บทบาทของโปรตีนคลิปโดเมนซีรีนโปรติเนส 2 *Pm*ClipSP2 ในการกระตุ้นระบบกระตุ้นโพรฟีนอลออกซิเดสในกุ้งกุลาดำ *Penaeus monodon*

นายนรัช คอรัตนกุลชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ROLE OF CLIP DOMAIN SERINE PROTEINASE 2, *Pm*ClipSP2, IN ACTIVATION OF PROPHENOLOXIDASE-ACTIVATING SYSTEM IN THE BLACK TIGER SHRIMP *Penaeus monodon*

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Biochemistry
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Thesis Title	ROLE OF CLIP DOMAIN SERINE PROTEINASE 2, *Pm*ClipSP2, IN ACTIVATION OF PROPHENOLOXIDASE-ACTIVATING SYSTEM IN THE BLACK TIGER SHRIMP *Penaeus monodon*
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นรัช คอรัตนกุลชัย : บทบาทของโปรตีนคลิปโดเมนซีรีนโปรติเนส 2 *Pm*ClipSP2 ในการกระตุ้นระบบกระตุ้นโพรฟีนอลออกซิเดสในกุ้งกุลาดำ *Penaeus monodon* (ROLE OF CLIP DOMAIN SERINE PROTEINASE 2, *Pm*ClipSP2, IN ACTIVATION OF PROPHENOLOXIDASE-ACTIVATING SYSTEM IN THE BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อัญชลี ทัศนาขจร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ปิติ อ่ำพายัพ, 86 หน้า.

คลิปโดเมนซีรีนโปรติเนส (ClipSPs) มีบทบาทสาคัญในระบบกระตุ้นโพรฟีนอลออกซิเดสในกุ้งกุลาดำ *Penaeus monodon* ClipSP ชื่อ *Pm*ClipSP2 มีรายงานก่อนหน้านี้แสดงให้เห็นว่า จับกับพอลิแซคคาไรด์ของจุลชีพได้แก่ ลิโพพอลิแซคคาไรด์ (LPS) และบีต้า-1,3-กลูแคน (β-1,3-glucan) และเป็นไปได้ว่ากระตุ้นระบบโพรฟีนอลออกซิเดส นอกจากนี้อาจมีส่วนเกี่ยวข้องในการรักษาสมดุลเม็ดเลือดโดยการจับและลดทอนพิษของลิโพพอลิแซคคาไรด์ เพื่อระบุบริเวณจับจุลชีพของโปรตีน *Pm*ClipSP2 ได้ทำการแยกโคลนส่วนคลิปโดเมนที่ปลายอะมิโน (Clip-*Pm*ClipSP) และส่วนซีรีนโปรติเนสโดเมนที่ส่วนปลายคาร์บอกซิล (SP-*Pm*ClipSP2) โปรตีนรีคอมบิแนนท์ถูกนำมาทดสอบ สมบัติการจับและความเกี่ยวข้องในการกระตุ้นโพรฟีนอลออกซิเดส แอคติวิตีต้านเชื้อจุลชีพ และการลดทอนพิษของลิโพพอลิแซคคาไรด์ จากการทดสอบการจับด้วย ELISA พบว่า rSP-*Pm*ClipSP2 แต่ไม่ใช่ rClip-*Pm*ClipSP2 ที่สามารถจับกับลิโพพอลิแซคคาไรด์และบีต้า-1,3-กลูแคนที่ตรึงไว้และกระตุ้นฟีนอลออกซิเดสแอคติวิตีอย่างมีนัยสำคัญ นอกจากนี้บริเวณจับของผนังเซลล์จุลชีพถูกเสนอว่าน่าจะเป็นบริเวณ Pattern sequence ที่มีรูปแบบเป็น (X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X) ที่อยู่ในบริเวณปลายคาร์บอกซิลของซีรีนโปรติเนสโดเมนของ *Pm*ClipSP2 จึงได้ทดสอบว่าโปรตีนรีคอมบิแนนท์ที่ตัดส่วน Pattern sequence ไม่สามารถจับกับลิโพพอลิแซคคาไรด์และบีต้า-1,3-กลูแคนได้ ในทางกลับกันโปรตีนรีคอมบิแนนท์ที่มี Pattern sequence (rPT-*Pm*ClipSP2-TRX) มีความสามารถในจับกับองค์ประกอบผนังเซลล์จุลชีพ แต่ไม่พบการต้านแบคทีเรียจากการทดสอบด้วย Radial diffusion assay ที่ความเข้มข้น 100 μM นอกจากนี้ r*Pm*ClipSP2 ไม่สามรถลดทอนพิษของลิโพพอลิแซคคาไรด์ได้โดยตรงใน เซลล์ S2 จากผลเหล่านี้ยืนยันหน้าที่ของ *Pm*ClipSP2 ในการจับแบคทีเรียและรา โดย Pattern sequence ที่ปลายคาร์บอกซิล แล้วนำไปสู่การกระตุ้นโพรฟีนอลออกซิเดสเป็นลำดับขั้น

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต	.....
สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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NARACH KHORATTANAKULCHAI: ROLE OF CLIP DOMAIN SERINE PROTEINASE 2, *Pm*ClipSP2, IN ACTIVATION OF PROPHENOLOXIDASE-ACTIVATING SYSTEM IN THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR: PITI AMPARYUP, Ph.D., 86 pp.

Clip domain serine proteinases (ClipSPs) play an important role in the prophenoloxidase-activating (proPO) system. In the shrimp *Penaeus monodon*, the ClipSP, *Pm*ClipSP2, has been previously shown to bind to microbial polysaccharides (LPS and β-1,3-glucan) and likely activates the proPO-system. Moreover, it may play key role in the hemocyte homeostasis by scavenging and neutralizing LPS. To reveal the binding site of the *Pm*ClipSP2 protein, the N-terminal clip domain (Clip-*Pm*ClipSP) and C-terminal SP domain (SP-*Pm*ClipSP2) were separately cloned. The recombinant proteins were then assayed for their binding properties and involvement in proPO activation, antimicrobial activity and LPS neutralization. According to the ELISA-based binding assay, rSP-*Pm*ClipSP2, but not rClip-*Pm*ClipSP, can bind immobilized LPS and β-1,3-glucan as well as significantly activate PO activity. The binding site at the SP domain is proposed to have a pattern sequence (X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X) that is located at the C-terminal region of the SP domain of *Pm*ClipSP2. Deletion of the pattern sequence abolished binding to LPS and β-1,3-glucan. Conversely, a recombinant protein containing the pattern sequence (rPT-*Pm*ClipSP2-TRX) had the ability to bind to cell wall components but did not exhibit antibacterial activity via radial diffusion assay at 100 μM. Furthermore, r*Pm*ClipSP2 cannot directly neutralize toxicity of LPS in S2 cells. These results confirm the function of *Pm*ClipSP2 in binding to microbial cell wall components of bacteria and fungi via the pattern sequence at the C-terminus SP domain, subsequently leading to activation of the proPO cascade.

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

°C	Degree celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Absorbance
AHPND	Acute Hepatopancreatic Necrosis Disease
AP	Alkaline phosphatase
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
ClipSP	Clip domain serine proteinase
C-terminal	Carboxy terminal
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMS	Early mortality syndrome
GFP	Green fluorescence protein
h	Hour
HLS	Hemocyte lysate supernatant
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
K_d	Dissociation constant

kDa	Kilodalton
LB	Luria-Bertani
LD_{50}	50% Lethal dose
L-DOPA	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
M	Molar
mg	Milligram
min	Minute
ml	Mililitre
N-terminal	Amino terminal
O.D.	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pm	*Penaeus monodon*
pmol	Picomole
PO	Phenoloxidase
PPAE	Prophenoloxidase activating enzyme
proPO	Prophenoloxidase
PRP	Pattern recognition protein
r	recombinant
RNA	Ribonucleic acid

RNAi	Ribonucleic acid interference
RT-PCR	Reverse transcription/polymerase chain reaction
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SP	Serine proteinase
SPH	Serine proteinase homolog
THC	Total hemocyte number
TRX	Thioredoxin
TSB	Tryptic soy broth
WSSV	White spot syndrome virus
YHV	Yellow head virus

CHAPTER I
INTRODUCTION

1.1 Shrimp Aquaculture and Production

Over the last few decades, aquaculture has been importantly increased to reach greater demands of the world consumption rate. Among those farming, shrimp aquaculture has become a main economic activity in many countries in Asia and South America. In Thailand, the shrimp farms distributed along the coastal areas, especially in Suratthani and Nakhornsrithammarat provinces, are the mainstream of shrimp production. The shrimp farms are also placed in the central and the eastern provinces such as Samutsongkharm, Samutsakhorn, Chachoengsoa and Chantaburi. Unfortunately, the production of the black tiger shrimp, *Penaeus monodon*, has dramatically decreased that caused by the outbreaks of severe pathogens. Then, farmer shifted species to the Pacific white shrimp *Litopenaeus vannamei* that have been successfully selected the tolerate diseases white shrimp and high survival rate. Thus, white shrimp become the major aquaculture species instead of black tiger shrimp and it will continuously increase up to 72% in 2018 of shrimp aquaculture in Asia as prediction by Global Outlook on Aquaculture Leadership 2016 (GOAL (2016)) (Figure 1.1). However, in recent years, diseases also have a great impact on the farming of the white shrimp particularly the Early mortality syndrome (EMS), officially named acute hepatopancreatic necrosis disease or AHPND (Tran et al., 2013). This outbreak caused huge economic losses in Southern China then expanded to Vietnam, Malaysia and reached Thailand in 2012 (Tran et al., 2013). According to resolve the diseases problems and maintain the production of black tiger shrimp, well farm management, well-organized domestication, genetic improvement and invention of effective disease control are immediately required. The knowledge is not only applied for protection of diseases but also for selective breeding of healthy shrimp for Thai industry.

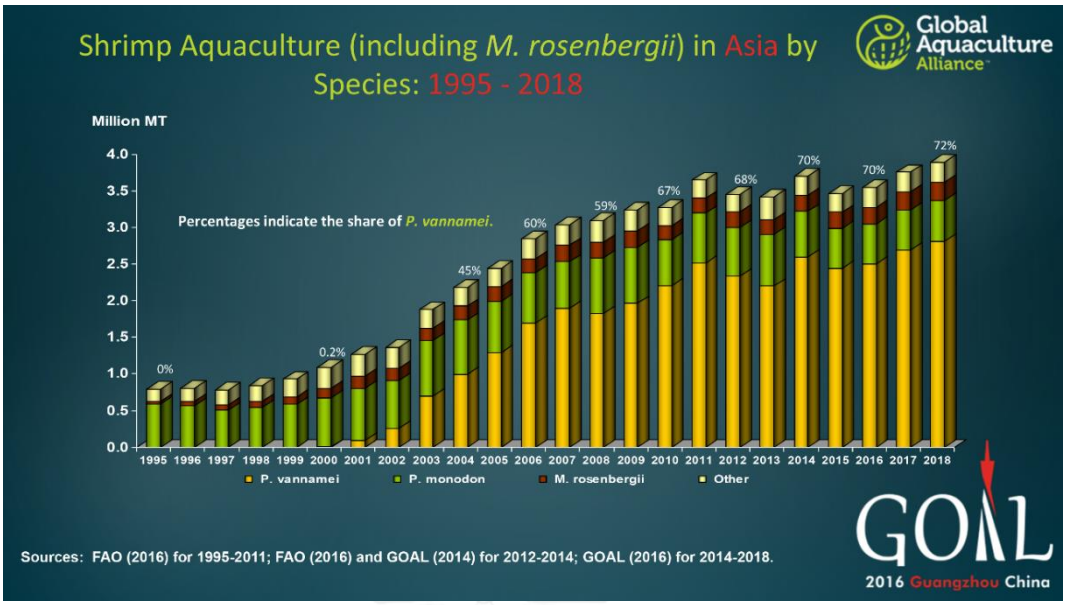


Figure 1.1 Shrimp aquaculture in Asia by shrimp species during 1995-2018. (Sources: FAO (2016) for 1995-2011; FAO (2016) and GOAL (2014) for 2012-2014; GOAL (2016) for 2014-2018).

1.2 Taxonomy of black tiger shrimp, *Penaeus monodon*

The black tiger shrimp *Penaeus monodon* is an economically important live stock in Thailand. Normally, *P. monodon* is widely distributed to the South-East Asia, South Africa, Australia, the Pacific and Indian Oceans. The appearance of the *P. monodon* are black and white or yellow stripes on their cuticles like striped tiger, tails and abdomens. The general body pattern is a head tail, five pairs of pleopods (swimming legs), five pairs of pereopods (walking legs) and presence of pair appendages and a protective exoskeleton that covers the whole body. The taxonomy is identified as below (Bailey-Brock et al., 1992).

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Order: Decapoda

Suborder: Natantia

Superfamily: Penaeoidea

Family: Penaeidae Rafinesque, 1985

Genus: *Panaeus* Fabricius, 1798

Species: *Penaeus monodon*

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

Common name: Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost prawn (Hong Kong), Jinga (India, Bombay region), Udang windu (Indonesia), Ushi-ebi (Japan), Kamba ndogo (Kenya), Kalri (Pakistan), Sugpo (Phillipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Tim sa (Vietnam).

The FAO names are Crevette giante tigre (French), Caramon tigre gigante (Spanish) and Giant tiger prawn (English) (Noel B. Solis, 1988).

Figure 1.2 Black tiger shrimp (*Penaeus monodon*)

(http://www.sea-ex.com/fishphotos/images/black-tiger-prawn.jpg)

1.3 Diseases

The most issues of shrimp production in all countries was still diseases problem (Figure 1.3). Shrimp diseases can be categorized into two groups, infectious and noninfectious etiologies (Lightner and Redman, 1998). Diseases can be caused by various pathogens for example fungi, bacterial, parasites and virus. Bacteria and virus are major serious causes of diseases in shrimp farming.

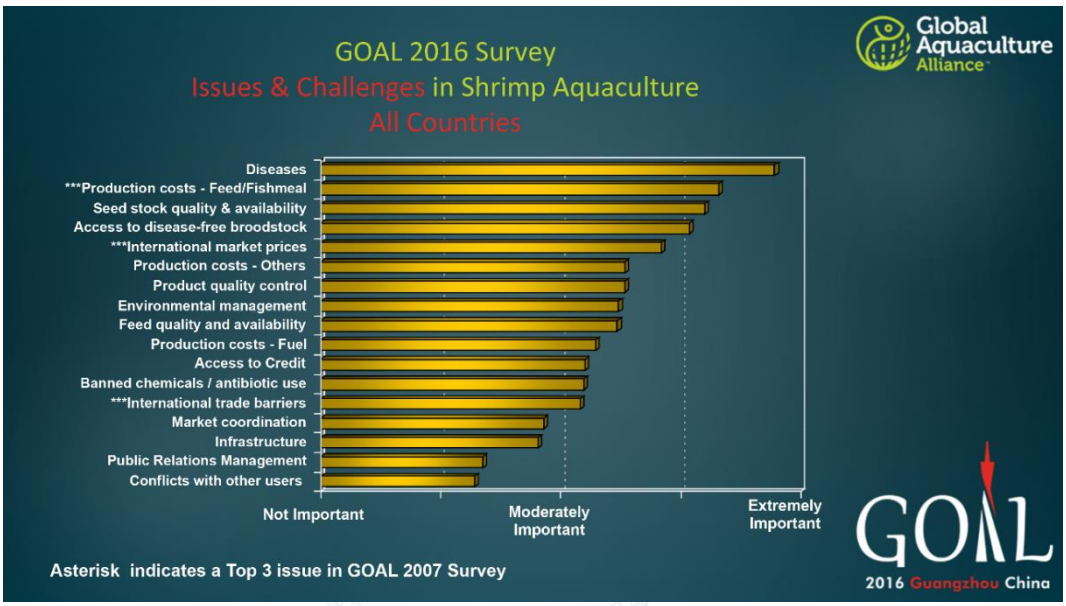


Figure 1.3 Factors affecting shrimp aquaculture in all countries (Source: GOAL 2016)

1.3.1 Bacterial diseases

1.3.1.1 Vibriosis

Vibriosis, caused by bacterium *Vibrio harveyi*, is one of the major diseases in shrimp farming. The disease contributes to morbidity and high mortality rate in shrimp (Austin and Zhang, 2006; Lightner and Lewis, 1975), which it results in almost 100% cumulative mortality which generally happened in post-larvae and young juvenile shrimp (Flegel, 2006), and also caused by other *Vibrio* species (Lightner, 1996).

V. haeveyi is a Gram-negative, motile, rod shape and luminescent (Lavilla-Pitogo et al., 1990). Disease transmission can occur via water or consumption of infective material. The clear symptoms of the *V. haeveyi* infected shrimp are called

luminous vibriosis (disorder development of basal tissues in the digestive system). The pathogen releases exotoxins to terminate the wall of gastrointestinal tract and the host's immune cells (Peddie and Wardle, 2005) and mainly caused in juvenile shrimp, moribund shrimp, which appear hypoxic and often come to the pond surface or edge, reddish discoloration and show black spots of melanization on the cephalothorax (Figure 1.4).

Figure 1.4 Vibriosis in shrimp farming. Black spots of melanization was observed on the cephalothorax. (http://agritech.tnau.ac.in/fishery/vibriosisshrimp.jpg)

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

Recently, an emerging disease known as early mortality syndrome or Acute Hepatopancreatic Necrosis Disease (EMS/AHPND) was first reported in China and consequently in Vietnam, Thailand and Malaysia. Mortalities can reach up to 100% in post-larvae shrimp during 20-30 days of culture (De Schryver et al., 2014) and *Vibrio parahaemolyticus* has been reported as caused of EMS/AHPND. Newly, the unique plasmid was identified from virulent strain of *V. parahaemolyticus* that encodes two genes, PirA and PirB. These gene are homologous to the *Photorhabdus* insect-related (Pir) toxin genes that are lethal to insect (Lee et al., 2015; Lightner, 2014; Yang et al., 2014). Nevertheless, the non-virulent strains carrying the plasmid without the PirA and PirB genes (Flegel and Lo, 2014; Sirikharin et al., 2014).

The PCR detection methods were developed in order to detect this disease by designed the AP1, AP2, AP3 and AP4 PCR primer pairs, which based on conserved sequences of the plasmid to detect AHPND-related *V. parahaemolyticus* (Flegel and Lo, 2014; Sirikharin et al., 2015; Sritunyalucksana et al., 2015).This bacterium can spread by oral, colony in the shrimp gastrointestinal tract and toxin production, causes tissue destruction and dysfunction of the shrimp digestive organ known as the hepatopancreas (Tran et al., 2013). The symptoms of this disease are slow growth, corkscrew swimming, soft texture of the exoskeleton, reduction size of hepatopancreas and discolored hepatopancreas (Figure 1.5).

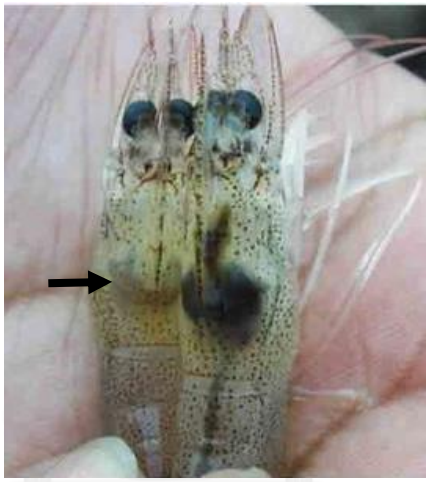


Figure 1.5 The infected shrimp caused by early mortality syndrome or Acute Hepatopancreatic Necrosis Disease (EMS/AHPND). The black arrow indicated the fade hepatopancreas of infected shrimp (left) compared to the brown hepatopancreas of normal shrimp (right) (Tran et al., 2013).

1.3.2 Viral diseases

1.3.2.1 Taura syndrome

In 1992, Taura Syndrome (TS) was first reported in Ecuador (Jimenez, 1992), then quickly transmitted to the USA (Lightner, 1996) and later to Southeast Asia. It is responsible for critical mortalities of penaeid shrimp in Taiwan (Yu and Song, 2000). The pacific white shrimp *Litopenaeus vannamei* is highly sensitive to TS (Lightner,

1996). Taura syndrome is caused by infected Taura Syndrome Virus (TSV) that is a small single RNA virus. The particles are non-enveloped 32 nm diameter icosahedral and replicate within the cytoplasm of host cells. The genome of TSV contains a linear, positive sense single-stranded RNA and two large open reading frames (Lightner, 2011). TSV can be spread by contaminated water or by horizontal transmission. Symptoms in shrimp are pale reddish coloration with the tail fan and pleopods appearing hyperpigmented, the soften cuticle, the gut empty and also exhibited irregularly shapes of melanised cuticular lessions (Figure 1.6).

Moribund shrimp can find at the pond surfaces and edges and typically result in cumulative mortalities between 40-100%. *L. vannamei* selective breeding program has been successfully developed to select the resistance TSV species. The result of breeding program increases survival rate to that equal to the levels of those prior to the initial TS outbreak (Cock et al., 2009) and has proven to be the most successful in preventing and controlling of TS disease. Currently, TSV is no longer considered as a main risk.

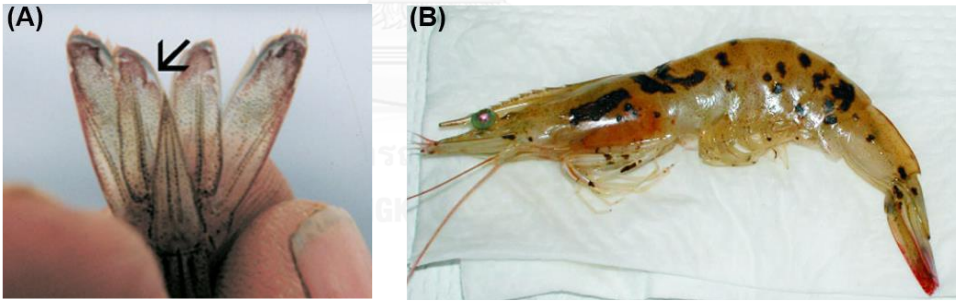


Figure 1.6 Taura syndrome disease in shrimp. (A) The characteristic of red tail fan was observed in infected shrimp. (B) The epidermal necrosis will cause black spots on the body of infected shrimp. (European Community Reference Laboratory for Crustacean Diseases Leaflet, 2008)

1.3.2.2 Yellow head disease

The yellow head disease, caused by the virus called yellow head virus (YHV), was first described as an epizootic from black tiger shrimp farms in Thailand

(Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Limsuwan, 1991). YHV is an invertebrate nidovirus, containing single-stranded RNA of about 22 kb, and is an enveloped rod-shaped particle (Sittidilokratna et al., 2002), and replicates in the cytoplasm of infected host's cells (Cowley et al., 1999).Three YHV structural proteins, including two envelope glycoproteins gp116 and gp64 and a nucleocapsid protein p20, have been discovered (Jitrapakdee et al., 2003; Sittidilokratna et al., 2006). The lymphoid organ is the major organ being infected by YHV (Chantanachookin et al., 1993; Khanobdee et al., 2002). The signs of yellow head disease are the development of yellowish cephalothorax and brown gills, increase in feeding and then rapidly decline and the mortality can extent to 100% within 3-5 days of the first appearance (Boonyaratpalin et al., 1993; Chantanachookin et al., 1993; Lightner, 1996). The symptoms of YHV-infected shrimp also display light yellowish of the dorsal cephalothorax area, has a generally pale or bleached appearance (Limsuwan, 1991) and spheroids at lymphoid organ (Pantoja and Lightner, 2001) The detection of YHV methods were developed such as RT-PCR (Cowley et al., 1999), *in situ* hybridization (Tang et al., 2002), loop mediated isothermal amplification (RT-LAMP) (Mekata et al., 2006) and real-time RT-LAMP (Mekata et al., 2009). A comprehensive assessment of host susceptibility to YHV has been completed by EFSA (EFSA 2008).

