การโคลนและลักษณะสมบัติของคลิปโดเมนซีรีนโปรติเนส *Pm*ClipSP1 จากกุ้งกุลาดำ *Penaeus monodon*

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CLONING AND CHARACTERIZATION OF CLIP DOMAIN SERINE PROTEINASE, *Pm*ClipSP1, FROM BLACK TIGER SHRIMP *Penaeus monodon*

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เกรียงพล วิริยะอัครเดชา : การโคลนและลักษณะสมบัติของคลิปโดเมนซีรีนโปรติเนส *Pm*ClipSP1 จากกุ้งกุลาดำ *Penaeus monodon* (CLONING AND CHARACTERIZATION OF CLIP DOMAIN SERINE PROTEINASE, *Pm*ClipSP1, FROM BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร.อัญชลี ทัศนาขจร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ปิติ อ่ำพายัพ, 134 หน้า.

คลิปโดเมนซีรีนโปรติเนสเป็นองค์ประกอบสำคัญในการควบคุมระบบภูมิคุ้มกันของสัตว์ที่ไม่มีกระดูกสันหลัง จากฐานข้อมูล EST ของกุ้งกุลาดำ (*Penaeus monodon*) ลำดับนิวคลีโอไทด์ที่สมบูรณ์ของยีน *Pm*ClipSP1 ประกอบไปด้วย open reading frame (ORF) จำนวน 1,101 คู่เบส ซึ่งสามารถแปลงเป็นโปรตีนที่มีกรดอะมิโน 366 ตัว โดยมี signal peptide จำนวน 25 กรดอะมิโน โครงสร้างของโปรตีน *Pm*ClipSP1 ประกอบด้วยคลิปโดเมนทางด้านปลายอะมิโนและซีรีนโปรติเนสโดเมนทางด้านปลายคาร์บอกซิล ในงานวิจัยนี้เราได้ทำการโคลนยีน *Pm*ClipSP1 ทั้งยีนและเฉพาะส่วนซีรีนโปรติเนสโดเมนเข้าสู่เวกเตอร์ pET22b(+) โดยได้มีการติด hexa-histidine tag เข้าที่บริเวณปลายอะมิโน รีคอมบิแนนท์โปรตีนจากยีน *Pm*ClipSP1 ทั้งยีนและเฉพาะส่วนซีรีนโปรติเนสโดเมนที่มีขนาด 37 และ 28 กิโลดาลตันถูกผลิตได้อยู่ในรูปที่ไม่ละลายน้ำ (inclusion body) ใน *Escherichia coli* โปรตีนที่ถูกผลิตสามารถทำให้บริสุทธิ์ด้วย Ni-NTA โครมาโทกราฟี จากการศึกษาหน้าที่ของรีคอมบิแนนท์โปรตีนทั้งสองพบว่าไม่มีคุณสมบัติของโปรติเนสแอกทิวิตีและไม่สามารถกระตุ้นฟีนอลออกซิเดสแอกทิวิตี จากการวิเคราะห์การแสดงออกของโปรตีนโดยวิธีเวสเทิร์นบลอต (Western blot) ด้วยแอนติบอดีที่จำเพาะต่อซีรีนโปรติเนสโดเมนของเอนไซม์ *Pm*ClipSP1 พบโปรตีน *Pm*ClipSP1 ในเม็ดเลือดกุ้งแต่ไม่พบในพลาสมา ในการยับยั้งการแสดงออกของยีน *Pm*ClipSP1 โดยการฉีดอาร์เอ็นเอสายคู่พบว่าประสบความสำเร็จในการลดระดับการเกิดทรานสคริปชัน แต่ไม่สามารถลดการทำงานของเอนไซม์ฟีนอลออกซิเดสในกุ้งที่ได้รับอาร์เอ็นเอสายคู่ แสดงว่ายีนนี้อาจจะไม่มีความเกี่ยวข้องกับระบบโพรฟีนอลออกซิเดส แต่อย่างไรก็ตามการลดการแสดงออกของยีน *Pm*ClipSP1 มีผลทำให้จำนวนของเชื้อแบคทีเรียในเลือดกุ้งเพิ่มขึ้นประมาณ 2.4 เท่า และมีอัตราการตายเพิ่มขึ้น 59% ในกุ้งที่ได้รับการฉีดด้วยเชื้อ *Vibrio harveyi* จากข้อมูลนี้แสดงถึงความสำคัญของยีน *Pm*ClipSP1 ในกระบวนการต่อต้านเชื้อแบคทีเรียในกุ้งกุลาดำ

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KRIANGPOL WIRIYAUKARADECHA: CLONING AND CHARACTERIZATION OF CLIP DOMAIN SERINE PROTEINASE, *Pm*ClipSP1, FROM BLACK TIGER SHRIMP *Penaeus monodon.* THESIS ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR: PITI AMPARYUP, Ph.D. 134 p.

