocyte. The results suggested that the Clip-SPs except *Pm*clipSp1 play a role in shrimp immunity and are involved in the activation of the proPO cascade. In this study, *Pm*Snake dsRNA injection could suppress both *Pm*Snake transcript and protein levels resulted in a significant

reduction of the hemolymph PO activity (36%) supporting that *Pm*Snake is likely to be involved in the activation of shrimp proPO.

To further investigate the function of *Pm*Snake *in vitro*, the recombinant *Pm*Snake was produced by *E. coli* expression system (Baneyx 1999). The mature recombinant *Pm*Snake protein was successfully expressed in *E. coli* (BL 21) system using pET 28 b(+) as expression vector. The protein was purified by Ni-NTA affinity chromatography and analyzed by SDS-PAGE. The r*Pm*Snake with predicted molecular mass of 36.7 kDa was expressed in inclusion body. Western blot analysis using rabbit polyclonal *Pm*Snake antibody, revealed that *Pm*Snake proteins are localized in shrimp hemocyte but not cell free plasma. Immunofluorescent labeling technique with a confocal laser scanning microscope showed that *Pm*Snake protein was localized in all three types of shrimp hemocyte cells (Hyalin, granular and semi-granular). In crustacean, protein in the proPO system are localized in semi-granular and granular hemocyte (Perazzolo and Barracco 1997). In crayfish, PPAE was found in in active form granule hemocyte cells when pathogens infect PPAE secreted into plasma and changed to active form (Aspán et al. 1990; Aspán et al. 1995; Aspán and Söderhäll 1991). In shrimp, it has been shown that the genes involved in the PO activation are mainly expressed in shrimp hemocytes and released into plasma upon activation.

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The *in vitro* assay of r*Pm*Snake in the activation of the PO activity showed that total shrimp hemolymph PO activity was significantly increased when adding r*Pm*Snake as compared to control BSA, suggesting that *Pm*Snake is involved in the proPO activation. In *P. monodon*, r*Pm*ClipSP2 exhibits the ability to activate the proPO system *in vitro* (Amparyup et al. 2013b). In addition, *in vitro* proteinase activity assay of r*Pm*Snake using B2133 as substrate showed a significant increase in the proteinase activity in shrimp hemolymph when adding r*Pm*Snake protein. The resute supported that r*Pm*Snake participated in proPO activating system in *P. monodon* shrimp via the serine proteinase cascade.

The mechanism of prophenoloxidase activation in *M. sexta* revealed that the proHP21 was cleaved by HP14 and then the active HP21 activates the final serine proteinase in the cascade, proPAP3, which in turn activates the conversion of proPO to PO. They also propose that proHP21 is activated by a conformational change that occurs when proHP21 binds to HP14 and proPAP3 (Gorman et al. 2007a; Gorman et al. 2007b). Although, *Pm*Snake exhibits the highest similarity to the insect proHP21, the mechanism by which this protein activates the *P. monodon* proPO system remains unknown and needs further investigation.

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CHAPTER V
CONCLUSIONS

A novel clip-SP, named snake-like serine proteinase (*Pm*Snake), from the shrimp *Penaeus monodon* was identified from the suppression subtractive hybridization cDNA library of proPO dsRNA treated hemocyte. An open reading frame of *Pm*Snake contains 1,068 bp encoding a predicted protein of 355 amino acid residues.

The sequence analysis by BlastX showed that the deduced amino acids of *Pm*Snake shared high similarities to snake-like Hemolymph Proteinase 21 (49% sequence similarity) of silkworm *Manduca sexta* that involved in the proPO system. The multiple amino acid sequence alignments of *Pm*Snake with other Clip-SPs in *P. monodon* and *M. sexta* showed the conservation of the six cysteine residues engaging three disulfide linkages of clip-domain at N-terminal region and conserved catalytic site (His, Asp and Ser) at C-terminal region of serine proteinase domain suggested that *Pm*Snake is a member of the clip-SPs. Phylogenetic analysis revealed that *Pm*Snake is closely related to snake-like serine proteinases in insects.

*Pm*Snake is highly expressed in shrimp hemocyte and changes in mRNA expression level were observed after systemic *Vibrio harveyi* infection supporting that it is an immune-responsive gene in shrimp hemocyte.

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Suppression of *Pm*Snake transcripts by injection of dsRNA resulted in significant reduction of hemolymph phenoloxidase (PO) activity (36.1%), suggesting that *Pm*Snake is likely involved in the activation of shrimp proPO system.

The recombinant *Pm*Snake protein (r*Pm*Snake) was successfully overexpressed after 6 hours IPTG induction in *E. coli* BL21 as a host cells and pET 28 b (+) as an expression vector The recombinant protein with predicted molecular mass of 36.7 kDa was produced as inclusion body protein and was successfully purified by Ni-NTA chromatography. *Pm*Snake proteins were found in all three types of shrimp hemocyte cells but not cell free plasma as analyzed by Western Blot and Immunofluorescent labeling technique.

In vitro PO activation and serine proteinase activity assay showed that r*Pm*Snake significantly enhance phenoloxidase activity and also serine proteinase activity of shrimp hemolymph suggested that r*Pm*Snake participates in the proPO activating system in *P. monodon* via the serine proteinase cascade.

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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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VITA

Miss Warunthorn Monwan was born in December 26, 1989 in Bangkok, Thailand. She graduated with the degree of Bachelor of Science (Second class Honor) from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2011. She has studied for the degree of Master of Science, Program in Biochemistry and Molecular biology, Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2012. Then, she got the scholarship from The Chulalongkorn University graduate scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej.

Conferences

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Proceeding Publication

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