Figure 1.7 Yellow head disease in shrimp. The white arrow indicated the YHV-infected shrimp which shows a yellowish discoloration of the cephalothorax compare to the black color of normal shrimp (right). (Source: AGDAFF-NACA (Photo D. V. Lightner))

1.3.2.3 White spot syndrome

White spot syndrome has been the most serious diseases in shrimp aquaculture worldwide, which causes by White Spot Syndrome Virus (WSSV) (Flegel, 1997; Lightner, 1996). The virus infects only crustaceans and it is not linked to another known viruses. In crustaceans, including shrimp, lobsters and crabs from marine, brackish or freshwater environment, are considered susceptible to infection (Lo et al., 1996). The WSSV is assigned by the ICTV to its own new genus, Whispovirus, and family *Nimaviridae* (Lightner, 2011) The virions are large rod-shape to elliptical, double-stranded DNA virus and with trilaminar envelope (Lightner, 2011; Yang et al., 2001) (Figure 1.8). The symptoms of this disease are development of white spots on the carapace of the infected shrimp, which caused by accumulation of calcium in cuticle but not all host species (Chou et al., 1995), lower food consumption, the body surface and appendages turning to red or pink, loosing shell and lethargy (Figure 1.9). This disease can reach to 80-100% mortalities within 2-10 days after infection (Flegel, 1997; Lo et al., 1996).

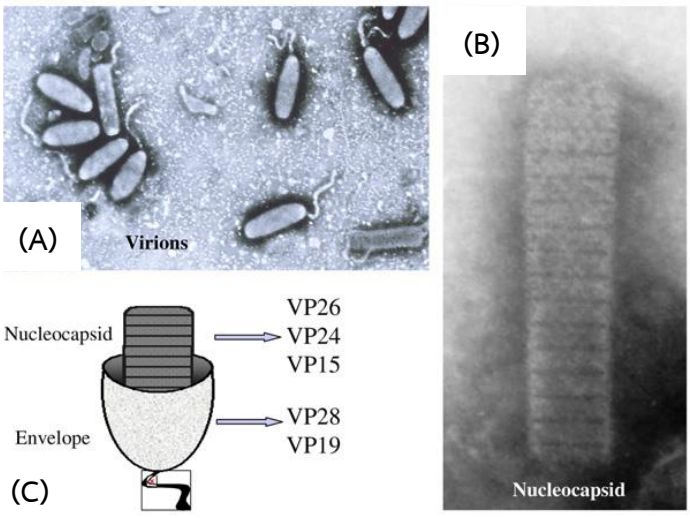


Figure 1.8 The structure of White spot syndrome virus (WSSV). (A) Transmission electron micrograph (TEM) of semi-purified WSSV virions concentrated from the hemolymph of infected *L. vannamei*. Intact virions have a tail-like appendage that extends from the envelope; (B) TEM of a nucleocapsid and (C) schematic showing in a cut-away view the outer envelope, the inner nucleocapsid, and the principal proteins associated with each (Lightner, 2011).

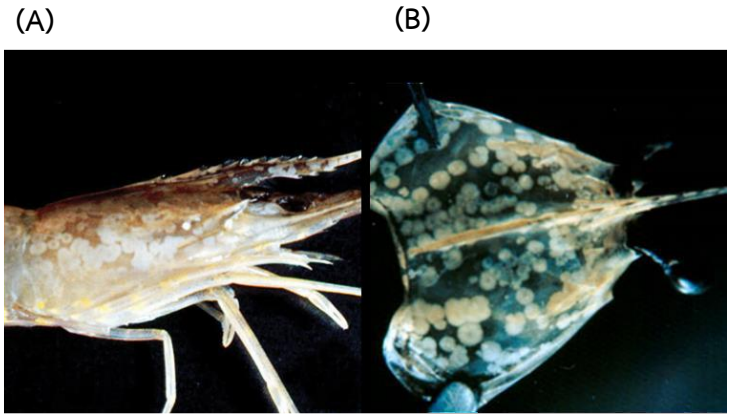


Figure 1.9 White spot syndrome disease. (A) and (B) The white spots occur on the cuticle of WSSV infected shrimp at the late phase of infection (Lightner, 1996).

1.4 Shrimp immunity

Shrimp lack adaptive immunity but they have an innate immunity, non-specific immune. Innate immunity is the first line of defense against pathogen infections, bacteria, fungi and viruses (Salzet, 2001), and relying on the cellular and humoral immune responses. 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, which faster than the development of adaptive immune responses. The cellular immune reactions include phagocytosis (Smith and Söderhäll, 1983), nodulation and encapsulation (Kobayashi et al., 1990; Pech and Strand, 2000; Sung et al., 1998). While, the humoral responses involve in 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 (Holmblad and Söderhäll, 1999; Jiravanichpaisal et al., 2006).

The circulatory system of shrimp consists of cellular and humoral immune responses which produce and store in the granules of hemocytes before being released into the hemolymph upon activation by bacterial and/or fungal cell wall

components (Tassanakajon et al., 2013). The overview of shrimp immune system is shown in Figure 1.10.

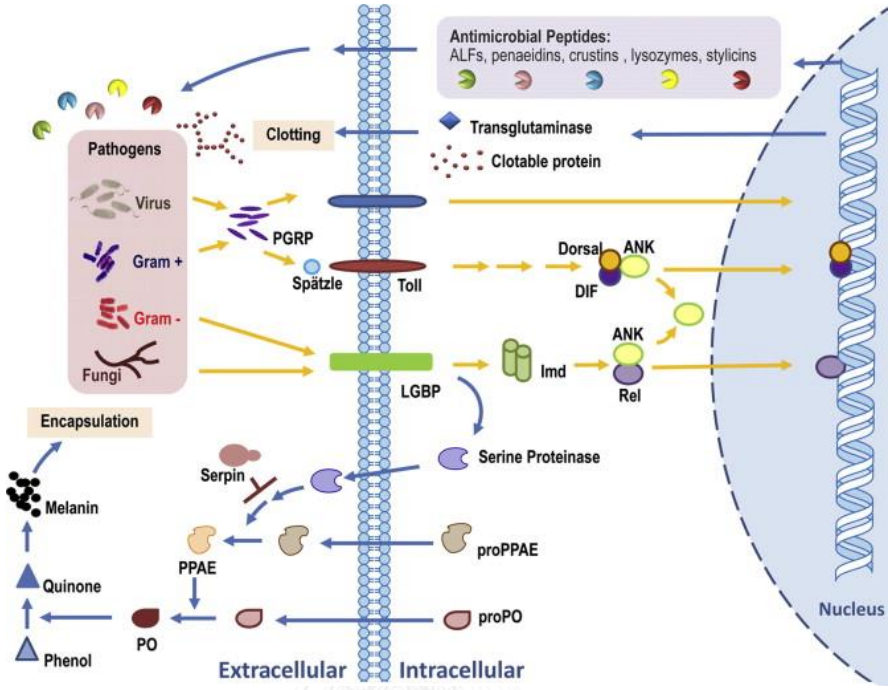


Figure 1.10 A schematic model of the shrimp immune system (Tassanakajon et al., 2013).

1.4.1 The prophenoloxidase-activating system

A major innate immunity in invertebrates is melanization, which is mediated by the prophenoloxidase (proPO)-activating system. Melanization protects the hosts from pathogenic infections and is essential for the clearance of various microbial pathogens (Amparyup et al., 2013a; Cerenius and Söderhäll, 2004; Kanost and Gorman, 2008). Phenoloxidase (PO), the key enzyme of the proPO-system, catalyzes the non-enzymatic conversion of phenolic substances to quinones, leading to the production of cytotoxic intermediates and melanin. Melanin encapsulates and kills pathogens (Christensen et al., 2005; Nappi et al., 1991). However, the melanization reaction needs to be tightly controlled; otherwise, excessive quinone as well as other intermediates produced during the procession of the proPO-system would be harmful to the host cells (Cerenius et al., 2008; Nappi and Christensen, 2005) (Figure 1.11).

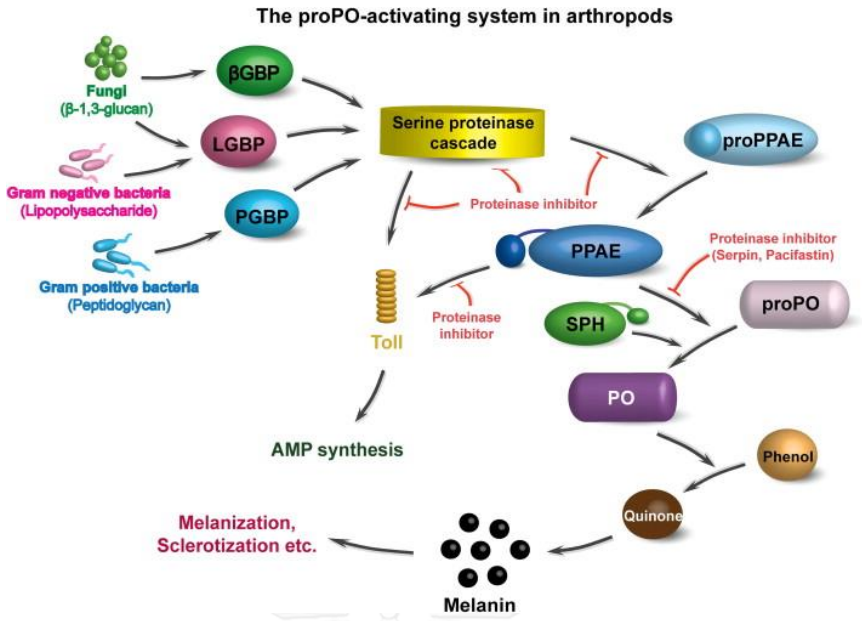


Figure 1.11 The prophenoloxidase-activating system in arthropods (Amparyup et al., 2013a)

Melanization is initially triggered by the pattern-recognition proteins (PRPs) that recognize molecular patterns found in microbial pathogens, known as pathogen-associated molecular patterns (PAMPs), leading to the activation of the clip domain serine proteinase (ClipSP) cascade. The terminal ClipSP, named the proPO-activating enzyme (PPAE), cleaves and activates proPO into active PO, which then oxidizes the phenolic substrates, resulting in melanin formation (Amparyup et al., 2013a; Cerenius et al., 2008; Söderhäll et al., 2013). Previously, several proPO-system components were identified and characterized in *Penaeus monodon*, including two proPOs (*Pm*proPO1 and *Pm*proPO2), two PPAEs (*Pm*PPAE1 and *Pm*PPAE2) and a few ClipSPs (see a review by Amparyup et al., 2013a). The *P. monodon* LPS and β-1,3-glucan binding protein (*Pm*LGBP) has been characterized as a PRP for LPS and β-1,3-glucan in the activation of the proPO-system (Amparyup et al., 2012). Moreover, the proPO-system has been shown to be an essential shrimp immune response against highly pathogenic microorganisms, the bacterium *Vibrio harveyi*, fungus *Fusarium solani* as well as the white spot syndrome virus (WSSV) (Amparyup et al., 2009; Charoensapsri et al., 2009, 2011; Sutthangkul et al., 2015).

1.4.1.1 Pattern Recognition Proteins (PRPs)

The PRPs recognizes the cell wall of pathogens and act as an initial step for the activation of the proPO cascade such as LPS, PGN and β-1,3glucan. The many types of PRPs in the proPO-system have been reported such as peptidoglycan recognition proteins (PGRPs) (Charroux et al., 2009; Kanost et al., 2004; Sumathipala and Jiang, 2010; Yoshida et al., 1996), C-type lectins (found to activate proPO in the cockroach hemolymph and enhance the laminarin-stimulated proPO-system activation) (Chen et al., 1995; Yu and Kanost, 2004; Yu et al., 2006), β-glucan-binding proteins (bGBPs) (firstly discovered 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 β-1,3-glucan binding proteins (LGBPs) (bound to LPS or β-1,3-glucan and activate the proPO-system) (Amparyup et al., 2012; Beschin et al., 1998; Lee et al., 2000). In *P. monodon*, *Pm*LGBP, a PRP involved in the shrimp proPO-system mediated melanization, reveals LPS and β-1,3-glucan binding activities, and initiate the proPO-system (Amparyup et al., 2012).

1.4.1.2 Clip domain serine proteinases (ClipSPs)

ClipSPs, found in the hemolymph of insects and crustaceans, are composed of one or more amino-terminal clip domains followed by a linker sequence and a carboxyl-terminal serine protease (SP) domain. 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; Kanost and Jiang, 2015; Veillard et al., 2016). ClipSPs can be categorized into catalytic SPs and non-catalytic SPs. The catalytic ClipSPs, a group of proteolytic enzymes, and the non-catalytic SPs, referred to as ClipSP homologues (ClipSPHs), are similar to an amino acid sequence but the serine residue in the active catalytic triads of ClipSPHs is replaced by glycine (Cerenius and Söderhäll, 2004).

Microbial infections stimulate the activation of zymogens of ClipSPs in hemolymph by specific cleavage at the amino terminus of the protease domain, creating an active enzyme, in which the clip domain and SP domain remain connected

by a disulfide bond (Figure 1.14). Several ClipSPs have been shown to participate in the proPO activation and Toll signaling pathway in insects and crustaceans (Amparyup et al., 2012; Gorman et al., 2007; Jang et al., 2006).

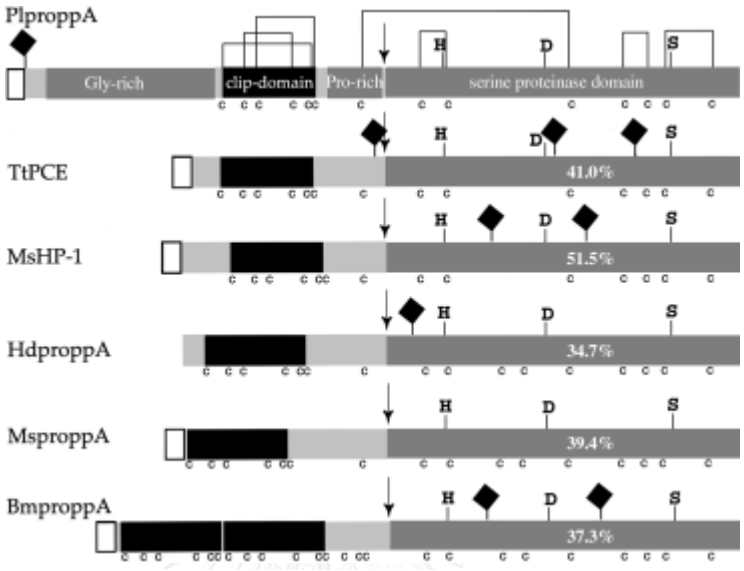


Figure 1.12 The structural feature of the crayfish proppA, insect proppAs and two similar serine proteinases. (Wang et al., 2001).

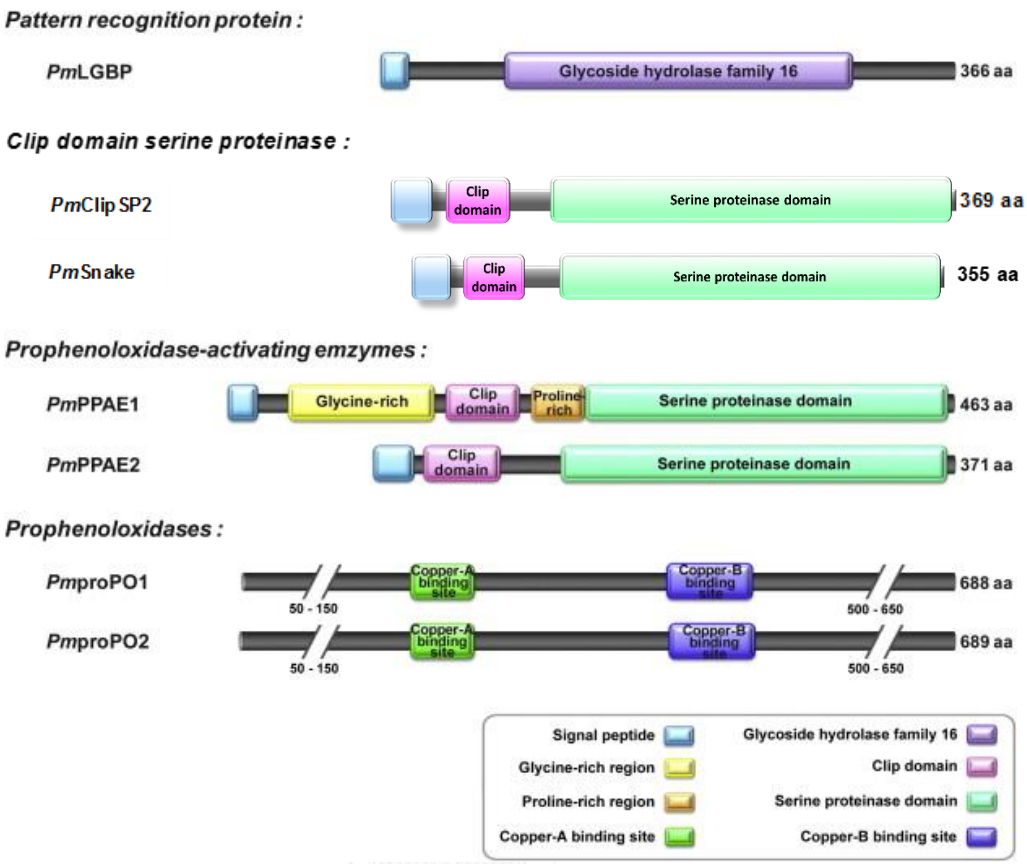


Figure 1.13 The structure of proteins in proPO-system of *P. monodon* (modified from Amparyup et al. 2013a)

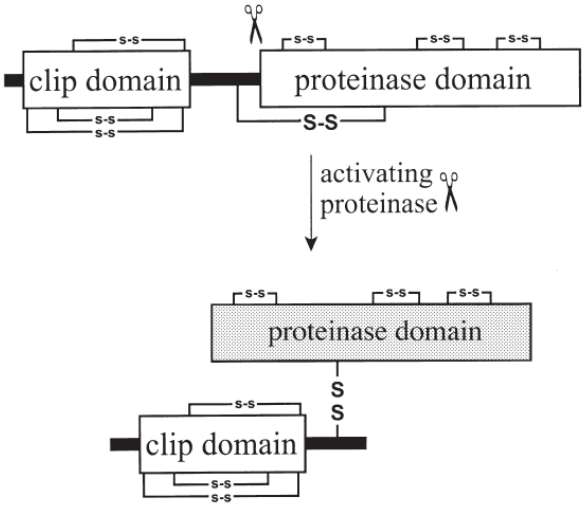


Figure 1.14 Domain organization of ClipSPs. ClipSPs contain the N-terminal clip domain and C-terminal proteinase domain linked by disulfide bridge as a zymogen or

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).

Serine proteinase (SP) cascades plays important roles in unique biological processes, along with response to infection of pathogens. The horseshoe crabs' (*Tachypleus tridentatus* and *Limulus polyphemus*) coagulation system, the most known function of SP cascades in arthropods (Theopold et al., 2004), has two ClipSPs (factor B and a proclotting enzyme) and two other proteinases (factors C and G) that involved in the proteolytic cascade and leads to the formation of a physical barrier at the site of infection. In this case, the SP zymogen factors C and G also independently act as PRPs for the bacterial LPS and fungal β-1,3-glucan (Ariki et al., 2004; Takaki et al., 2002). In *Drosophila*, the melanization protease-1 (MP1) and MP2, clipSPs, of SP cascade are involved in activation of the proPO cascade (Castillejo-López and Häcker, 2005; Tang et al., 2006). In *Tenebrio molitor*, three SPs (modular serine proteinase (MSP), spätzle-processing enzyme- activating enzyme (SAE) and spätzle-processing enzyme (SPE)) are the key in the initiation of the proPO-system, which then finally cleaves SP homologue 1 (SPH1) and proPO to produce a stable melanization complex for melanization (Kan et al., 2008; Kim et al., 2008; Park et al., 2007). In *Manduca sexta*, two SP cascades are involved in activation of the proPO pathways. The first contains of the hemolymph proteinase 14 (HP14), HP21, proPO-activating proteinase 2 (PAP2) and PAP3 (Gorman et al., 2007), while the second cascade is involved in the activation of two SPs (HP6 and PAP1) (An et al., 2009). In addition to the activation of the SP cascade, zymogen HP14 also serves as a PRP in pathogen recognition. Autocatalysis of HP14 is induced either by forming a complex with β-1,3-glucans and β-1,3-glucan recognition protein-2 (βGRP2) or by binding directly to bacterial PGNs without another binding protein (Ji et al., 2004; Wang and Jiang, 2006). In crustaceans, several ClipSPs have been reported the functions in the activation of the proPO-system, including one PPAE from the freshwater crayfish *Pacifastacus leniusculus* (Wang et al., 2001), two PPAEs from the black tiger shrimp *Penaeus monodon* (Charoensapsri et al., 2009, 2011), and one PPAE from the white shrimp *Litopenaeus vannamei* (Jang et al., 2011).

Moreover, the mannose-binding lectin (*Pl*-MBL), a C-type lectin, from *P. leniusculus* has been shown to act as a PRP for LPS and also to function as a scavenger for LPS and stop LPS spreading in the hemolymph and avoid spatially (systemic) or temporally extended activation of the proPO-system (Wu et al., 2013).