Clip domain serine proteinases (clip-SPs) are the essential components of signaling cascades in the innate immune system of invertebrates. From the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th), a full-length cDNA of *Pm*ClipSP1 was characterized. It contains an open reading frame (ORF) of 1,101 bp encoding a predicted protein of 364 amino acids including a 25 amino acid signal peptide. The mature protein of *Pm*ClipSP1 exhibits a characteristic sequence structure of clip-SPs consisting of an N terminal clip domain and a C terminal SP domain. The mature protein and SP domain of *Pm*ClipSP1 were cloned into the pET22b(+) vector with an N terminal hexa-histidine tag fused in-frame, and expressed in *Escherichia coli* as 37 kDa and 28 kDa proteins, respectively. The recombinant proteins were successfully purified by Ni-NTA chromatography. Functional analysis revealed that the recombinant proteins lack a proteolytic activity and could not activate of phenoloxidase (PO) activity. Western blot analysis using the antibody raised against the SP domain of *Pm*ClipSP1 revealed that *Pm*ClipSP1 was present in hemocytes, but not in cell free plasma. Knockdown of the *Pm*ClipSP1 gene by double-stranded RNA (dsRNA) of *Pm*ClipSP1 gene, significantly reduced *Pm*ClipSP1 transcript levels, but did not significantly reduced the total PO enzyme activity, suggesting that *Pm*ClipSP1 might not involved in the proPO system. However, silencing of the *Pm*ClipSP1 gene led to a significant increase in the number of viable bacteria in the hemolymph (~2.4 fold) and in the mortality rate (59%) of shrimp systemically infected with *Vibrio harveyi*. These findings suggest that *Pm*ClipSP1 plays a role in the antibacterial defense mechanism of *P. monodon* shrimp.

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LIST OF ABBREVIATIONS

A	absorbance
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
clipSPs	clip domain serine proteinases
C-terminal	carboxyl terminal
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double stranded ribonucleic acid
dTTP	deoxythymidine triphosphate
EF1α	elongation factor 1 alpha
EST	expressed sequence taq
EtBr	ethidium bromide
GFP	green fluorescence protein
HLS	hemocyte lysate supernatant
HP	hemolymp protease
hr	hour
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L-dopa	L-3,4-dihydroxyphenylalanine
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar

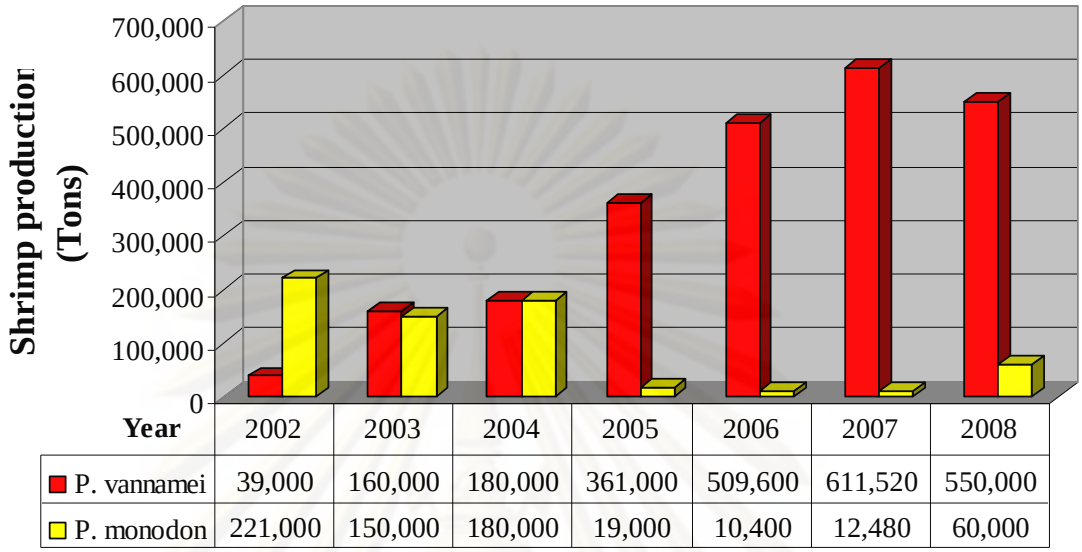
MP	melanization protease
ng	nanogram
nm	nanometer
N-terminal	amino terminal
OD	optical density
^{o}C	degree Celsius
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAP, PPAE, ppA	prophenoloxidase activating enzyme
PCR	polymerase chain reaction
*Pm*ClipSP1	serine proteinase 1 of *Penaeus monodon*
PO	phenoloxidase
PPAF	prophenoloxidase activating factor
proPO	prophenoloxidase
rm*Pm*ClipSP1	recombinant *Penaeus monodon* serine proteinase 1 mature protein
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
rSP-*Pm*ClipSP1	recombinant serine proteinase domain of *Penaeus monodon* serine proteinase 1
RT	reverse transcription
SDS	sodium dodecyl sulfate
s	second
SP	serine proteinase
SPH	serine proteinase homolog
SP-like domain	serine proteinase like domain
UTR	untranslated region
WSSV	white spot syndrome virus
YHV	yellow head virus
μg	microgram
μl	microliter
μM	micromolar