In the shrimp *P. monodon*, two ClipSPs, *Pm*PPAE1 and *Pm*PPAE2, are involved in the proPO-system and likely function as terminal SPs that convert proPO to PO (Amparyup et al., 2009; Charoensapsri et al., 2009, 2011; Sutthangkul et al., 2017). ClipSPHs, named *Pm*ClipSP1, *Pm*MasSPH1 and *Pm*MasSPH2, have been identified as a component of the shrimp antibacterial defense system. Moreover, the *Pm*MasSPH1 also shows a specific interaction with the *Pm*PPAE and may also be involved in the cross-talk regulation of the melanization cascade and antimicrobial peptide synthesis in shrimp (Amparyup et al., 2010; Jearaphunt et al., 2015) (Figure 1.16). On the other hand, *Pm*ClipSP2 and *Pm*Snake have been demonstrated to be the crucial serine proteinases in the activation of the proPO-system (Amparyup et al., 2013b; Monwan et al., 2017).

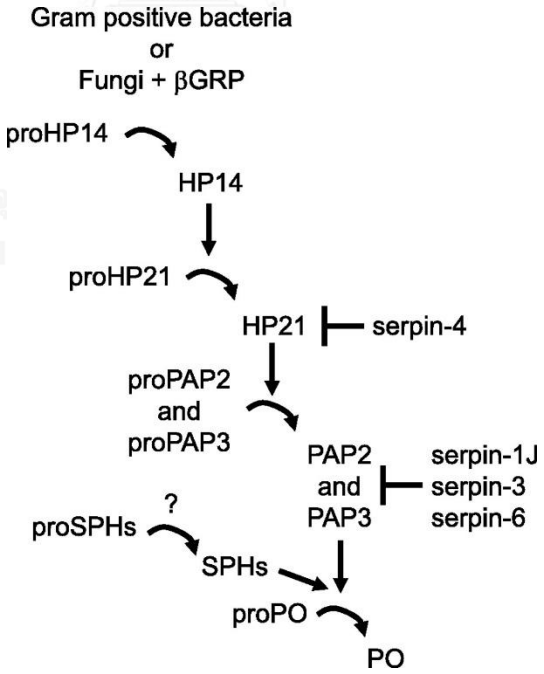


Figure 1.15 The proPO-activating system in *M. sexta.* (Gorman et al., 2007).

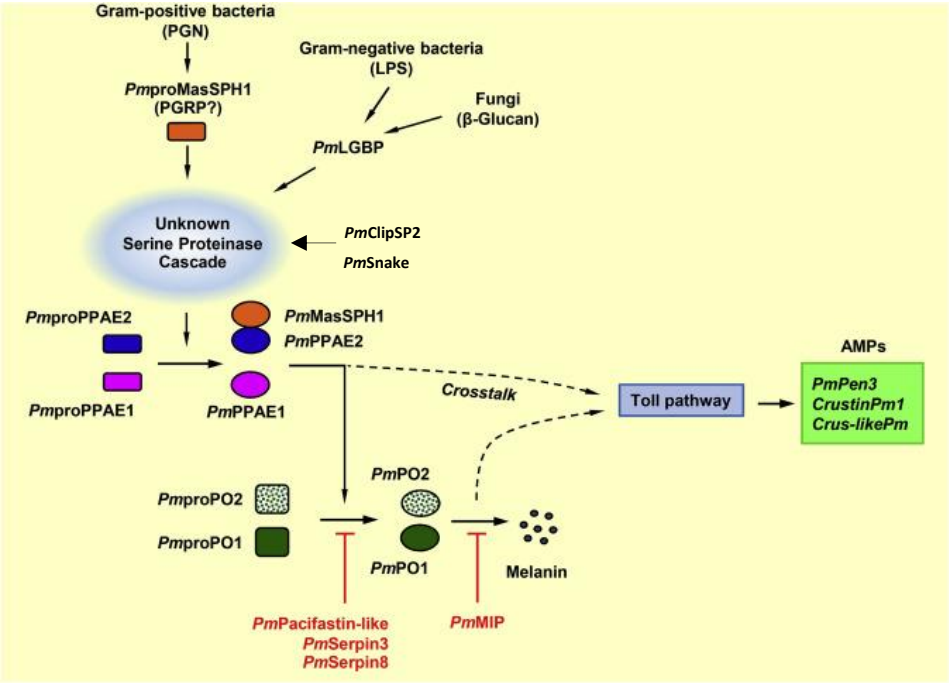


Figure 1.16 Activation of the proPO cascade and crosstalk with antimicrobial peptide (AMP) gene synthesis pathway in shrimp *Penaeus monodon*. (modified from Tassanakajon et al., 2017).

1.5 The pattern sequence in S1 family protease

From the previous study, the C-terminals of human thrombin, and other related coagulation factors, comply with a pattern sequence X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X (Kasetty et al., 2011) found not only in these proteases of the coagulation system, but also present in the various S1 family proteases. The C-terminal sequence contains conserved hydrophobic residues, which given an α-helical conformation (characterizing all structurally determined S1 peptidases). The wide range of C-terminal peptide sequences of serine proteases, mainly from the coagulation and kallikrein systems, share characteristics common antimicrobial peptides (Figure 1.17) and inhibit macrophage response LPS. In mice, selected peptides are defensive against lipopolysaccharide-induced shock. Moreover, these S1-derived host defense peptides exhibit helical structures upon binding to lipopolysaccharide and also permeabilize liposomes (Kasetty et al., 2011). Surprisingly, we found that the C-terminal SP domain

of *Pm*ClipSP2 matches with the pattern sequence (FPGVYTSVSHYRSWVE; except for the amino acid change from I/L to V (underlined)) (Amparyup et al., 2013b). This region may be responsible for binding property of *Pm*ClipSP2.

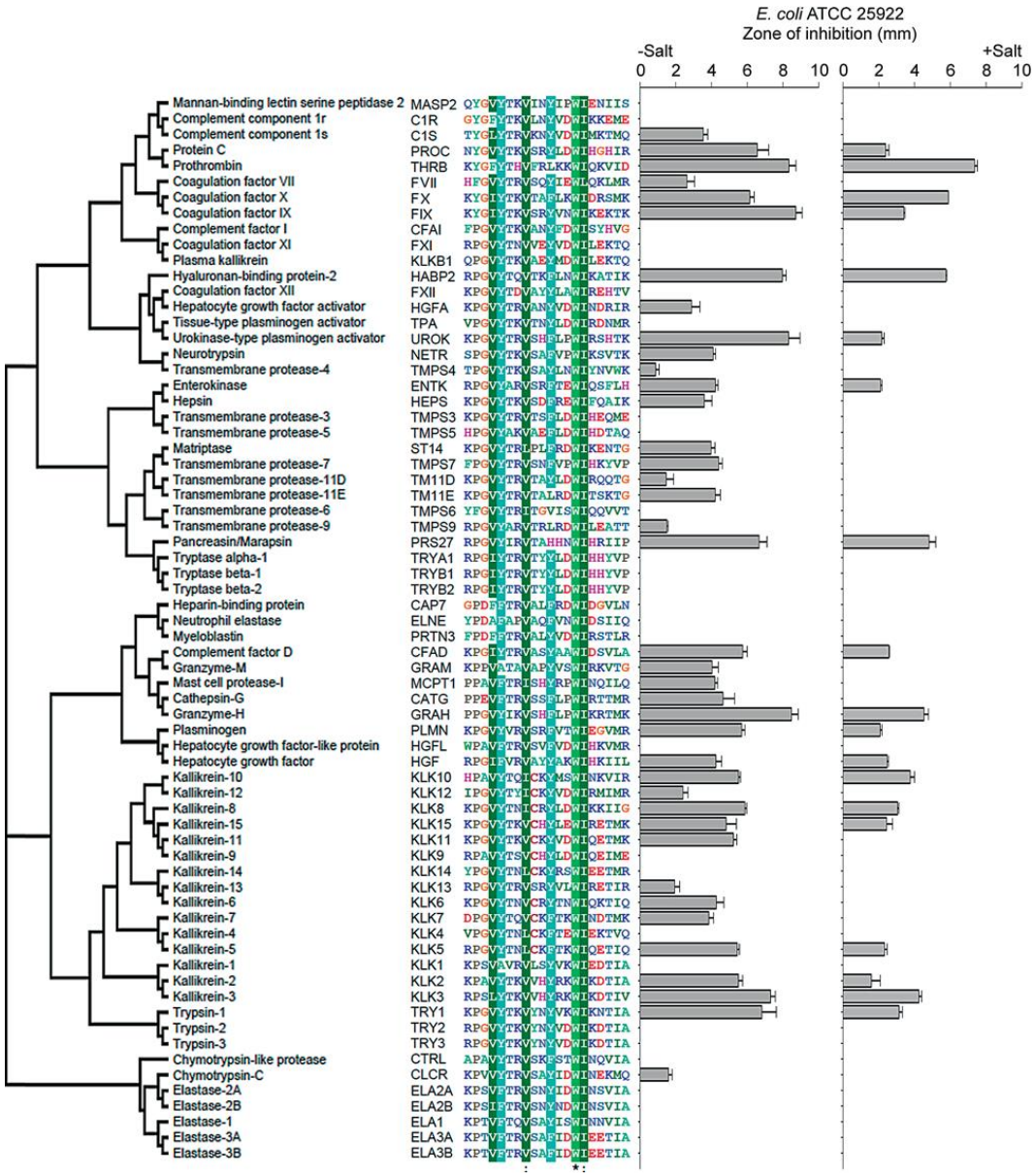


Figure 1.17 Activities of S1-derived peptides comprising the pattern sequence (X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X). (Kasetty et al., 2011).

1.6 The objective of research

From our previous study, a recombinant protein of *Pm*ClipSP2 binds to both immobilized LPS and laminarin (β-1,3-glucan) and activates the proPO-system, suggesting that *Pm*ClipSP2 might act as a pattern-recognition protein. Moreover, *Pm*ClipSP2-knockdown shrimp had significantly increased mortality rate in LPS and/or β-1,3-glucan injection and decreased in the number of total hemocytes, which suggested that *Pm*ClipSP2 may play essential role in the hemocyte homeostasis by scavenging LPS and neutralizing its toxicity (Amparyup et al., 2013b).

Here, we further investigated the binding ability of *Pm*ClipSP2 to LPS and β-1,3-glucan to determine which domain is responsible for the binding. Recombinant proteins of each domain, as well as those containing specific regions were expressed and investigated for their binding to PAMPs and the ability to activate the shrimp proPO-system. Moreover, *Pm*ClipSP2 was constructed with specific mutations at cleavage site, which can produce active form of *Pm*ClipSP2, to study activation of other proteins in the proPO-system. Finally, LPS neutralizing property of *Pm*ClipSP2 was examined.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipment and Chemicals

2.1.1 Equipment

-20°C Refrigerator Freezer (SHARP), -80°C Freezer (Thermo Forma)

24-well plate Costar® (Corning Incorporation)

96-well plate Costar® (Corning Incorporation)

Amicon Ultra-15 Centrifugal Filter Unit with Utracel-3-membrane, 10-membrane (Millipore, MERCK)

Automatic micropipette (Gilson Medical Electrical S.A.)

Balance AB204-5 (METTLER TOLEDO)

Balance PB303-S (METTLER TOLEDO)

Bright-Line™ Hemacytometer (SIGMA)

Cell culture flask, plug cap, 25 ml (SPL Life Sciences)

Centrifuge tube CentriStarTM 15 ml, 50 ml (Corning Incorporation)

Dry Bath Incubator (BOEKEL)

FiveEasy™ pH meter (METTLER TOLEDO)

Gel documentation (SYNGENE)

Gene Pulser (BIO-RAD)

Hybridization oven (Hybrid)

IKA mini G (IKA Works)

i-MyRun Electrophoresis Products (Cosmo Bio USA)

Incubator (Memmert)

Innova 4000 incubator shaker (New Brunswick Scientific)

Insulin syringes U 100 (Becton, Dickinson and Company)

Labo Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets (NuAire, Inc.)

Microcentrifuge tube (Axygen Scientific)

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

Mini-PROTEAN® Glass Plates (BIO-RAD)

Mini-PROTEAN® Short Plates (BIO-RAD)

Mini-PROTEAN® Tetra System (BIO-RAD)

NanoDrop 2000c (Thermo Scientific)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

Laboratory film (PARAFILM)

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

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

PD-10 Column (GE Healthcare)

Pipetman ClassicTM (Gilson Incorporation)

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

Power supply, Power PAC 3000 (BIO-RAD)

Semi-dry Trans-Blot® (BIO-RAD)

Sorvall™ Legend™ Micro 21R Centrifuge (Thermo Scientific)

SpectraMax M5 Multi-Mode Microplate Readers (Molecular Devices)

Sterring hot plate (Fisher Scientific)

Stuart See-saw rockers SSL4 (Stuart Equipment)

Touch mixer Model # 232 (Fisher Scientific)

Ultra Sonicator (SONICS Vibracell)

Universal 320R (Hettich Zentrifugen)

Water bath (Memmert)

2.1.2 Chemicals, Reagents and Biological substance

1 kb GeneRuler™ (Thermo Scientific)

100 bp PlusGeneRuler™ (Thermo Scientific)

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

2-Mercaptoethanol (Fluka)

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

6x-His Tag Monoclonal Antibody (Thermo Scientific)

Absolute ethanol, CH_3CH_2OH (HAYMAN)

Acrylamide/Bis Solution (BIO-RAD)

Agar powder (HIMEDIA)

Agarose (Research organics)

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

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

Ammonium persulfate (USB)

Amplicillin (BioBasic)

Anti-His antiserum (GE Helthcare)

Boric acid (MERCK)

Bovine serum albumin (BSA) (SIGMA)

Bromophenol blue sodium salt (USB)

Calcium chloride (MERCK)

Cellfectin® Reagent (Invitrogen)

Chloramphenicol (SIGMA)

Coomassie brilliant blue G-250, R-250 (BIO BASIC INC.)

Di-Sodium hydrogen orthophosphate anhydrous (Carlo Erba)

Dithiothereitol (DTT) (BIO BASIC INC.)

Ethanol (MERCK)

Ethylene diamine tetraacetic acid disodium salt dehydrate (EDTA) (Ajax Finechem)

Glacial acetic acid (MERCK)

Glycerol (Scharlau)

Glycine (Scharlau)

Hydrochloric acid fuming 37% (MERCK)

Imidazole (Fluka)

Isopropyl-β-D-thiogalactoside (IPTG) (Thermo Scientific)

Kanamycin (BIO BASIC Inc.)

L-3,4-dihydroxyphenylalanine (L-DOPA) (SIGMA)

Laminarin from *Laminaria digitata* (β-1,3-glucan) (L9634) (SIGMA)

Lipopolysaccharide (LPS) of *E.coli* serotype 0111:B4 (L4130) (SIGMA)

Magnesium chloride (MERCK)

Methanol (MERCK)

N, N, N, N-Tetramethylethylenediamine (TEMED) (BDH)

N, N-dimethyl formamide (Carlo Erba)

N, N-methylenebisacrylamide (Fluka)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nickle sulphate (KEMAUS)

Nitrobluetetrazolium (NBT) (Fermentas)

Normal saline (0.9% NaCl) (Klean & Kare)

pET28b(+) vector (Novagen)

pET32a(+) vector (Novagen)

pIZT/V5-His vector (Invitrogen)

Potassium chloride, KCl (Ajax Finechem)

Potassium dihydrogen orthophosphate (Ajax Finechem)

Prestained protein molecular weight marker (Fermentas)

RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology)

SFX-Insect Serum-Free Insect Cell Culture Medium (HyClone™)

Skim milk powder (HIMEDIA)

Sodium acetate (Carlo Erba)

Sodium cacodylate trihydrate (SIGMA)

Sodium chloride (Ajax Finechem)

Sodium citrate (Carlo Erba)

Sodium dihydrogen orthophosphate (Carlo Erba)

Sodium dodecyl sulfate (Vivantis)

Sodium hydroxide (MERCK)

Tris (Vivantis)

Triton® X-100 (MERCK)

Trypan blue (Millipore, MERCK)

Tryptic soy broth (HACH®)

Tryptone type I (HIMEDIA)

Tween™ 20 (Ajax Finechem)

Urea (Ajax Finechem)

V5 Tag Antibody (Invitrogen)

Yeast extract powder (HIMEDIA)

Zeocin™ (Invitrogen)

2.1.3 Enzymes and Kits

AccuPower® ProFi Taq PCR PreMix (Bioneer)

Advantage® 2 Polymerase Mix (Clontech)

AP Substrate Kit (BIO-RAD)

DNase I (RNase-free) (Biolabs® Inc.)

Expin™ Combo GP (GeneAll®)

FavorPrep™ Plasmid Extraction Mini Kit (FAVORGEN BIOTECH CORP.)

*Nco*I (New England Biolabs® Inc.)

*Not*I (New England Biolabs® Inc.)

PCR-Select™ cDNA Subtraction Kit (Clontech)

RBC T&A Cloning Vector (RBC Bioscience)

T4 DNA ligase (New England Biolabs® Inc.)

Taq DNA polymerase (RBC Bioscience)

*Xba*I (New England Biolabs® Inc.)

*Xho*I (New England Biolabs® Inc.)

2.1.4 Microorganisms

Drosophila S2 cells (Invitrogen)

Escherichia coli strain 363

Escherichia coli strain JM109

Escherichia coli strain Rosetta (DE3)

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/)

EMBOSS Pairwise Alignment (http://www.ebi.ac.uk/Tools/emboss/align/)

GENETYX 7.0.3 program (GENETYX Corporation)

GraphPad Prism 6 (GraphPad Software, Inc.)

GeneSnap (SYNGENE)

NanoDrop 2000 (Thermo Scientific)

Primer Premier 5 Software (Premier Biosoft)

ScanProsite ExPASy (http://prosite.expasy.org/scanprosite)

SECentral program (Scientific & Education Software)

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

2.2 Shrimp samples

Healthy black tiger shrimp, *Penaeus monodon*, of about 2-5 and 8-10 g bodyweight were obtained from the Marine Shrimp Broodstock Research Center II (MSBRC-2), Charoen Pokphand Foods PCL in Phetchaburi province, Thailand. Shrimp were maintained in tanks with aerated water with a salinity of 20 ppt for at least 7 days before use. Shrimp were fed 1 time/day in close container and for LPS injection and hemocyte collection.

2.3 Sequence analysis

Nucleotide and amino acid sequences of *Pm*ClipSP2 obtained from GenBank database (GenBank: FJ620687 and ACP19561.1) were analyzed with NCBI Conserved Domain Search. The pattern sequence was searched by ScanProsite with modified motif (X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL<u>V</u>]-X, underlined is changed from original).

2.4 Primer design

All primers were designed base on nucleotide sequence of *Pm*ClipSP2 cDNA (GenBank: FJ620687) 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 (Table 2.1).

Table 2.1 Nucleotide sequence of the primers

Primer	Sequence (5'-3')	
SP-*Pm*ClipSP2 DLT-F	CATGCCATGGGAAGAGTGGTAGGAGGCGAAGA	Used for recombinant proteins expression
SP-*Pm*ClipSP2 DLT-R	CCGCTCGAGTCCTTTCTGGCCGCAACTCGT	
PT-*Pm*ClipSP2-F	CATGCCATGGGATTCCCTGGCGTCTATACCG	
PT-*Pm*ClipSP2-R	CCGCTCGAGCTCGACCCAGGACCTATAAT	
S2-*Pm*ClipSP2-F	ATAAGAATGCGGCCGCGCCATGGCCAACAAACAGAGACCA AGCACA	
S2-*Pm*ClipSP2-R	GCTCTAGACCCGGCCTAAGAGTTTCCTC	
S2-*Pm*ClipSP2$_{Xa}$-F	GACGCCATAGAAGGGCGGGCAGTTCTGGGATATCGAATT	
S2-*Pm*ClipSP2$_{Xa}$-R	AACTGCCCGCCCTTCTATGGCGTCGATTGGGGCGT	
OpIE2-F	CGCAACGATCTGGTAAACAC	
OpIE2-R	GACAATACAAACTAAGATTTAGTCAG	

2.5 Expression and purification of recombinant proteins

2.5.1 Amplification of recombinant SP-*Pm*ClipSP2 DLT, PT-*Pm*ClipSP2, S2-*Pm*ClipSP2 and S2-*Pm*ClipSP2$_{Xa}$

Gene specific primers (name SP-*Pm*ClipSP2 DLT-F/ SP-*Pm*ClipSP2 DLT-R, PT-*Pm*ClipSP2-F/ PT-*Pm*ClipSP2-R, S2-*Pm*ClipSP2-F/S2-*Pm*ClipSP2-R and S2-*Pm*ClipSP2$_{Xa}$-F/ S2-*Pm*ClipSP2$_{Xa}$-R) (Table 2.1) with restriction enzyme site (*Nco*I and *Xho*I for SP-*Pm*ClipSP2 DLT and PT-*Pm*ClipSP2, *Not*I and *Xba*I for S2-*Pm*ClipSP2 and S2-*Pm*ClipSP2$_{Xa}$) were designed from nucleotide sequence of *P. monodon* to amplify the rSP-*Pm*ClipSP2 DLT (SP domain of *Pm*ClipSP2 with deletion of the pattern sequence (349_369delFPGVYTSVSHYRSWVEETLRP; underlined), rPT-*Pm*ClipSP2 (the pattern sequence of *Pm*ClipSP2), S2-*Pm*ClipSP2 (*Pm*ClipSP2 gene for S2 cells host system) and S2-*Pm*ClipSP2$_{Xa}$ (*Pm*ClipSP2 gene for S2 cells host system with mutation the cleavage site for Factor Xa). The PCR conditions of 20 µl amplification reaction were consisted of 1 tube of AccuPower® ProFi Taq PCR PreMix (Bioneer) per reaction, 1 µM each primer and 2 µl pET-28b(+)-r*Pm*ClipSP2 (for SP-*Pm*ClipSP2 DLT and PT-*Pm*ClipSP2) and cDNA from normal shrimp sample (for S2-*Pm*ClipSP2 and S2-*Pm*ClipSP2$_{Xa}$). The PCR thermal cycling conditions for SP-*Pm*ClipSP2 DLT were 94°C for 1 min, 35 cycles of 94°C for 1 min, 55°C for 30 sec and 70°C for 1 min, and then a final extension at 72°C 10 min; PT-*Pm*ClipSP2 were 94°C for 1 min, 30 cycles of 94°C for 1 min, 50°C for 30 sec and 72°C for 30 sec, and then a final extension at 72°C 10 min; S2-*Pm*ClipSP2 were 94°C for 1 min, 35 cycles of 94°C for 30 sec, 50°C for 1 min and 68°C for 1 min, and then a final extension at 72°C 10 min; the first step of S2-*Pm*ClipSP2$_{Xa}$ were 94°C for 1 min, 35 cycles of 94°C for 30 sec, 50°C for 15 sec and 68°C for 50 sec, and then a final extension at 72°C 10 min; and the second step of S2-*Pm*ClipSP2$_{Xa}$ were 94°C for 1 min, 35 cycles of 94°C for 1 min, 55°C for 20 sec and 68°C for 1.5 min, and then a final extension at 72°C 10 min.

2.5.2 Agarose gel electrophoresis

The PCR products were analyzed by using either 1.2% or 2% agarose gel electrophoresis. Agarose powder was dissolved in 1X TBE buffer (Tris-HCl, Boric acid,

EDTA), boiled the solution. After that, RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology) was added (1:20,000). 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 loaded on each well. The size was determined by comparing with DNA Marker (100 bp or 1 kb). Electrophoresis was performed at 100-135 mV 30 min and DNA fragment was detected by UV transilluminator and photographed.

2.5.3 Purification PCR product from agarose gel

The expected bands were cut from agarose gel and DNA was purified using Expin™ Combo GP (GeneAll®). Briefly, the 3 volume (µl) times of gel weight (mg) of GB Buffer was added to dissolved gel and melted at 55°C for 10 min or until gel completely dissolved. The solution was transferred into Expin™ column and then centrifuged at 12,000 rpm for 1 min room temperature. The supernatant was removed, washed by adding 700 µl of NW buffer and centrifuged at 12,000 rpm for 1 min 2 times to remove ethanol from the column. The ultrapure water (40 µl) was used as elution buffer and centrifuged at 12,000 rpm for 2 min, then stored at -20°C.

2.5.4 Construction of S2-*Pm*ClipSP2 and S2-*Pm*ClipSP2$_{Xa}$ to T&A vector

The purified PCR was ligated into T&A cloning vector (RBC Bioscience) (Figure2.1). The ligation product was transformed into *E. coli* strain JM109, the positive colonies were confirmed by colony PCR and digested with *Not*I/*Xba*I restriction enzyme. Recombinant plasmid was extracted by FavorPrep™ Plasmid Extraction Mini Kit (FAVORGEN BIOTECH CORP.).

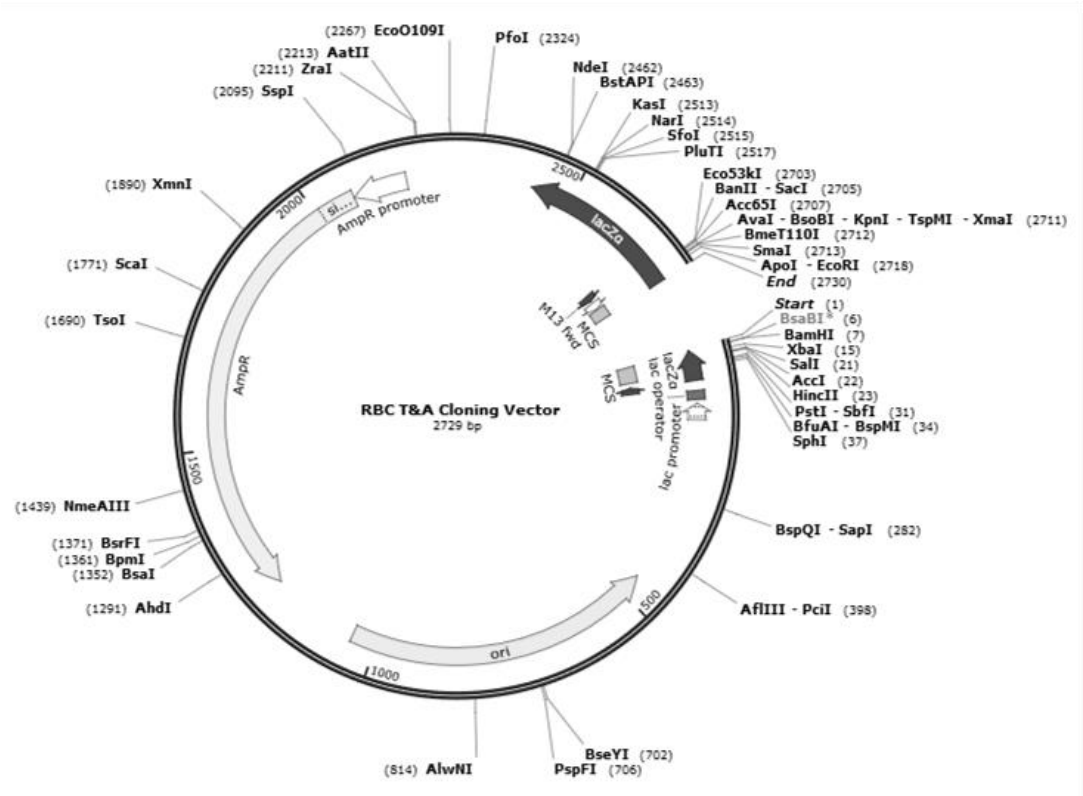


Figure 2.1 The map of RBC TA cloning vector (www.snapgene.com)

2.5.5 Expression and purification of recombinant proteins in *E. coli* system

After the correct sequences were confirmed, the SP-*Pm*ClipSP2 DLT, PT-*Pm*ClipSP2, pET-28b(+) vector and pET-32a(+) vector (Novagen) (Figure 2.2 and 2.3) were digested with *Nco*I and *Xho*I. SP-*Pm*ClipSP2 DLT was cloned into the pET-28b(+) vector and PT-*Pm*ClipSP2 was cloned into the pET-32a(+) vector The fragments were ligated by using T4 DNA ligase (New England Biolabs® Inc.) and incubated at 16 °C overnight. The ligation mixture was transformed into *E. coli* JM109 and confirmed by nucleotide sequencing (Bioneer, Korea). The recombinant plasmid pET-28b(+)-SP-*Pm*ClipSP2 DLT and pET-32a(+)-PT-*Pm*ClipSP2 were transformed into *E. coli* Rosetta (DE3)-pLysS cells (Novagen) by heat shock method. The recombinant clones (*Pm*ClipSP2, Clip-*Pm*ClipSP2, SP-*Pm*ClipSP2, SP-*Pm*ClipSP2 DLT, PT-*Pm*ClipSP2 and pET-32a(+)) were 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 7,000 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 recombinant proteins was analyzed by 12.5% SDS-PAGE and strained with coomassie brilliant blue.

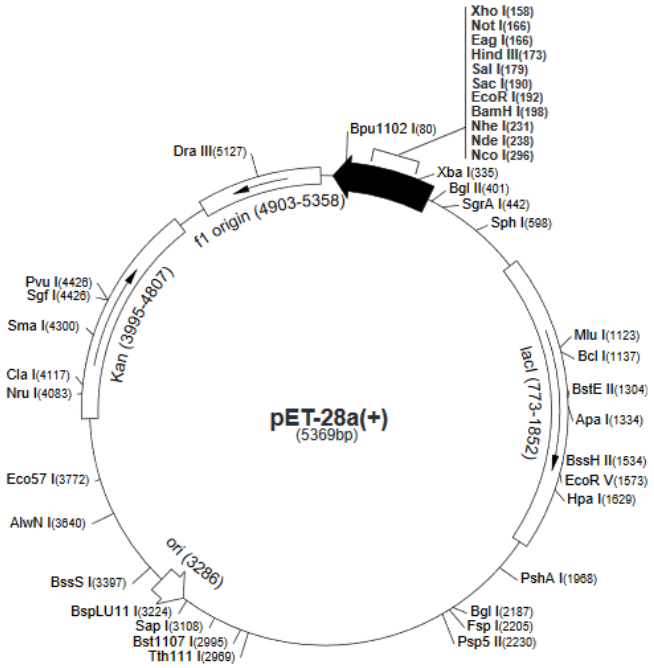


Figure 2.2 The map of pET-28b(+) expression vector. (Novagen)

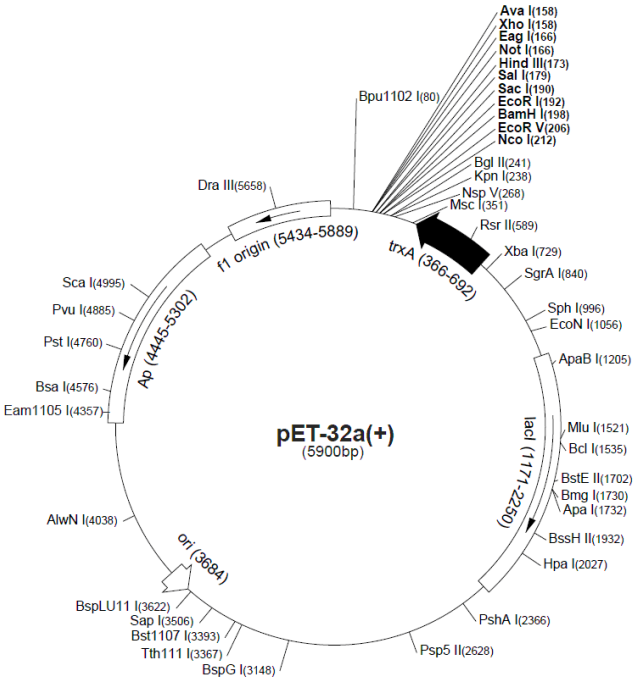


Figure 2.3 The map of pET-32a(+) expression vector. (Novagen)

The inclusion bodies of recombinant proteins were solubilized with 8 M Urea for completely dissolved before purification but the soluble fractions of recombinant proteins were not added 8 M Urea. The recombinant proteins were purified by using Ni-NTA affinity chromatography (Ni Sepharose 6 Fast Flow (GE Healthcare)). The crude recombinant proteins were pre-incubated with Ni-NTA bead and collected flow through. After that, Ni-NTA bead was washed with 4 ml lysis buffer (50 mM NaH_2PO_4 pH 8.0, 10 mM Immidazole, 8 M Urea and 0.3 M NaCl) 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 50 mM NaH_2PO_4 pH 8.0, 250 mM Immidazole, 8 M Urea and 0.3 M NaCl, for soluble fractions same buffer were used except 8 M Urea. The purified recombinant proteins were analyzed by 12.5% SDS-PAGE and coomassie brilliant blue for staining. The fractions of purified recombinant proteins were dialyzed with 20 mM Tris-HCl pH 8.0 and concentrated with Amicon Ultra-15 Centrifugal Filter Unit (Millipore, MERCK) with Utracel-3-membrane for rClip-*Pm*ClipSP2 and 10-membrane for r*Pm*ClipSP2, rSP-*Pm*ClipSP2, rSP-*Pm*ClipSP2 DLT, rPT-*Pm*ClipSP2-TRX and rTRX. The concentration of recombinant proteins was measured by Bradford assay.

2.5.6 Expression of recombinant proteins in *Drosophila* S2 cells system

S2-*Pm*ClipSP2, S2-*Pm*ClipSP2$_{Xa}$ and pIZT/V5-His vector (Invitrogen) (Figure 2.4) were digested with *Not*I and *Xba*I. The fragments were ligated with pIZT/V5-His vector and cut with the same restriction enzymes by using T4 ligase and incubation at 16 °C overnight. The ligation mixture was transformed into *E. coli* JM109 and confirmed by nucleotide sequencing (Bioneer, Korea).

Before transfection, recombinant plasmids (pIZT/V5-His-rS2-*Pm*ClipSP2 and pIZT/V5-His-rS2-*Pm*ClipSP2$_{Xa}$) were extracted by FavorPrep™ Plasmid Extraction Mini Kit (FAVORGEN BIOTECH CORP.). The concentration of recombinant plasmid was measured by using NanoDrop 2000c (Thermo Scientific). Then, recombinant plasmids (1 μg) were incubated with Cellfectin® Reagent (Invitrogen) (8 μl) and SFX-Insect Serum-Free Insect Cell Culture Medium (HyClone™) (500 μl) and shaken on Orbital shaker SO3 (Stuart Scientific, Great Britain) at room temperature for 30 min. After that transfection media mixture was replaced to old media of S2 cells, which were seeded 5×10^5 cells/well of 24-well plate Costar® (Corning Incorporation) in 500 μl media for overnight, and then shaken at room temperature for 7 hr. After incubation, transfection mixture was removed and then added new media (300 μl) and incubated at 27 °C for 48 hr. Finally, Cell and media were separately harvested, analyzed by SDS-PAGE and confirmed by Western blot with mouse anti-V5 tag antibody (Invitrogen).

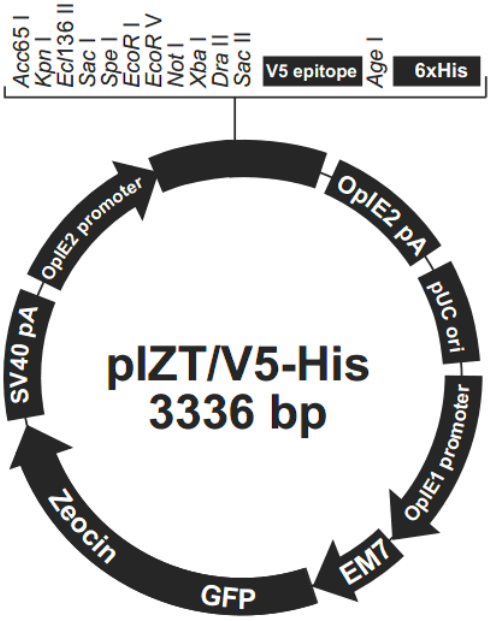


Figure 2.4 The map of pIZT/V5-His vector (www.lifetechnologies.com)

2.6 Binding assay of recombinant proteins

Enzyme-linked immunosorbent assay (ELISA) was performed to examine binding domain of r*Pm*ClipSP2. First, either lipopolysaccharide (LPS) or laminarin (β-1,3-glucan from *Laminaria digitata*) (2 µg/well) was coated onto each well of 96-well plate (Costar) and incubated at 37 °C overnight for drying plate. Then, plate was fixed at 60 °C for 1 hr. After that 5% bovine serum albumin (BSA) (SIGMA) was incubated for blocking non-specific binding site at room temperature for 3 hr. Next, phosphate buffer saline (PBS, pH 7.4) containing 0.1% (v/v) Tween 20 (PBST) was used as wash buffer 3 times. Various concentrations of recombinant proteins (0-10 µM) 100 µl were incubated onto each well at 4 °C overnight. After washing, the bound protein was immunochemically detected. 100 µl of a 1:3000 dilution of the mouse anti-His Tag monoclonal antibody (Thermo Scientific) (primary antibody) in PBS was incubated at room temperature for 3 hr, washed and then for 1 hr with 100 µl of a 1:5000 dilution of the alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) (secondary antibody) in PBS and washed. Finally, 50 µl of AP

Substrate Kit (p-nitrophenyl phosphate in diethanolamine buffer) was added and incubated for 30 min in the dark and then stopped the reaction by using 100 µl of 0.4 M NaOH. The alkaline phosphatase antibody-protein complex was measured at A_{405nm} with SpectraMax M5 Multi-Mode Microplate Readers (Molecular Devices).

2.7 Radial diffusion assay

The single colony of *E. coli* 363 (Gram-negative bacteria), *Staphylococcus aureus* (Gram-positive bacteria) and *Vibrio harveyi* (Gram-negative bacteria; pathogen of shrimp) from (3% w/v) Tryptic soy broth (HACH®) agar (3% TSA) were picked and diluted in normal saline (0.9% NaCl) (Klean & Kare) to $OD_{625\ nm}$ 0.0.8-0.1, and then they were swabbed into 6.5 ml 3% TSA (9-cm diameter culture plate) with punched 7-mm diameter wells. After air-dried, 35 µl of rPT-*Pm*ClipSP2-TRX (25, 50 and 100 µM), rTRX (50 and 100 µM), chloramphenicol (SIGMA) (2 µg) as positive control and 20 mM Tris-HCl pH 8.0 as negative control were added in each well. Plates were incubated at room temperature for 3 h to allow peptide diffusion. Antimicrobial activity of a peptide was visualized as a zone of clearing around each well after 18–24 h of incubation at 37 ° C.

2.8 PO activation assay of recombinant proteins

Hemocyte lysate supernatant (HLS) was prepared from hemocyte's normal shrimp. Hemolymph was withdrawn with 1:1 dilution with anti-coagulant (140 mM NaCl, 30 mM citric acid, 110 mM glucose, 30 mM Na-citrate, pH 5.6) and then collected hemocyte by centrifugation at 800 xg for 10 min and discarded plasma fraction. After that hemocyte was washed with CAC buffer (10 mM Na-cacodylate, 5 mM $CaCl_2$) 2 times. Then, hemocyted was homogenized in CAC buffer, centrifuged at 12,000 rpm for 15 min and collected supernatant fraction (HLS). The concentration of HLS was measured by Bradford assay.

The 80 µl reaction was pre-incubated for 30 min, which consisted of HLS (30 µg) recombinant proteins (10 µM) and with or without LPS or laminarin (20 µg). After

incubating, 20 µl of 3 mg/ml L-3,4-dihydroxyphenylalanine (L-DOPA) (SIGMA) was added and incubated at room temperature for 30 min in the dark, and then the absorbance at 490 nm was measured using a spectrophotometer. One unit of PO activity was defined as ΔA_{490nm}/mg total protein/min. Each experiment was performed in triplicate.

2.9 LPS neutralization activity of recombinant proteins

2.9.1 LPS neutralization of recombinant protein in normal shrimp

Normal shrimp (2-5 g) was injected with various concentration of LPS (0-80 µg/g shrimp) to find LC50 of LPS and observed mortality. Then LC50 of LPS injected shrimp with or without pre-incubating r*Pm*ClipSP2 for appropriate time point was collected hemocyte. Alive hemocyte was stained with trypan blue (Millipore, MERCK) and counted by hemacytometer (SIGMA). Each experiment was performed in 3 times.

2.9.2 LPS neutralization of recombinant protein in S2 cells

S2 cells (1.6×10^5 cells/well) were seeded (50 µl) in 96-well plate. The various dose of LPS (0-10 µg/well) was incubated in each well to determine LD50 that decrease live S2 cells in 48 hr. LD50 of LPS was incubated to S2 cells with or without 10 µM r*Pm*ClipSP2 or 50 µl of rS2-*Pm*ClipSP2 media. After 48 hr, S2 cells were collected and live cells were counted by hemacytometer. Each experiment was performed in triplicate.

CHAPTER III

RESULTS

3.1 Sequence analysis and conserved domains of *Pm*ClipSP2

Previously, a cDNA encoding an open reading frame (ORF) of clip domain serine proteinase 2 (*Pm*ClipSP2) gene was identified from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon et al., 2006). The complete cDNA sequence of *Pm*ClipSP2 (accession no. FJ620687) is obtained 1,317 bp and ORF encoding a polypeptide of 369 amino acid residues (ACP19561.1). The molecular mass of mature protein is 40.18 kDa with the predicted isoelectric point (pI) of 5.14.

Using the NCBI Conserved Domain Search, *Pm*ClipSP2 contains N-terminal clip domain (residue 32-78) and C-terminal serine proteinase domain (residue 105-366), which has predicted cleavage site at V105. Surprisingly, identification of sequences (349FPGVYTSVSHYRSWVE364; except for the amino acid change from I/L to V (underlined)) matchs to the pattern sequence (X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X (Kasetty et al., 2011)) that has been reported to function in the antibacterial and LPS binding activities in the C-terminal derived peptide from human thrombin and S1 family proteinase (Kasetty et al., 2011; Papareddy et al., 2010).

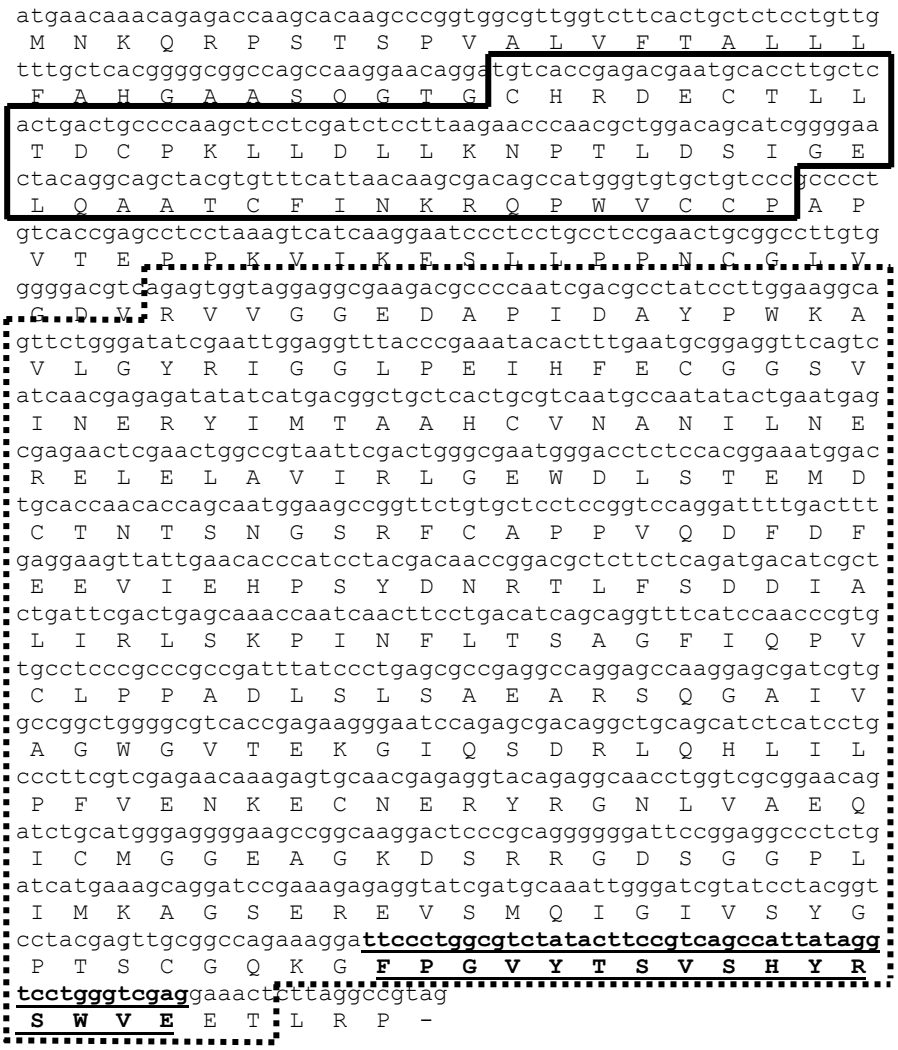


Figure 3.1 The nucleotide and deduced amino acid sequences of *Pm*ClipSP2. The N-terminal clip domain, black line box**;** SP domain, dashed line box; and bold and the pattern sequence , underlined.

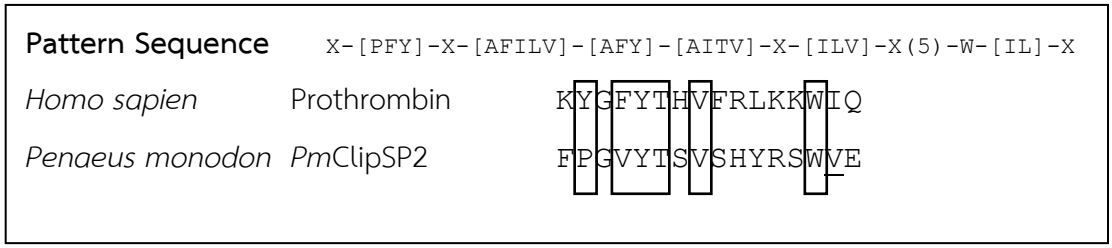


Figure 3.2 The comparation of the pattern sequence from human prothrombin and shrimp *Pm*ClipSP2. Match residues are shown with boxes and mismatch residue is undelined.