CHAPTER I
INTRODUCTION

1.1 General introduction

Around 80% of shrimp products come from Asia with Thailand, China, Indonesia and India as the main producers. Shrimp farming in Thailand is a multi-billion dollar industry contributing a major income to the country. Thai shrimp farming began in the early 1980s and widely distributed to different areas in the mid 1980s (Source: http://www.american.edu/ted/THAISHMP.HTM). Shrimp farms and hatcheries are mainly located along the coastal areas of Thailand. Southern provinces (Nakorn Sri Thammarat, Surat Thani) account for the majority while those in the East (Chanthaburi) and Central regions (Samut Sakhon, Samut Songkhram) comprise the minority in terms of number of farms.

The shrimp production was seriously affected by the outbreaks of infectious disease. Generally, the causes of infectious diseases in shrimp are mainly virus and bacteria (Bachère, 2000). Diseases are now rapidly spreading over the world as a result of expansion and globalization of the shrimp producing countries (Rönnbäck, 2001). Previously, black tiger shrimp, *Penaeus monodon*, account for more than half of the total shrimp aquaculture. Whereas the other commercial shrimp product are *P. vannamei, P. indicus, P. merguiensis* and *P. chinensis*. Since 2005, the production of black tiger shrimp in Thailand has been decreased while the shrimp culture was favor to the pacific white shrimp, *P. vannamei* (Figure 1.1) because of its advantage. Genetic selection is successfully performed in the white shrimp leading to the effective growth rate, disease resistance and high survival rate during larval rearing. Whereas so far breeding of black tiger shrimp has not been successful (Wyban, 2007).



Year	2002	2003	2004	2005	2006	2007	2008
P. vannamei	39,000	160,000	180,000	361,000	509,600	611,520	550,000
P. monodon	221,000	150,000	180,000	19,000	10,400	12,480	60,000

Figure 1.1 The black tiger shrimp and white shrimp production in Thailand from 2002 to 2008. (Source: http://www.shrimpcenter.com)

1.2 Taxonomy of *Penaeus monodon*

Penaeus monodon, is a shrimp species that was classified into the largest phylum in the animal kingdom, the Arthropoda. The taxonomic definition of *Penaeus monodon* is as follows (Baily-Brook et al., 1992):

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Subclass Eumalacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

Scientific name: *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(Philipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Timsa (Vietnam).

FA.O. Names: Giant tiger prawn, Crevette giante tigre, Camaron tigre gigante.

1.3 Shrimp diseases

Almost from the beginning disease was remembered as a biological threat to the shrimp farming, and some diseases caused severe economic losses. The major causes of infectious disease in *P. monodon* are virus and bacteria (Lightner et al., 1998). The severe problem of shrimp diseases in Thailand was begun in 1993. From 1993 to 1994, the yellow head disease was reported in center and southern of Thailand (Hasson et al., 1995). Whereas the white spot syndrome disease was initial begun between 1994 to 1996. As a result, shrimp export producing was reduced from 1992 high of 115,000 metric tons to 35,000 metric tons (Flegel, 1997). Vibriosis is the most prevalent bacteria disease causing mass mortalities in shrimp farming. The major virulent strains of *Vibrio* in shrimp are *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and *V. anguillaram* (Sunaryanto et al., 1986). The application of modern biotechnology to penaeid shrimp diseases has been essential for rapid and sensitive diagnosis. Therefore, the prevention and control of diseases turned into a priority for shrimp production. To deal with this problem, besides the development of farm management, shrimp immunity and defense effectors responded to pathogen should be elucidated.