3.2 Construction, expression and purification of recombinant proteins in *E. coli* expression system

To further characterize the functional domains of *Pm*ClipSP2, recombinant (r)*Pm*ClipSP2 proteins were expressed in the *E. coli* system. Three clones (mature protein, r*Pm*ClipSP2; clip domain, rClip- *Pm*ClipSP2; and serine proteinase domain, rSP-*Pm*ClipSP2) were constructed by Amparyup et al. and the other two clones (serine proteinase domain with deletion of the pattern sequence (349FPGVYTSVSHYRSWVE364), rSP-*Pm*ClipSP2 DLT; the pattern sequence of *Pm*ClipSP2 fused with thioredoxin, rPT-*Pm*ClipSP2-TRX; and thioredoxin, rTRX) were constructed in this research, which has six histidine tag at C-terminus to facilitate purification and detection step.

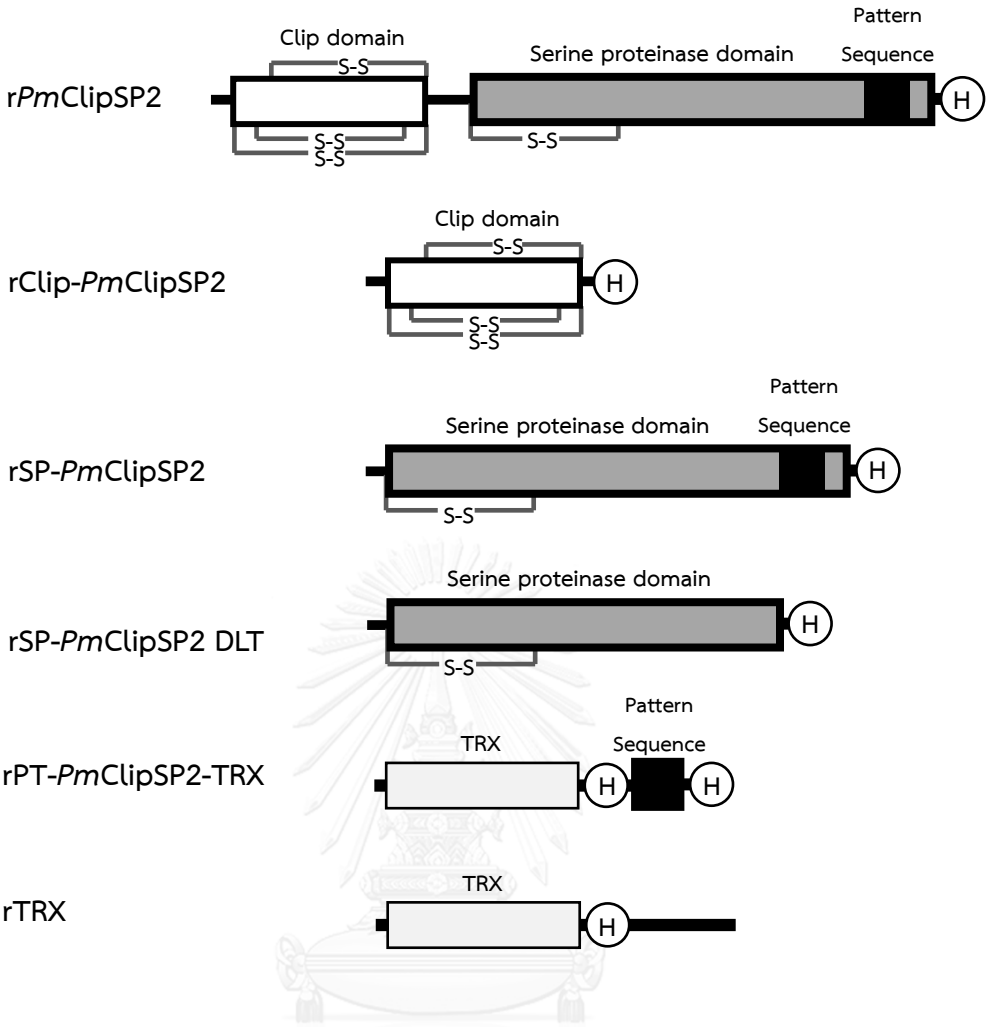


Figure 3.3 Schematic diagram showing recombiant proteins. r*Pm*ClipSP2, rClip-*Pm*ClipSP2, rSP-*Pm*ClipSP2, rSP-*Pm*ClipSP2 DLT, rPT-*Pm*ClipSP2-TRX and rTRX. Connected line with S-S is disulfide bridge, H in circle is 6×His tag.

3.2.1 Construction of rSP-*Pm*ClipSP2 DLT.

The SP domain *Pm*ClipSP2 without the pattern sequence was synthesized from pET-28b(+)-*Pm*ClipSP2 by using gene specific primers (SP-*Pm*ClipSP2 DLT-F/ SP-*Pm*ClipSP2 DLT-R). The SP-*Pm*ClipSP2 DLT was cloned and expressed in pET-28b(+)-SP *Pm*ClipSP2 DLT as an expression vector. A single band of SP-*Pm*ClipSP2 DLT with approximate size of 756 bp was successfully detected by agarose gel electrophoresis. Then, the PCR product was cloned and sequenced (Figure 3.4).

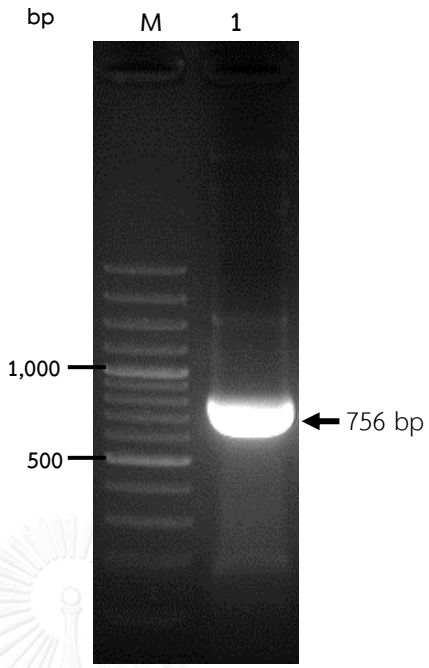


Figure 3.4 The amplification of gene coding for SP-*Pm*ClipSP2 DLT by PCR and agarose gel electrophoresis. Lane 1 is the PCR product of SP-*Pm*ClipSP2 DLT with size ~756 bp detected on 1.5% agarose gel electrophoresis, Lane M is 100 bp DNA marker.

The SP-*Pm*ClipSP2 DLT was cloned into expression vector pET-28b(+) at *Nco*I and *Xho*I sites. After ligation, the recombinant plasmid of SP-*Pm*ClipSP2 DLT was transformed into *E.coli* JM109 (Figure 3.5). Then, the recombinant plasmid was extracted, confirmed by colony PCR and sequenced.

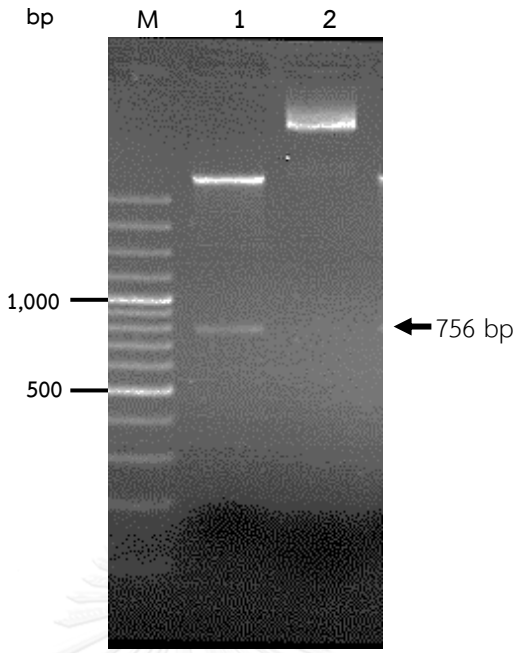


Figure 3.5 Screening of the pET-28b(+)-SP-*Pm*ClipSP2 DLT. The SP-*Pm*ClipSP2 DLT gene was cloned into pET-28b(+), digested with *Nco*I and *Xho*I and analyzed by 1.5% agarose electrophoresis. Lane M is 100 bp DNA ladder, Lane 1 is the digestion with *Nco*I and *Xho*I, Lane 2 is pET-28b(+)-SP-*Pm*ClipSP2 DLT.

3.2.2 Construction of rPT-*Pm*ClipSP2-TRX and rTRX

The rTRX was expressed from pET-32a(+) vector as control with rPT-*Pm*ClipSP2-TRX and the pattern sequence of *Pm*ClipSP2 was synthesized from pET-28b(+)-*Pm*ClipSP2 by using gene specific primers (PT-*Pm*ClipSP2-F/ PT-*Pm*ClipSP2-R). The PT-*Pm*ClipSP2 was cloned and expressed in pET-32a(+)-SP *Pm*ClipSP2 as an expression vector, which contains thioredoxin (TRX) fusion protein at the N-terminus. A single band of PT-*Pm*ClipSP2 with approximate size of 69 bp was detected by agarose gel electrophoresis. Then, the product was cloned and sequenced (Figure 3.6).

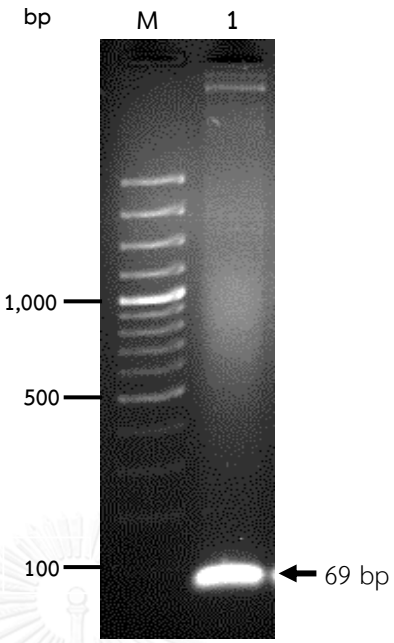


Figure 3.6 The amplification of gene coding for PT-*Pm*ClipSP2 by PCR and agarose gel electrophoresis. Lane 1 is the PCR product of PT-*Pm*ClipSP2 with size ~69 bp detected on 2.0% agarose gel electrophoresis, Lane M is 100 bp DNA marker.

The PT-*Pm*ClipSP2 was cloned into expression vector pET-32a(+) at *Nco*I and *Xho*I sites. After ligation, the recombinant plasmid of PT-*Pm*ClipSP2 was transformed into *E.coli* JM109 (Figure 3.7). Then, the recombinant plasmid was extracted, confirmed by colony PCR and sequenced.

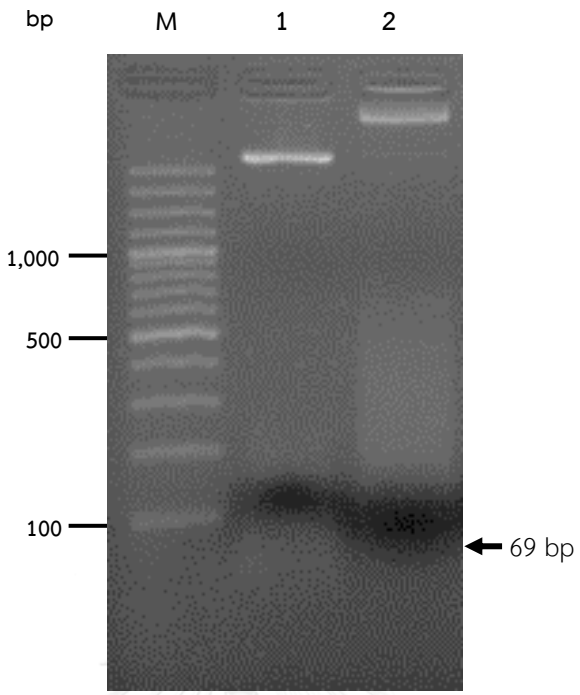
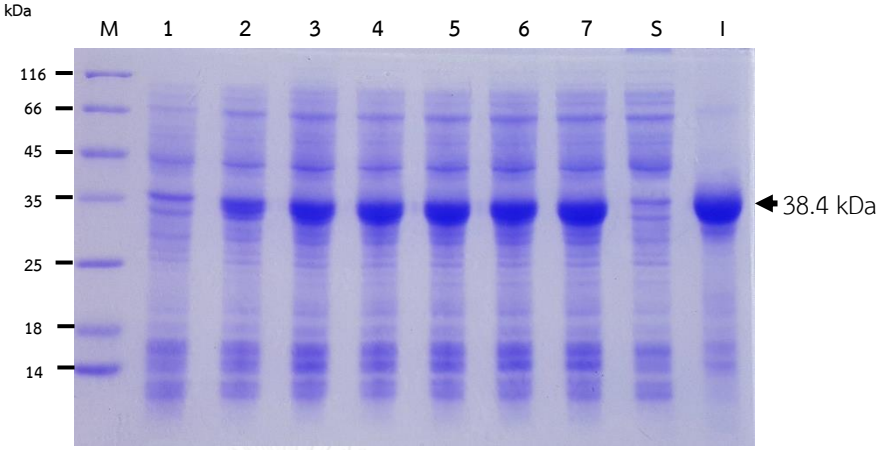


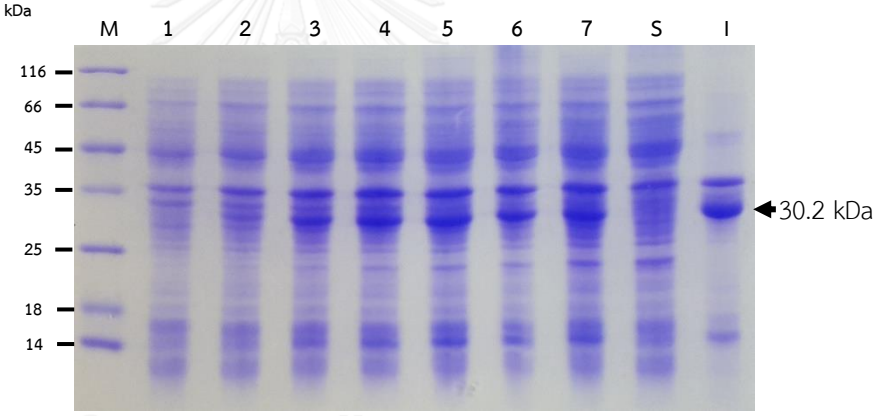
Figure 3.7 Screening of the pET-32a(+)-PT-*Pm*ClipSP2. The PT-*Pm*ClipSP2 was cloned into pET-32a(+), digested with *Nco*I and *Xho*I and analyzed by 2.0% agarose electrophoresis. Lane M is 100 bp DNA ladder, Lane 1 is the digestion with *Nco*I and *Xho*I, Lane 2 is pET-32a(+)-PT-*Pm*ClipSP2.

The corrected recombinant plasmids were transformed into *E. coli* Rosetta (DE3)-pLysS cells for protein expression analysis. The single colonies were selected to grow in LB broth containing kanamycin (for r*Pm*ClipSP2, rClip-*Pm*ClipSP2, rSP-*Pm*ClipSP2 and rSP-*Pm*ClipSP2 DLT) and ampicillin (for rPT-*Pm*ClipSP2-TRX and rTRX). 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 successfully detected by 12.5% SDS-PAGE gel and the coomassie brilliant blue staining (Figure 3.8).

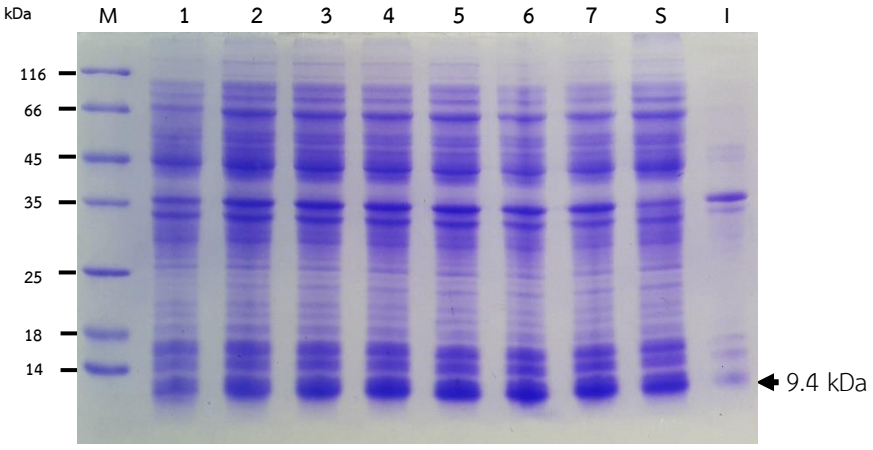
r*Pm*ClipSP2



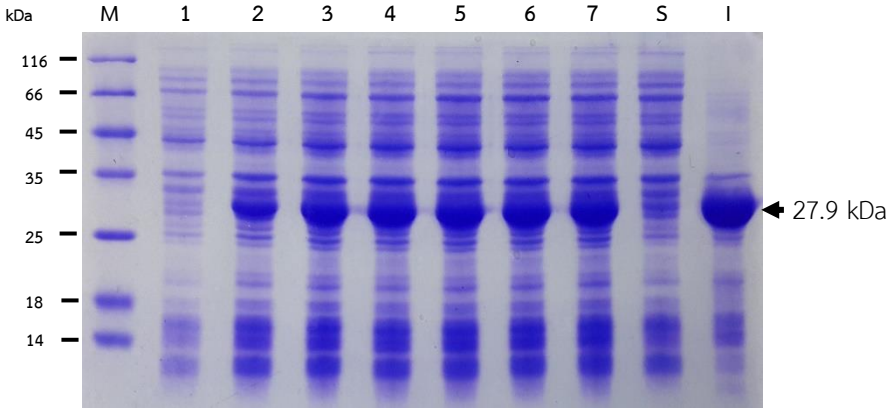
rSP-*Pm*ClipSP2



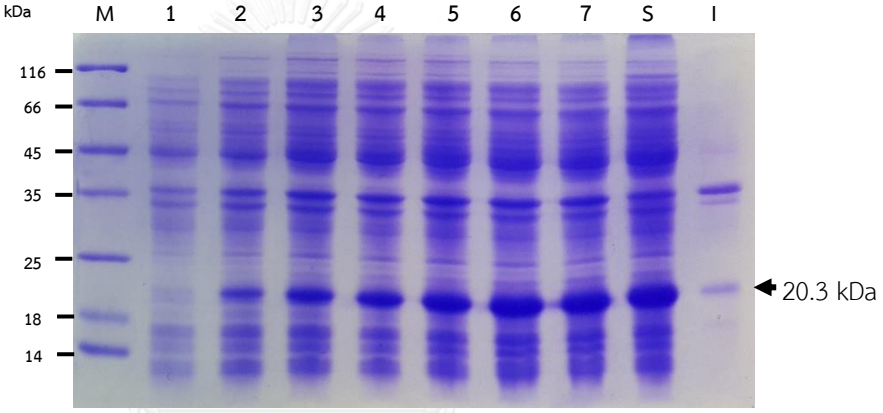
rClip-*Pm*ClipSP2



rSP-*Pm*ClipSP2 DLT



rPT-*Pm*ClipSP2-TRX



rTRX

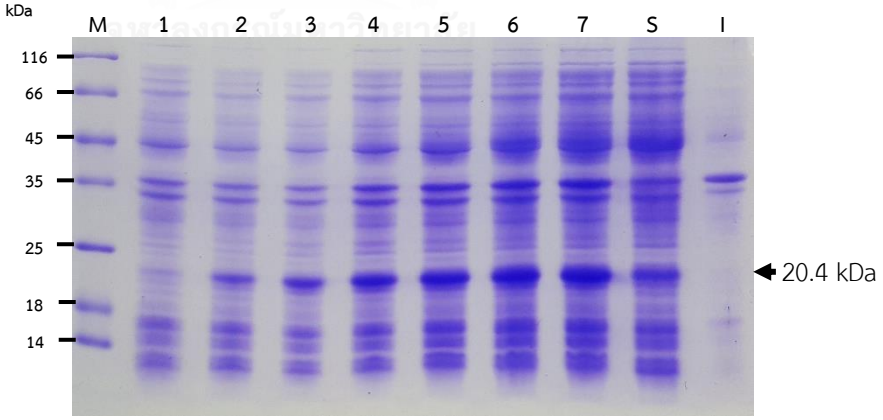
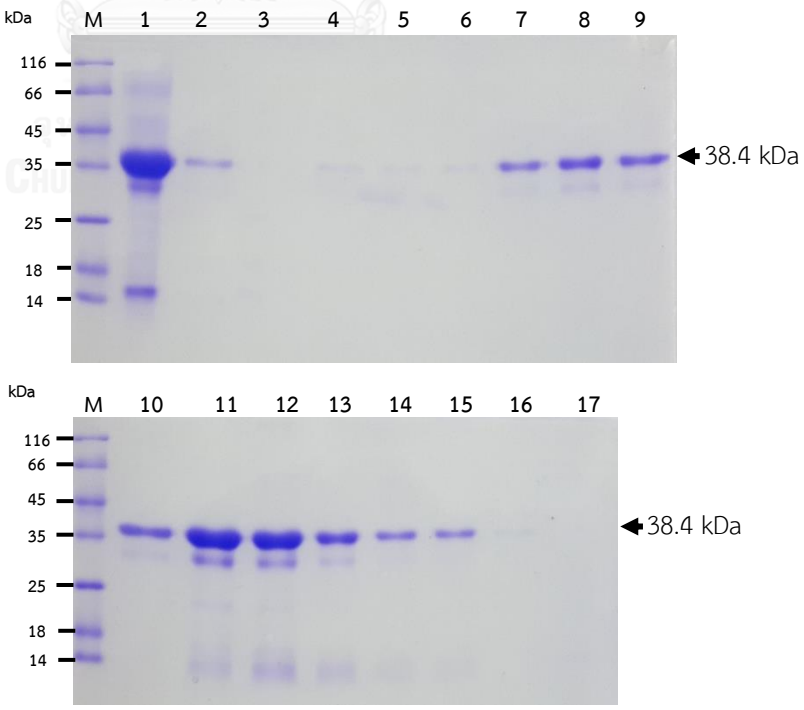


Figure 3.8 The expression of recombinant proteins in *E. coli* Rosetta. The cells were harvested and induced with IPTG at 0, 1, 2, 3, 4, 5 and 6 hours. Lane M is Unstained protein marker, Lanes 1-7 : The expressed proteins after IPTG induction at 0 to 6 hours. Lane S is the soluble fraction. Lane I is the inclusion body fraction, arrow indicates recombinant proteins.

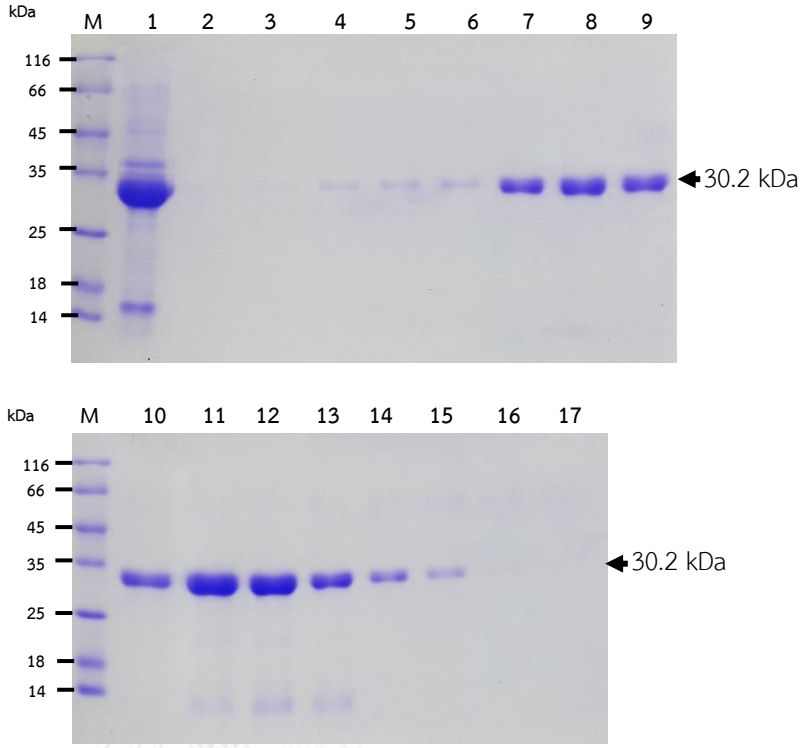
3.2.3 Purification of recombinant proteins by Ni-NTA affinity chromatography

The expressed recombinant proteins were purified from inclusion body (for r*Pm*ClipSP2, rSP-*Pm*ClipSP2 and rSP-*Pm*ClipSP2 DLT), pellets were dissolved in 8M urea before purification, and soluble fraction (for rClip-*Pm*ClipSP2, rPT-*Pm*ClipSP2-TRX and rTRX) by Ni-NTA affinity chromatography. The differential concentration of imidazole was used to wash and elute recombinant proteins after that each fraction was analyzed by 12.5% SDS-PAGE. The expected recombinant proteins were successfully purified with Ni-NTA chromatography (Figure 3.9). The purified proteins were dialyzed in 20 mM Tris-HCl pH 8.0 buffer and the protein concentration was measured by Bradford assay and determined the purity by 12.5% SDS-PAGE and Western blot analysis (Figure 3.10). The major band of purified r*Pm*ClipSP2, rClip-*Pm*ClipSP2, rSP-*Pm*ClipSP2, rSP-*Pm*ClipSP2 DLT, rPT-*Pm*ClipSP2-TRX and rTRX were indicated and the theoretical molecular mass of recombinant proteins are 38.4 kDa, 9.4 kDa, 30.2 kDa, 27.9 kDa, 20.3 kDa and 20.4 kDa, respectively.

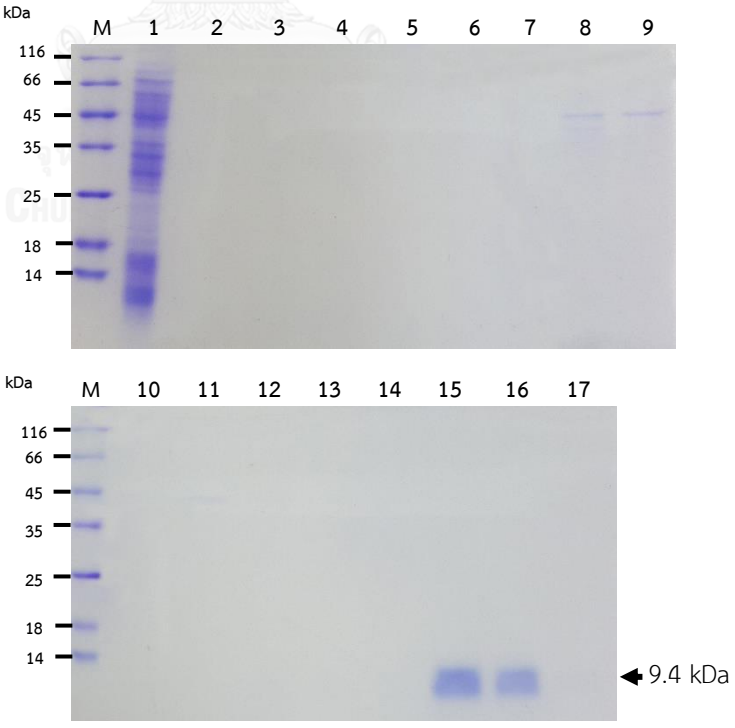
r*Pm*ClipSP2



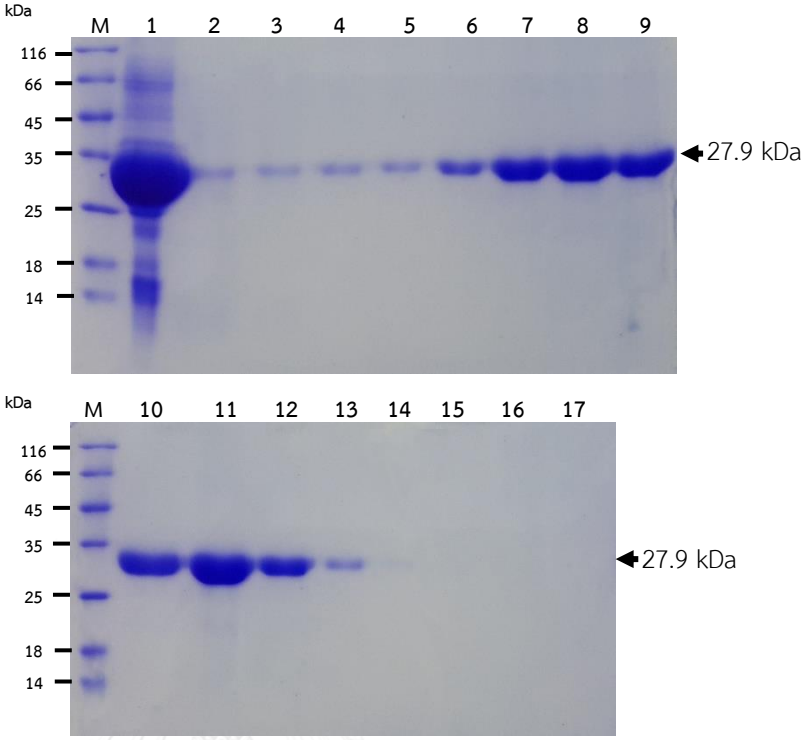
rSP-*Pm*ClipSP2



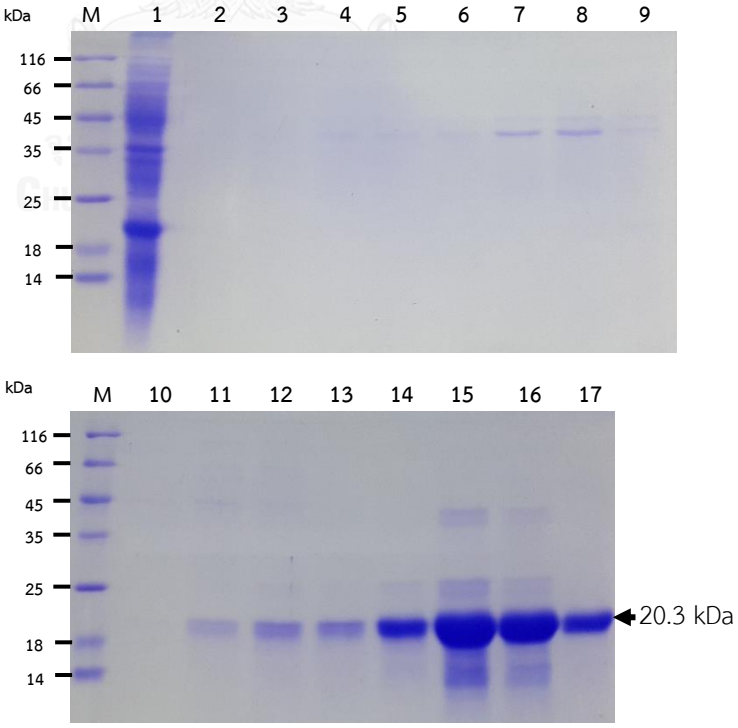
rClip-*Pm*ClipSP2



rSP-*Pm*ClipSP2 DLT



rPT-*Pm*ClipSP2-TRX



rTRX

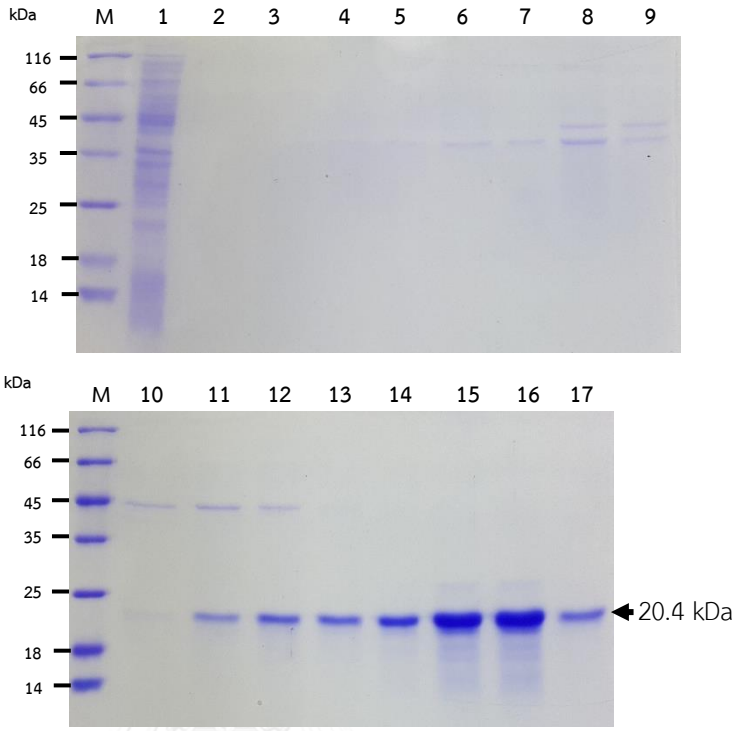


Figure 3.9 Purification of recombinant proteins using Ni-NTA chromatography. Lane M is Unstained protein marker, Lane 1 is Flow through, Lanes 2-5 and 6-9 are washed fractions containing 20 mM and 50 mM imidazole, respectively, Lanes 10-13 and 14-17 are eluted fractions containing 100 mM and 250 mM imidazole, respectively.

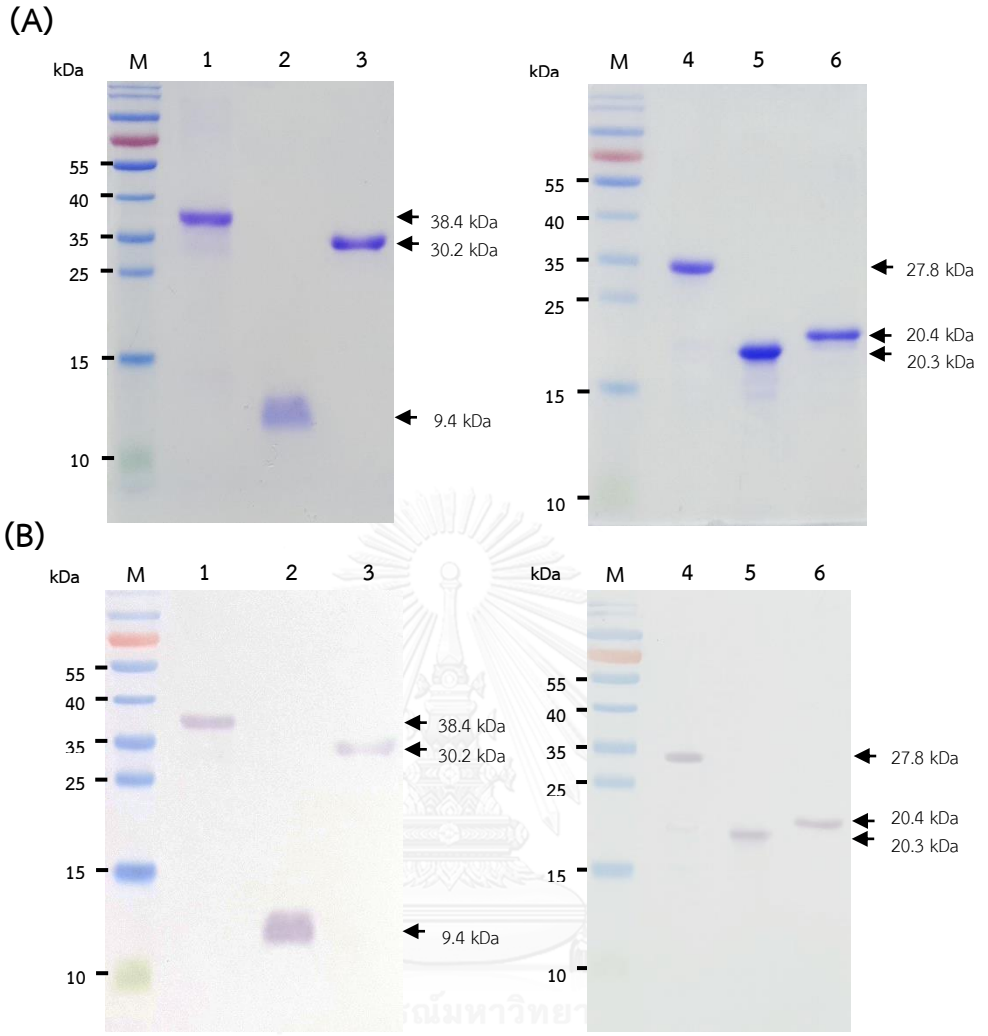


Figure 3.10 The SDS-PAGE and Western blot analysis of purified recombinant proteins. (A) 50 pmol of purified recombinant proteins were run on 12.5% SDS-PAGE and staining with coomassie brilliant blue. (B) 50 pmol of purified recombinant proteins were run on 12.5% SDS-PAGE, transferred into nitrocellulose membrane and detected by mouse anti-His tag antibody. Lane M is PageRuler™ prestained protein ladder, Lane 1 is r*Pm*ClipSP2, Lane 2 is rClip-*Pm*ClipSP2, Lane 3 is rSP-*Pm*ClipSP2, Lane 4 is rSP-*Pm*ClipSP2 DLT, Lane 5 is rPT-*Pm*ClipSP2-TRX, Lane 6 is rTRX, arrows indicate recombinant proteins.

3.3 Binding activity assay of each domain of *Pm*ClipSP2

From the previous study, r*Pm*ClipSP2 bound to both LPS and β-1,3-glucan by ELISA technique (Amparyup et al., 2013). In this research, binding activities of each domain of *Pm*ClipSP2 (rClip-*Pm*ClipSP2 and rSP-*Pm*ClipSP2) were further determined. LPS and β-1,3-glucan were coated and then added various concentration (0-10 μM) of r *Pm*ClipSP2, rClip-*Pm*ClipSP2 and rSP-*Pm*ClipSP2 into each well of a 96-well plate. The bound recombinant proteins were detected by mouse anti-His tag antibody as primary antibody and goat anti-mouse conjugated AP antibody as secondary antibody. The results clearly showed that r*Pm*ClipSP2 and rSP-*Pm*ClipSP2 bound to both immobilized LPS and β-1,3-glucan. On the contrary, rClip-*Pm*ClipSP2 did not bind to either LPS or β-1,3-glucan (Figure 3.11). Analysis of the binding interactions reveals that rSP-*Pm*ClipSP2 bound to LPS with a 1.3-fold higher affinity (dissociation constant (K_d) of 1.54 × 10^{-6} M) than r*Pm*ClipSP2 (K_d of 2.00 × 10^{-6} M) and β-1,3-glucan with a 1.1-fold higher affinity (K_d of 1.96 × 10^{-6} M) than r*Pm*ClipSP2 (K_d of 2.13 × 10^{-6} M). These high affinity binding of both LPS and β-1,3-glucan were dose-dependent. This result indicates that *Pm*ClipSP2 uses C-terminal SP domain to bind both LPS and β-1,3-glucan.

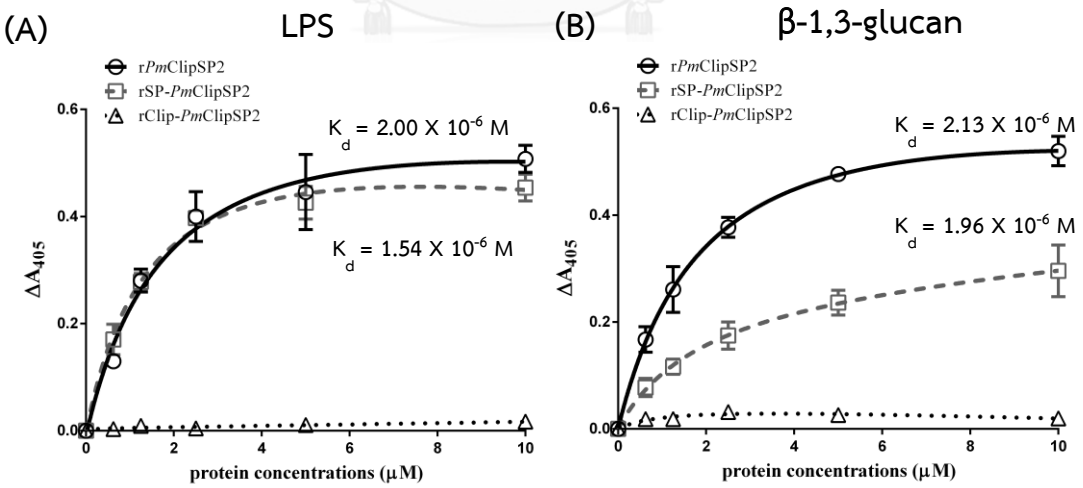


Figure 3.11 Binding ability of r*Pm*ClipSP2 (⊖), rSP-*Pm*ClipSP2 (▭) and rClip-*Pm*ClipSP2 (△) to immobilize LPS (A) and β-1,3-glucan (B). Binding activity was examined by using ELISA technique which used dose-dependent binding of recombinant proteins (0–10 μM), probing with mouse anti-His tag antibody as primary

antibody and goat anti-mouse conjugated AP antibody as secondary antibody for detection and ρ-nitrophenyl phosphate as substrate. Data are shown dissociation constant (K_d) near line and the mean ±1 SEM, derived from triplicate experiments. The data were curve fitted using a one site binding model with R^2 of *Pm*ClipSP2, rClip-*Pm*ClipSP2 and rSP-*Pm*ClipSP2 for LPS are 0.96, 0.98 and 0.50, respectively, and for β-1,3-glucan are 0.99, 0.96 and 0.87, respectively.

3.4 Activation of PO by recombinant proteins

More recently, it has been reported that *Pm*ClipSP2 may act as a PRP by binding to both LPS and β-1,3-glucan and then activating the shrimp proPO-system (Amparyup et al., 2013). To further study PO activation by each domain of *Pm*ClipSP2, hemocyte lysate supernatant (HLS) was prepared from *P. monodon* hemocyte. The recombinant proteins were incubated with LPS or β-1,3-glucan in HLS and then L-DOPA (L-3,4-dihydroxyphenyalanine) was used as a substrate. After that, PO activity was measured at $A_{490\ nm}$. The result indicated that addition of r*Pm*ClipSP2 and rSP-*Pm*ClipSP2 significantly increased PO activity in the presence of LPS or β-1,3-glucan, but not rClip-*Pm*ClipSP2 (Figure 3.12). These *in vitro* results are in accord with the notion that r*Pm*ClipSP2 interacts with both LPS and β-1,3-glucan and induces the proPO activation system in *P. monodon* shrimp using the SP domain.

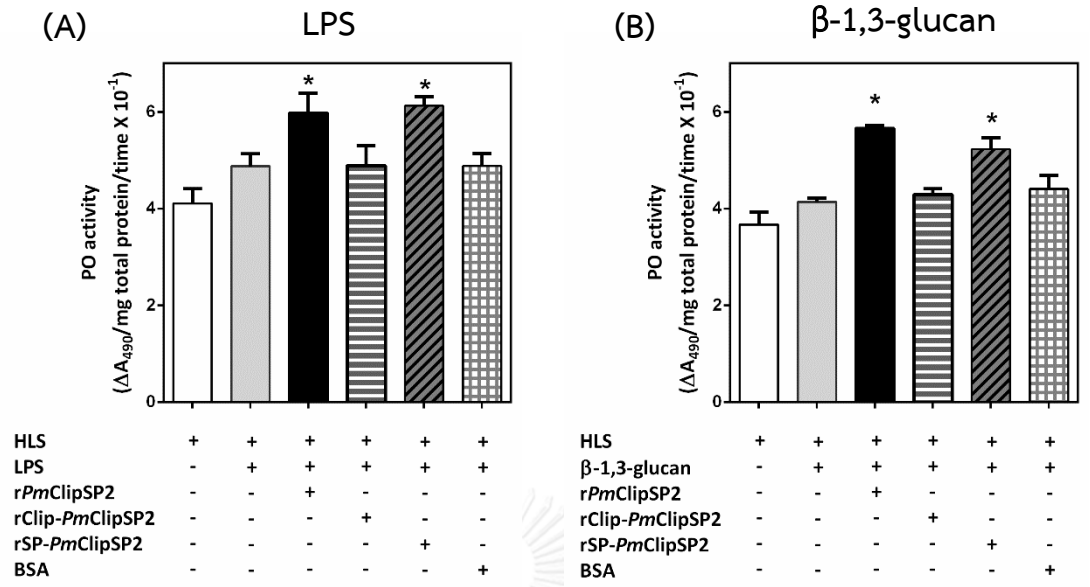


Figure 3.12 PO activation of r*Pm*ClipSP2, rClip-*Pm*ClipSP2 and rSP-*Pm*ClipSP2. Hemocyte lysate supernatant (HLS) (30 µg) was incubated with 10 µM of each of the recombinant proteins (r*Pm*ClipSP2 (■) rClip-*Pm*ClipSP2 (▬) and rSP-*Pm*ClipSP2 (▨)) in the presence of 20 µg of pre-incubated LPS or β-1,3-glucan. Controls (□) were performed as above except without the recombinant proteins and BSA (▦) was used as negative control. The PO activity was measured by spectrophotometry, and then after 30 min L-DOPA was used as the substrate. Data are shown as the mean ±1 SEM, derived from triplicate experiments. Means with an asterisk are significantly different ($P < 0.05$).

3.5 Binding activity assay of the pattern sequence of *Pm*ClipSP2

From the above results, we found that the C-terminal SP domain of *Pm*ClipSP2 interacts with PAMPs and activates the shrimp proPO-system. Sequence analysis of this binding region identified the sequences (FPGVYTSVSHYRSWVE; except for the amino acid change from I/L to V (underlined)) which match to the pattern sequence (X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X (Kasetty et al., 2011)) (Figure 3.2) that has

been reported to function in the antibacterial and LPS binding activities in the C-terminal derived peptide from human thrombin and S1 family proteinase (Kasetty et al., 2011; Papareddy et al., 2010). This region may be responsible for binding to LPS and laminarin (β-1,3-glucan), so a recombinant SP-domain protein which contained deletion of the pattern sequence (rSP-*Pm*ClipSP2 DLT) and tested whether this deletion effect the binding ability of the SP-domain of *Pm*ClipSP2, was constructed. The result of ELISA showed that rSP-*Pm*ClipSP2 DLT did not bind to both LPS and β-1,3-glucan, while rSP-*Pm*ClipSP2 as a control could bind in a dose-dependent manner (Figure 3.13A and 3.13B). To further confirm this result, we produced a recombinant protein which contains the pattern sequence. However, due to the short sequence, the recombinant protein was fused with thioredoxin (TRX), rPT-*Pm*ClipSP2-TRX and the fusion protein was tested for its binding ability by ELISA using rTRX as a control (Figure 3.13C and 3.13D). The result revealed that rPT-*Pm*ClipSP2-TRX bound to both LPS and laminarin (β-1,3-glucan) in a dose-dependent manner with higher affinity (K_d of 7.24 X 10^{-7} M for LPS and 9.82 X 10^{-7} M for laminarin) than the rTRX (K_d of 2.29 X 10^{-6} M for LPS and 1.12 X 10^{-6} M for β-1,3-glucan). These results confirm that the pattern sequence of *Pm*ClipSP2 is microbial cell wall components binding site.

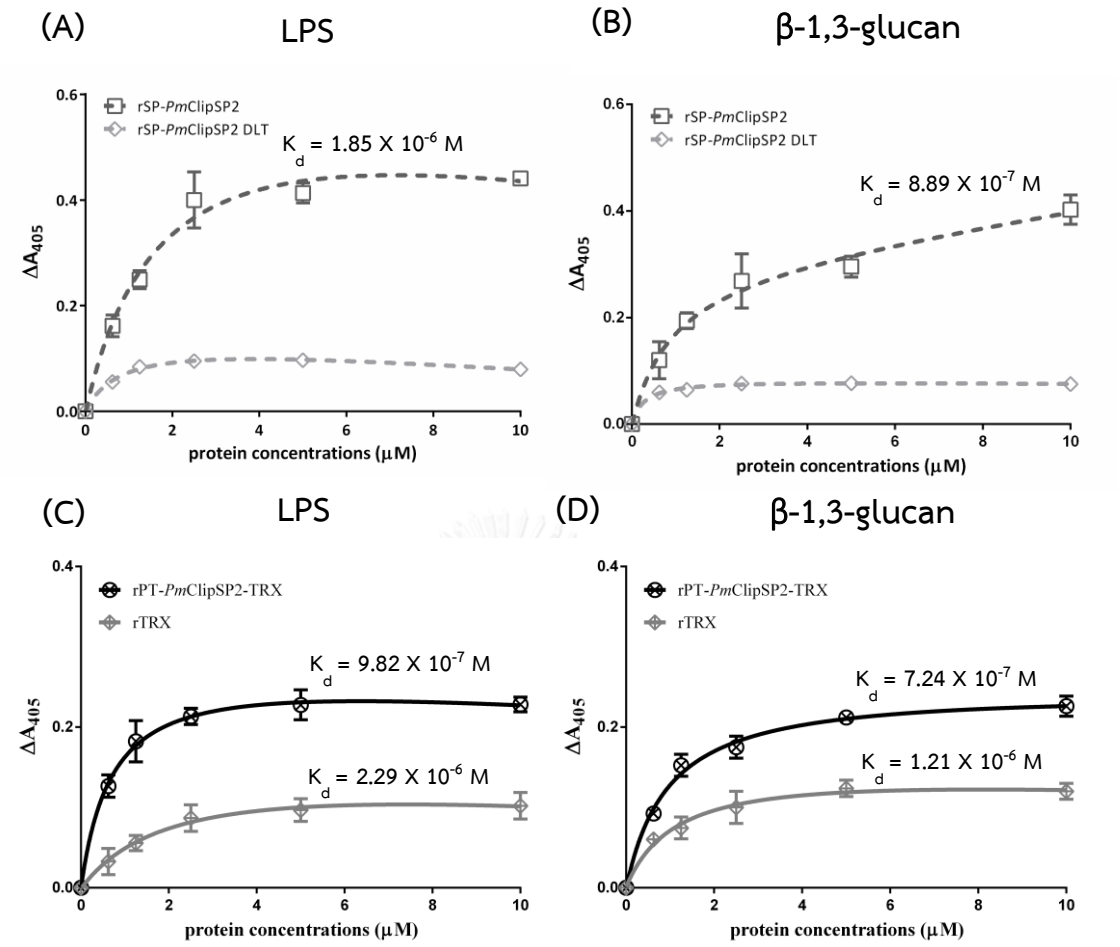


Figure 3.13 Binding ability of rSP-*Pm*ClipSP2 (), rSP-*Pm*ClipSP2 DLT (), rPT-*Pm*ClipSP2-TRX () and rTRX () to immobilize LPS (A, C) and β-1,3-glucan (B, D). Binding activity was examined by using ELISA technique which used dose-dependent binding of recombinant proteins (0–10 μM), probing with mouse anti-His tag antibody as primary antibody and goat anti-mouse conjugated AP antibody as secondary antibody for detection and ρ-nitrophenyl phosphate as substrate. Data are shown dissociation constant (K_d) near line and the mean ±1 SEM, derived from triplicate experiments. The data were curve fitted using a one site binding model with R^2 of rSP-*Pm*ClipSP2, rSP-*Pm*ClipSP2 DLT, rPT-*Pm*ClipSP2-TRX and rTRX for LPS are 0.97, 0.97, 0.98 and 0.91, respectively, and for β-1,3-glucan are 0.96, 0.98, 0.98 and 0.94, respectively

3.6 Antimicrobial activity of the pattern sequence of *Pm*ClipSP2

In addition, the pattern sequence C-terminal derived peptide from human thrombin and S1 family proteinase did not only bind to LPS, but also had antibacterial activities (Kasetty et al., 2011; Papareddy et al., 2010). We examined the antimicrobial activities of the pattern sequence from *Pm*ClipSP2 (rPT-*Pm*ClipSP2-TRX). In this experiment, *E. coli* 363 (gram-negative bacteria), *S. aureus* (gram-positive bacteria) and *V. harveyi* (gram-negative bacteria; pathogen of shrimp) were selected. Bacteria were swabbed into 3% TSA with punched wells, then rPT-*Pm*ClipSP2-TRX (50 and 100 μM), rTRX (50 and 100 μM) as control tag, chloramphenicol (SIGMA) (2 μg) as positive control and 20 mM Tris-HCl pH 8.0 as negative control were added in each well. After 18 hr of incubation, the result showed that rPT-*Pm*ClipSP2-TRX did not have clear zone around well (Figure 3.14).

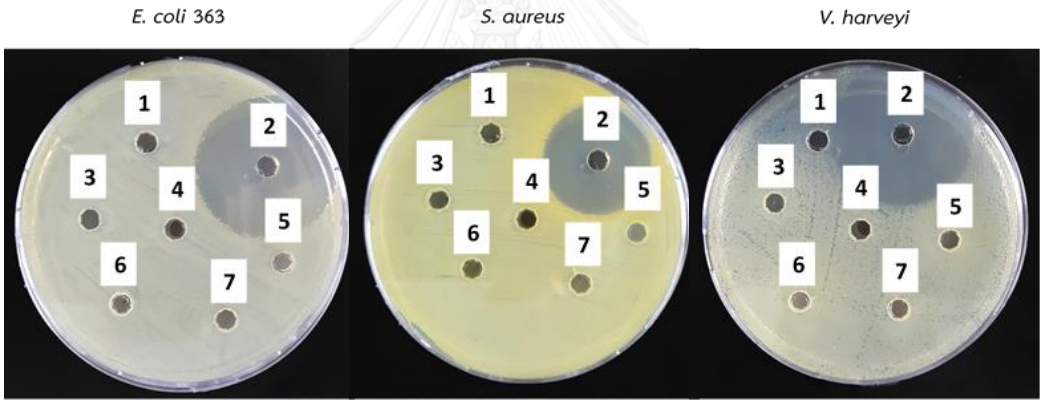


Figure 3.14 Antibacterial activities of rPT-*Pm*ClipSP2-TRX. Well 1 is 20 mM Tris-HCl pH 8.0, Well 2 is 2 μg chloramphenicol, Well 3-5 are 25, 50 and 100 μM rPT-*Pm*ClipSP2-TRX, Well 6, 7 are 50 and 100 μM rTRX.

3.7 Construction, expression and PO activation of recombinant proteins in *Drosophila* S2 cells

To further characterize the function of *Pm*ClipSP2, we also produced the constructs and expressed the recombinant *Pm*ClipSP2 in *Drosophila* S2 cells host system (rS2-*Pm*ClipSP2) and the recombinant protein that contains mutated cleavage site for Factor X_a to produce active form of *Pm*ClipSP2 (S2-r*Pm*ClipSP2$_{Xa}$) for study interaction with other proteinase in proPO-system.

3.7.1 Construction of rS2-*Pm*ClipSP2

The *Pm*ClipSP2 gene coding for mature protein with signal peptide and Kozak sequence was synthesized from cDNA of normal shrimp by gene specific primers (S2-*Pm*ClipSP2-F/ S2-*Pm*ClipSP2-R). A single band of S2-*Pm*ClipSP2 with approximate size of 1,137 bp was detected by agarose gel electrophoresis. Then, the product was cloned and sequenced (Figure 3.15).

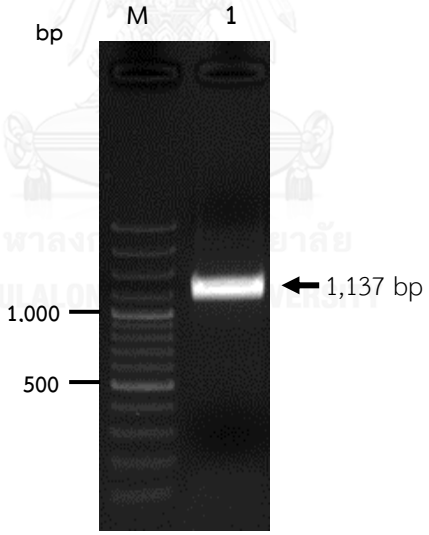


Figure 3.15 The amplification of gene coding for S2-*Pm*ClipSP2 by PCR and agarose gel electrophoresis. Lane 1 is the PCR product of S2-*Pm*ClipSP2 with size ~1,137 bp detected on 1.5% agarose gel electrophoresis, Lane M is 100 bp DNA marker.

The S2-*Pm*ClipSP2 was cloned into TA Cloning Vector by TA Cloning Kits. After ligation, the recombinant plasmid of S2-*Pm*ClipSP2 was transformed into *E.coli* JM109 (Figure 3.16). Then, the recombinant plasmid was extracted, confirmed by colony PCR.

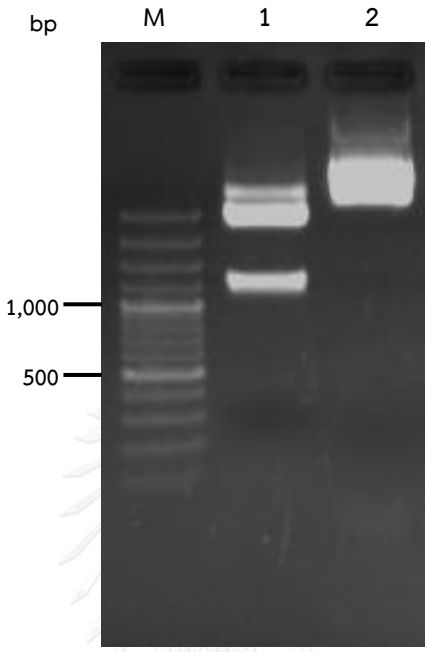


Figure 3.16 Screening of the TA-S2-*Pm*ClipSP2. The S2-*Pm*ClipSP2 gene was cloned into TA Cloning Vector, digested with *Not*I and *Xba*I and analyzed by 1.5% agarose electrophoresis. Lane M is 100 bp DNA ladder, Lane 1 is the digestion with *Not*I and *Xba*I, Lane 2 is TA-S2-*Pm*ClipSP2.

TA-S2-*Pm*ClipSP2 was subcloned into an expression vector pIZTV5-His at the *Not*I and *Xba*I sites fused with V5 epitope and six His encoded nucleotides at the C terminus. After that the recombinant plasmid pIZTV5-His-S2-*Pm*ClipSP2 was transformed into *E. coli* JM109. The recombinant plasmid was extracted and confirmed by restriction enzymes, *Not*I and *Xba*I, digestion. The recombinant plasmid screening was detected by agarose gel electrophoresis (Figure 3.17).

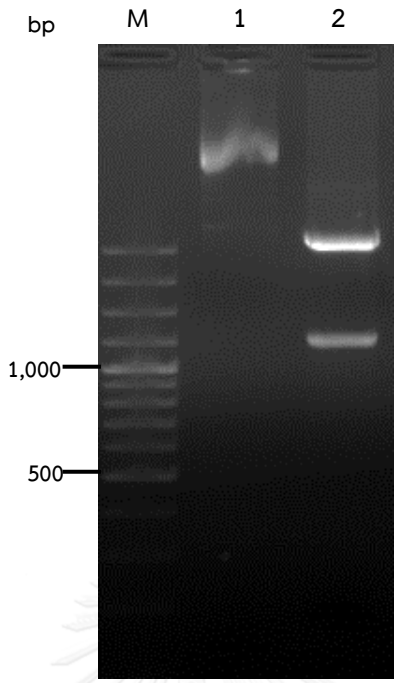


Figure 3.17 Screening of the pIZTV5-His-S2-*Pm*ClipSP2. The S2-*Pm*ClipSP2 gene was cloned into pIZTV5-His, digested with *Not*I and *Xba*I and analyzed by 1.5% agarose electrophoresis. Lane M is 100 bp DNA ladder, Lane 1 is pIZTV5-His-S2-*Pm*ClipSP2, Lane 2 is the digestion with *Not*I and *Xba*I.

3.7.2 Construction of rS2-*Pm*ClipSP2$_{Xa}$

After pIZTV5-His-S2-*Pm*ClipSP2 was constructed, the mutation at cleavage site for Factor Xa of S2-*Pm*ClipSP2 was synthesized by PCR-based site-directed mutagenesis. First, S2-*Pm*ClipSP2 was amplified into 2 parts, Left-arm and Right-arm, by 2 pairs of specific primers (S2-*Pm*ClipSP2-F/ S2-*Pm*ClipSP2$_{Xa}$-R and S2-*Pm*ClipSP2$_{Xa}$-F/ S2-*Pm*ClipSP2-R, respectively). A single band of 2 parts with approximate size of 400 and 789 bp were detected by agarose gel electrophoresis. Then, these 2 parts were combined and amplified using S2-*Pm*ClipSP2-F/ S2-*Pm*ClipSP2-R primers. A single band with approximate size 1,137 bp was detected by agarose gel electrophoresis. Then, the product was cloned and sequenced (Figure 3.18).

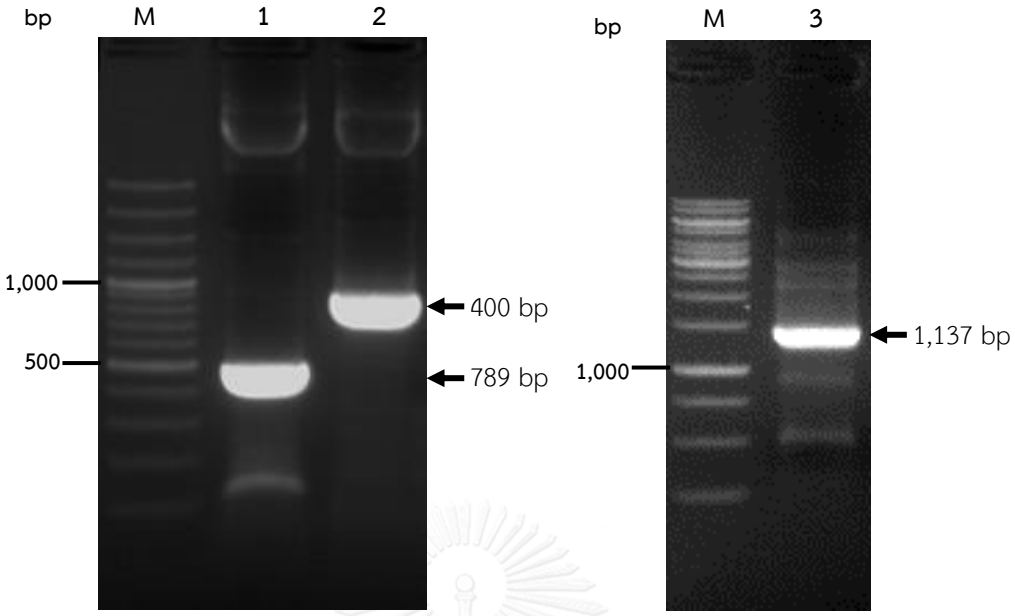


Figure 3.18 The amplification of gene coding for S2-*Pm*ClipSP2$_{Xa}$ by PCR and agarose gel electrophoresis. Lane 1 and 2 are the PCR products of Left-arm ad Right-arm with size ~400 and 789 bp, respectively, Lane 3 is the PCR product of S2-*Pm*ClipSP2$_{Xa}$ with size ~1,137 bp detected on 1.5% agarose gel electrophoresis, Lane M, left and right, are 100 bp DNA marker and 1kbp DNA marker, respectively.

The S2-*Pm*ClipSP2$_{Xa}$ was cloned into TA Cloning Vector by TA Cloning Kits. After ligation, the recombinant plasmid of S2-*Pm*ClipSP2$_{Xa}$ was transformed into *E.coli* JM109 (Figure 3.19). Then, the recombinant plasmid was extracted, confirmed by colony PCR.

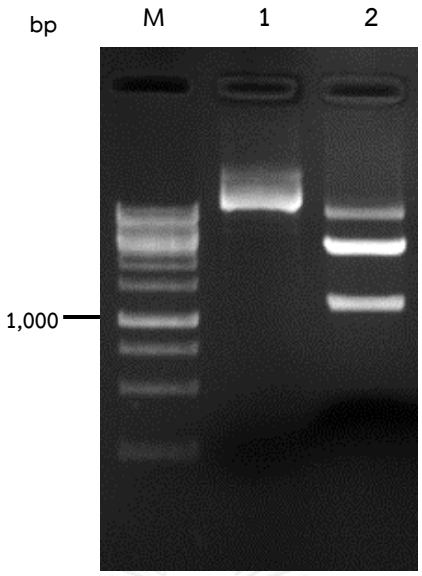


Figure 3.19 Screening of the TA-S2-*Pm*ClipSP2$_{Xa}$. The S2-*Pm*ClipSP2$_{Xa}$ gene was cloned into TA Cloning Vector, digested with *Not*I and *Xba*I and analyzed by 1.5% agarose electrophoresis. Lane M is 1 kbp DNA ladder, Lane 1 is TA-S2-*Pm*ClipSP2$_{Xa,}$ Lane 2 is the digestion with *Not*I and *Xba*I.

TA-S2-*Pm*ClipSP2$_{Xa}$ was subcloned into an expression vector pIZTV5-His at the *Not*I and *Xba*I sites fused with V5 epitope and six His encoded nucleotides at the C terminus. After that the recombinant plasmid pIZTV5-His-S2-*Pm*ClipSP2$_{Xa}$ was transformed into *E. coli* JM109. The recombinant plasmid was extracted and confirmed by restriction enzymes, *Not*I and *Xba*I, digestion. The recombinant plasmid screening was detected by agarose gel electrophoresis (Figure 3.20).

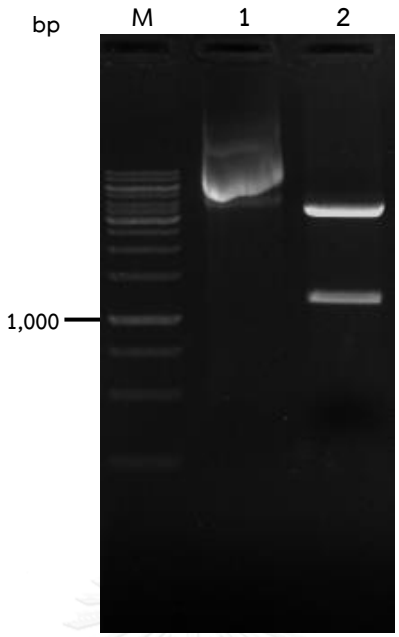


Figure 3.20 Screening of the pIZTV5-His-S2-*Pm*ClipSP2$_{Xa}$. The S2-*Pm*ClipSP2$_{Xa}$ gene was cloned into pIZTV5-His, digested with *Not*I and *Xba*I and analyzed by 1.5% agarose electrophoresis. Lane M is 1 kbp DNA ladder, Lane 1 is pIZTV5-His-S2-*Pm*ClipSP2$_{Xa}$, Lane 2 is the digestion with *Not*I and *Xba*I.

The corrected recombinant plasmids were extracted and transfected into S2 cells for recombinant protein expression. S2 cells were grown over night after that recombinant plasmid (pIZTV5-His-S2-r*Pm*ClipSP2 and pIZTV5-His-S2-r*Pm*ClipSP2$_{Xa}$) with Cellfectin® II were added to transfect S2 cells for 48 hr. Then, transfected S2 cells and media were collected. The expressed proteins were detected by 12.5% SDS-PAGE gel and Western blot analysis. The major band of rS2-*Pm*ClipSP2 was found in media fraction and the theoretical molecular mass of recombinant proteins is 43.7 kDa (Figure 3.21). However, we unsuccessfully expressed rS2-*Pm*ClipSP2$_{Xa}$ (Figure 3.22).

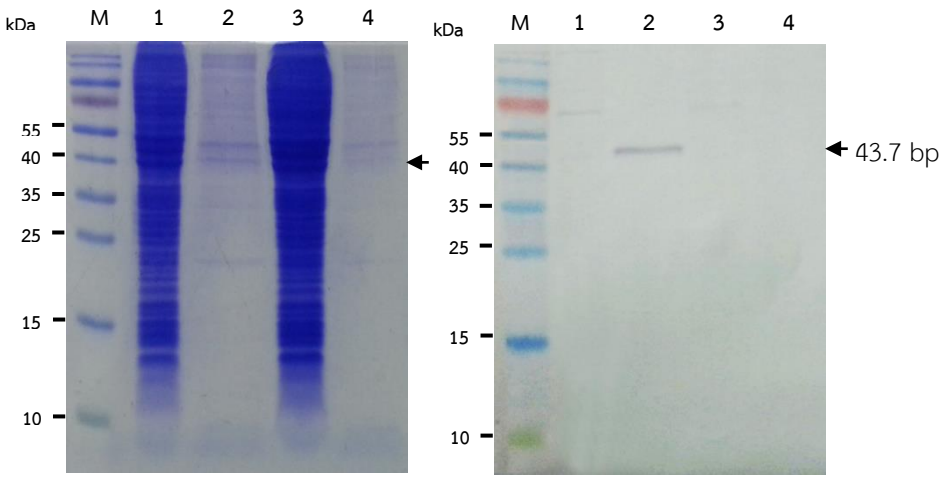


Figure 3.21 The SDS-PAGE and Western blot analysis of rS2-*Pm*ClipSP2. Twenty microliters of cell and media fractions were run on 12.5% SDS-PAGE and staining with coomassie brilliant blue (right). Followed by transferring into nitrocellulose membrane and detected by mouse anti-V5 tag antibody (Left). Lane M is PageRuler™ prestained protein ladder, Lane 1 is cell fraction of rS2-*Pm*ClipSP2, Lane 2 is media fraction of rS2-*Pm*ClipSP2, Lane 3 is cell fraction of control cell, Lane 4 is media fraction of control cell, arrow indicates recombinant protein.

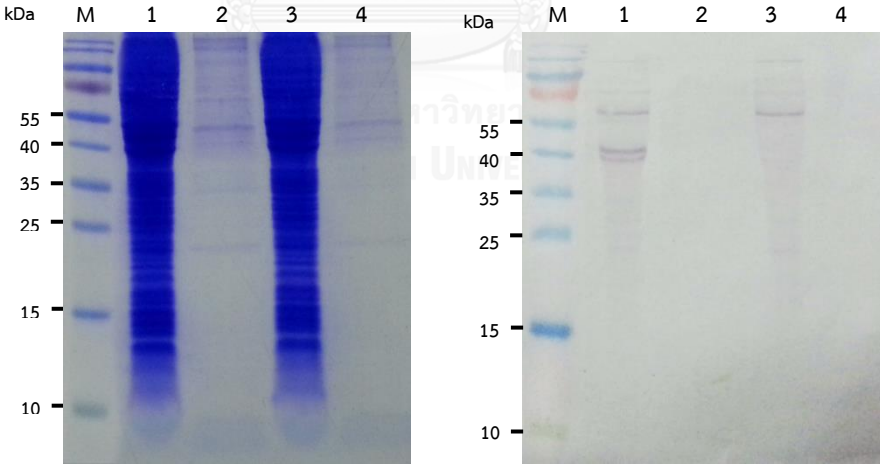


Figure 3.22 The SDS-PAGE and Western blot analysis of rS2-*Pm*ClipSP2$_{Xa}$. Twenty microliters of cell and media fractions were run on 12.5% SDS-PAGE and staining with coomassie brilliant blue (right). Follow by transferred into nitrocellulose membrane and detected by mouse anti-V5 tag antibody (Left). Lane M is PageRuler™ prestained protein ladder, Lane 1 is cell fraction of rS2-*Pm*ClipSP2$_{Xa}$, Lane 2 is media fraction of

rS2-*Pm*ClipSP2$_{Xa}$, Lane 3 is cell fraction of control cell, Lane 4 is media fraction of control cell, arrow indicates recombinant protein.

3.7.3 Activation of PO by rS2-*Pm*ClipSP2

The recombinant *Pm*ClipSP2 expressed from S2 cells (rS2-*Pm*ClipSP2) was preliminary examined PO activation in HLS. Media fraction from rS2-*Pm*ClipSP2 transfected S2 cells was incubated with 25 µg of HLS in the presence of LPS and media fraction from normal S2 cells was used as control. L-DOPA (L-3,4-dihydroxyphenyalanine) was used as substrate. After that, PO activity was measured at $A_{490\ nm}$. The result showed that rS2-*Pm*ClipSP2 media could activate PO activity as compare to the control media (Figure 3.23).

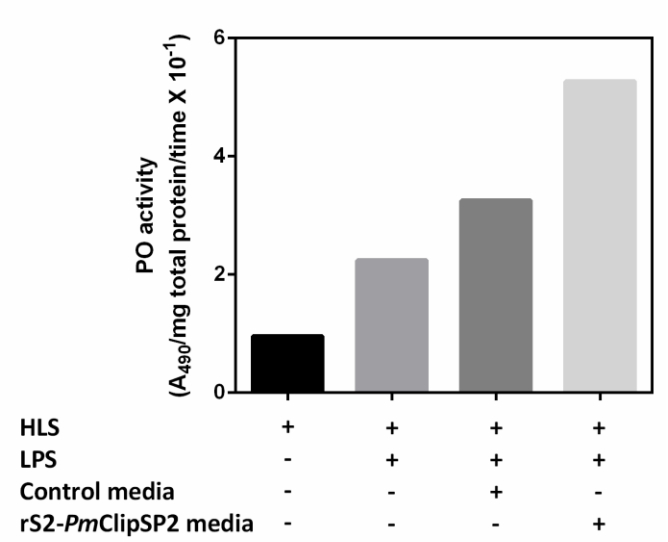


Figure 3.23 PO activation of rS2-*Pm*ClipSP2 with LPS. HLS (25 µg) was incubated with 20 µl of media fraction from rS2-*Pm*ClipSP2 transfected S2 cells and normal cells as control in the presence of 20 µg of pre-incubated LPS. The PO activity was measured by spectrophotometry, and then after 30 min L-DOPA was used as the substrate. Data are derived from duplicate experiments.

3.8 LPS neutralization of *Pm*ClipSP2

The *Pm*ClipSP2 knock down shrimp showed increased mortality rate and hemocyte number was decreased when shrimp were injected with LPS (Amparyup et al., 2013). To further test the LPS neutralization function of r*Pm*ClipSP2 firstly, LPS dosage (1-10 µg) that caused 50% mortality rate of shrimp was determined. Unfortunately, all shrimp died in one day after LPS injection at 10 µg/g shrimp (Figure 3.24) but the previous research reported that shrimp could tolerate up to 50 µg/g shrimp. The variation of LPS dosage as well as the shrimp samples might cause variation of the experiment so the LPS neutralization property was performed using Drosophila S2 cell instead of shrimp.

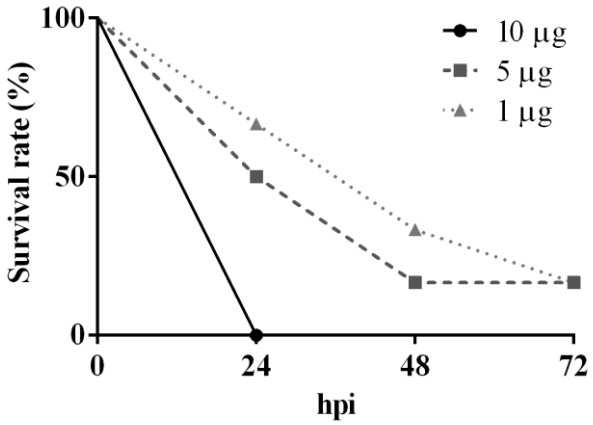


Figure 3.24 Survival rate of shrimp after LPS injection. Normal healthy shrimp (6 shrimp/group) was injected with vary dose of LPS (0, 5 and 10 µg/g shrimp) and observed mortality for 72hour post injection (hpi).

LD_{50} of LPS that reduces live S2 cells by 50% in 48 hr was determined (Figure 3.25). Five µg/well of LPS was then used in the incubation with S2 cells in the presence or absence of r*Pm*ClipSP2 and rS2-*Pm*ClipSP2 media. The result showed that neither r*Pm*ClipSP2 nor rS2-*Pm*ClipSP2 reduced the toxicity of LPS to S2 cells (Figure 3.26) suggested that *Pm*ClipSP2 had no ability to neutralize the toxic effect of LPS to S2 cells.

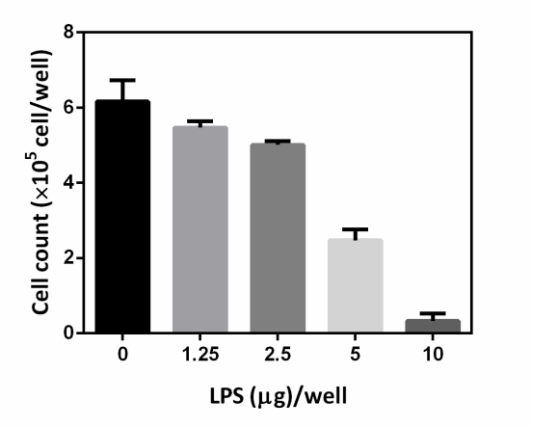


Figure 3.25 Determination of LD_{50} of LPS to S2 cells. S2 cells (1.6×10^5 cells/well) were incubated with various dose of LPS (0-10 µg/well) for 48 hr. Live cells were stained with trypan blue and counted by hemocytometer. Data are shown as the mean ±1 SEM, derived from triplicate experiments.

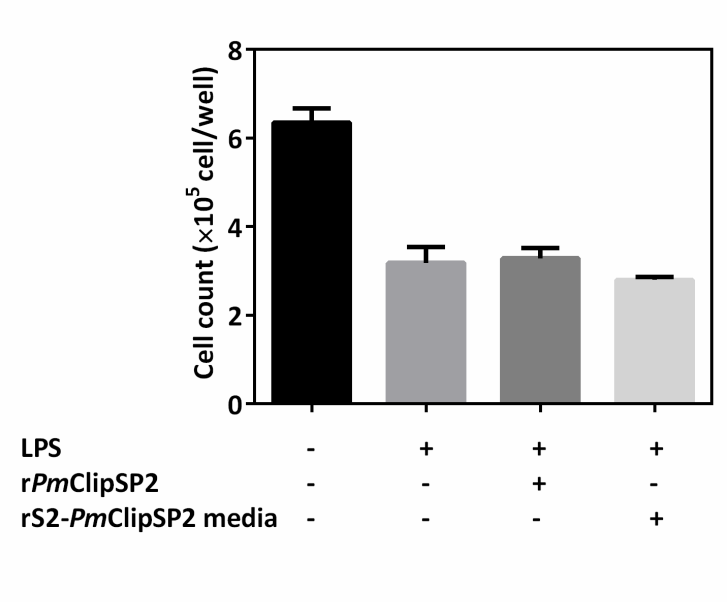


Figure 3.26 LPS neutralization of r*Pm*ClipSP2 in S2 cells. S2 cells (1.6×10^5 cells/well) were incubated with LPS (5 µg/well) with or without r*Pm*ClipSP2 (10 µM) and rS2-*Pm*ClipSP2 media (200 µl) for 48 hr. Live cells were stained with trypan blue and counted by hemocytometer. Data are shown as the mean ±1 SEM, derived from triplicate experiments.

CHAPTER IV
DISCUSSIONS

In the proPO-activating system, proteins, which are involved in the melanization cascade (PRPs, proPOs, proteinases and inhibitors), have been characterized and their functions have been studied in numerous invertebrate species (Amparyup et al., 2013a; Cerenius et al., 2008; Cerenius and Söderhäll, 2004; Kanost and Gorman, 2008). The proPO-system in crustaceans is reported to play a key role in the immune response against pathogenic bacteria and fungi (Amparyup et al., 2009, 2013a; Charoensapsri et al., 2009, 2011; Fagutao et al., 2009; Jang et al., 2011; Liu et al., 2007). Moreover, the recognition of pathogen cell wall components by PRPs is a key step in the activation of the proPO-system. Recent evidence in insects suggests that certain SPs act as PRPs and are required for the activation of the melanization cascade to recognize the microorganisms (Ji et al., 2004; Wang and Jiang, 2006). The SP domain of ClipSPs is classified as the trypsin-like SP family, which has been identified in both vertebrates and invertebrates. This proteinase family has biological functions, which have been widely characterized and have been found to be involved in immunity and homeostasis (Jang et al., 2008; Jiang and Kanost, 2000).

In our previous study, *Pm*ClipSP2 was identified from the shrimp *P. monodon*, and its important functions were reported (Amparyup et al., 2013b). Sequence analysis of *Pm*ClipSP2 showed an N-terminus clip domain and a C-terminus SP domain and is a member of the ClipSP family (Jang et al., 2008; Jiang and Kanost, 2000). *Pm*ClipSP2 was found to be expressed mainly in hemocytes, which corresponds to the expression of the other known proPO-associated transcripts in shrimp *P. monodon* (*Pm*proPO1, *Pm*proPO2, *Pm*PPAE1, *Pm*PPAE2 and *Pm*LGBP) that are mainly detected in the hemocytes (Amparyup et al., 2009; Amparyup et al., 2012; Charoensapsri et al., 2009, 2011). *Pm*ClipSP2-knockdown shrimp showed a significant reduction of PO activity, increased susceptibility to *V. harveyi* infection and increased mortality of low dose (1 μg/g shimp) microbial cell wall components (LPS and β-1,3-glucan) injection. In addition, it may also play an essential role in hemocyte homeostasis by scavenging

LPS and neutralizing its toxicity. Interestingly, it has been revealed that recombinant *Pm*ClipSP2 binds to LPS and β-1,3-glucan and activates PO activity, suggesting that *Pm*ClipSP2 may act as a PRP and activate the shrimp proPO-system. (Amparyup et al., 2013b).

In this study, we further investigated the binding domain of *Pm*ClipSP2 to bacterial cell wall components by separately expressing *Pm*ClipSP2 (N-terminal clip domain, rClip-*Pm*ClipSP2 and C-terminal serine proteinase domain, rSP-*Pm*ClipSP2) and examining the binding properties with LPS and β-1,3-glucan and PO activation in HLS. The results demonstrated that rSP-*Pm*ClipSP2 bound to both LPS and β-1,3-glucan with high affinity (K_d of 1.54×10^{-6} M and K_d of 1.96×10^{-6} M, respectively), but the clip domain did not bind. Moreover, the SP domain could activate the proPO-system in HLS of *P. monodon* in the presence of LPS Thus, the SP domain of *Pm*ClipSP2 plays a recognition role with LPS and β-1,3-glucan and induces the proPO-system.

In the horseshoe crab coagulation cascade, the factor C, SP zymogen, acts as a PRP for pathogens. The tripeptide motif (-Arg36-Trp37-Arg38-), which located at the N-terminal Cys-rich region, of factor C interacts with LPS in the lipid A portion (Ariki et al., 2004; Koshiba et al., 2007). However, the N-terminal clip domain of *Pm*ClipSP2 does not contain this motif.

The involvement of SPs in the recognition processes of the horseshoe crab (*Limulus polyphemus and Tachypleus tridentatus*) coagulation cascade system (factor C and factor G) and the tobacco hornworm (*Manduca sexta*) proPO cascade system (HP14), which interact with the microbial cell wall components, have also been reported (Ariki et al., 2004; Ji et al., 2004; Takaki et al., 2002; Theopold et al., 2004; Wang and Jiang, 2006). However, *Pm*ClipSP2 displays only a single predicted N-terminal clip and a C-terminal SP domain with no extra domains like those found in factor C, factor G and HP14, which contain additional individual domains involved in the immune recognition of pathogens.

Recently, C-terminal peptides from the SP domain of human thrombin, a key enzyme in the coagulation cascade, and numerous S1 family members were reported

to bind to LPS and display bactericidal and anti-inflammatory functions, suggesting a new role of thrombin in regulating host defense peptides (Kasetty et al., 2011; Papareddy et al., 2010). Interestingly, a similar C-terminal sequence pattern to that of thrombin and the S1 family (X-[PFY]-X-[AFILV]-[AFY]-[AITV]-X-[ILV]-X(5)-W-[IL]-X (Kasetty et al., 2011)), which exhibit important functions in the antibacterial and LPS binding activities, was also found in the C-terminal region of *Pm*ClipSP2 (FPGVYTSVSHYRSWVE, except for the amino acid change from I/L to V (underlined)). Therefore, we expressed the recombinant proteins, one which contains the SP domain with deletion of the pattern sequence (rSP-*Pm*ClipSP2 DLT) and another fusion protein containing the pattern sequence with TRX-tag (rPT-*Pm*ClipSP2-TRX). The ELISA-based binding result reveal that the pattern sequence participates in binding property of *Pm*ClipSP2, as rPT-*Pm*ClipSP2-TRX bound to both LPS and β-1,3-glucan but not rSP-*Pm*ClipSP2 DLT. These results demonstrated that the pattern sequence of *Pm*ClipSP2 was responsible for binding to microbial cell wall components. Nevertheless, the pattern sequence did not exhibit antimicrobial activity from Radial Diffusion Assay with *E. coli* 363, *S aureus* and *V. harveyi*, which showed the same as some the S1 derived peptides (Kasetty et al., 2011).

Others ClipSPs in *P. monodon* have been identified and characterized, *Pm*PPAE1 and *Pm*PPAE2 are involved in the proPO-system and likely function as terminal SPs that convert proPO to PO (Amparyup et al., 2009; Charoensapsri et al., 2009, 2011; Sutthangkul et al., 2017). A homolog of ClipSP, named *Pm*ClipSP1, has been identified as a component of the shrimp antibacterial defense system, but it is not involved in the activation of the proPO-system (Amparyup et al., 2010). On the other hand, *Pm*ClipSP2 and *Pm*Snake have been demonstrated to be the crucial serine proteinases in the activation of the proPO-system (Amparyup et al., 2013b; Monwan et al., 2017). However, the sequential activation of ClipSPs cascade in the shrimp proPO-system is still unknown. Therefore, we expressed *Pm*ClipSP2 (rS2-*Pm*ClipSP2) as template for the mutation of cleavage site (YPWK) to Factor Xa cleavage site (IEGR) (rS2-*Pm*ClipSP2$_{Xa}$), which allowed active form of *Pm*ClipSP2 by Factor Xa, in S2 cells express system. The active form *Pm*ClipSP2 was planned to incubate with PRP and

other ClipSPs, which involved in shrimp proPO-system, and observed the activation of ClipSPs to reveal the sequential activation of SP cascade. Unfortunately, rS2-*Pm*ClipSP2$_{Xa}$ was not successfully expressed unlike the report by Sutthangkul et al. (2015), *Pm*proPPAE2$_{Xa}$ was successfully produced and showed to be the enzyme that converts *Pm*proPOs to active *Pm*POs. However, rS2-*Pm*ClipSP2 could activate PO activity in preliminary examination.

LPS and β-glucan are known to exhibit immunostimulant activities in many animals. In crustaceans, these molecules can cause a severe reduction in the circulating total hemocyte counts (THC) and viability of the animal (Lorenzon et al., 1999; Söderhäll et al., 2003). From our previous study, LPS- or β-1,3-glucan injection increased the mortality in *Pm*ClipSP2 knockdown shrimp that mediated by the interference of hemocyte homeostasis. Moreover, THC of LPS-stimulated *Pm*ClipSP2 knockdown shrimp were reduced and significantly changed morphological in the remaining hemocytes. Thus, *Pm*ClipSP2 is also to control hemocyte homeostasis in the presence of LPS.

PRPs represent an essential first line of defense against pathogenic microorganisms and can be divided into the two groups that are signal an immune activation and scavenger of pathogens and their toxic products. Dectin-1, a receptor for β-glucan, is a PRP for both signaling and scavenger receptors (Taylor et al., 2005). The mannose-binding lectin of the crayfish *P. leniusculus* (*Pl*-MBL) was report to play a key role in the proPO-system by acting as a scavenger receptor to avoid the spread of LPS in the hemolymph and control the place of the proPO activation (Wu et al., 2013). In contrast to *Pl*-MBL, *Pm*ClipSP2 does not contain a carbohydrate recognition domain (CRD) in the C-terminus that occurs in C-type lectin.

In this study, *Pm*ClipSP2 was examine the LPS neutralization in S2 cells by pre-incubation *Pm*ClipSP2 with LPS. However, *Pm*ClipSP2 did not show directly neutralize toxicity of LPS. However, the proPO-system is a complex biochemical process that is still unknown widely. Therefore, continued effort to study the function of proteins in the proPO-system is important for an understanding of the shrimp immune system.

CHAPTER V

CONCLUSIONS

1. The recombinant proteins, r*Pm*ClipSP2, rClip-*Pm*ClipSP2, rSP-*Pm*ClipSP2, rSP-*Pm*ClipSP2 DLT, rPT-*Pm*ClipSP2-TRX and rTRX, were successfully overexpressed in *E. coli* with predicted molecular mass of 38.4 kDa, 9.4 kDa, 30.2 kDa, 27.9 kDa, 20.3 kDa and 20.4 kDa, respectively.

2. The ELISA-based binding assay revealed that *Pm*ClipSP2 binds to both LPS and β-1,3-glucan via the C-terminal pattern sequence (FPGVYTSVSHYRSWVE) of the SP domain.

3. *In vitro* PO activation assay showed that rSP-*Pm*ClipSP2 significantly enhances phenoloxidase activity of shrimp HLS in the presence of LPS and β-1,3-glucan. These results suggested that *Pm*ClipSP2 acts as PRP and participates in the proPO-activating system in *P. monodon* via the SP domain.

4. LPS neutralization showed that *Pm*ClipSP2 could not reduce LPS toxicity to S2 cells.

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APPENDIX

VITA

Mr. Narach Khorattanakulchai was born in March 2, 1992 at Bangkok, Thailand. He graduated with the degree of Bachelor of Science (B.Sc.) in the major of Biochemistry (First class Honor) from the department of Biochemistry, Faculty of Science, Chulalongkorn University in 2013. Then, he has studied for the degree of Master of Science, Program in Biochemistry and Molecular biology, Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2014. Then, he got the scholarship from The Chulalongkorn University graduate scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej, the 90th Anniversary of Chulalongkorn University Fund and also the Young Scientist and Technologist Program : YSTP.

Conference

1. Khorattanakulchai N., Amparyup, P., and Tassanakajon, A., 2016. Role of clip domain serine proteinase 2, PmClipSP2, in activation of prophenoloxidase-activating system in the black tiger shrimp, Penaeus monodon. The 21st Biological Science Graduate Congress. 15th - 17th December 2016, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. (Poster presentation)

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