1.3.1 Viral disease

Disastrous failures have occurred in the shrimp farming industry in Thailand over a decade mostly due to virus infection. White spot syndrome virus (WSSV) and Yellow-head virus (YHV) are the important virus species that have been reported in *P. monodon*. They cause white spot syndrome disease (WSS) and yellow-head disease (YH), respectively (Boonyaratpalin et al., 1993; Wongteerasupaya et al., 1995). Moreover, infectious hypodermal and hematopoeitic virus (IHHNV), hepatopancreatic parvovirus (HPV) and monodon baculovirus (MBV) infections are related to the impeding of shrimp growth. In Thailand, Taura syndrome and (TSV) and Laem Singh virus (LSNV) are now important infectious virus in shrimp farming. The outbreak of these viruses causes a great loss in the shrimp industry in several countries including Thailand.

1.3.1.1 White spot syndrome virus (WSSV)

White spot syndrome virus (WSSV) is an enveloped DNA virus of bacilliform to cylindrical morphology with an average size of 120 x 275 ± 22 nm and has a tail-like projection at one end of the particle (Kasornchandra et al., 1995; Wongteerasupaya et al., 1995). WSSV was first called a baculovirus because of its cylindrical morphology and the histological lesions that resembled those of "non-occluded" baculoviruses (Wongteerasupaya et al., 1995).This virus is a member of the virus family Nimaviridae (genus *Whispovirus*). The enveloped virus that infects a broad range of crustacean species (Wang et al., 1998). The viral genome contains double-stranded DNA of ~292 to 305 kb in length from the three different virus isolate (WSSV-TH, WSSV-CN and WSSV-TW) (Van Hulten et al., 2001; Yang et al., 2001). In cultured shrimp, WSSV infection can cause a cumulative mortality of up to 100% within 3–10 days, leading to large economic losses to the shrimp-culture industry. The apparent sign of WSSV infection is the white inclusion of various size embedded in shrimp cuticle at the last stages of infection. The causative agent was a new bacilliform virus (Takahashi et al., 1994). Thus, white spots in the cuticle are unreliable for diagnosis of WSSV.

WSSV was first discovered in the Chinese province Fujian in 1992, from where it spread quickly (Flegel, 1997). Whereas the first reported epidemic due to this virus is from Taiwan in 1992 (Chen, 1995). However; reports of losses due to white spot disease came from China in 1993 (Huang et al., 1995), where it led to a virtual collapse of the shrimp farming industry. This was followed by outbreaks in Japan and Korea in the same year, Thailand, India and Malaysia in 1994 and by 1996 it had severely affected East Asia and South Asia. In late 1995, it was reported in the USA, 1998 in Central and South America, 1999 in Mexico and in 2000 in the Philippines. Currently, it is known to be present in all shrimp growing regions except Australia. Nowadays, the virus has spread to almost all major shrimp-farming areas of the world. In 1996, Lightner pointed out that no significant resistance to this disease had been reported for any species of shrimp, and this still remains true today. The causative agent of WSS, WSSV is extremely virulent and has a wide host range (Lo et al., 1996).

The disease is thought to spread by means of contaminated water, decomposing fecal matter or tissue, cannibalism and fluid from infected females. Direct transmission can occur between unrelated crustacean species. Shrimp may be indirectly exposed to the disease through expose to previous hatchery or pond growing cycles, contaminated water supplies (new or previously utilized) contaminated food (through unlikely), equipment surfaces and clothing, or animals who have ingested diseased shrimp. Humans may also facilitate transmission of the disease by global transportation of viruses in infected frozen imported shrimps. Shrimp, which survive the infection, are suspected to be life-long carriers of WSS. It is difficult to prevent and inhibited the WSSV infection because this virus is survive for long time in the environment (2 years in a shrimp pond).

Rapid and specific diagnosis of the virus is carried out using two step-nested polymerase chain reactions (PCR). Histopathological changes in infected shrimps include prominent intranuclear eosinophilic to basophilic inclusions in the infected cells and cellular degeneration with hypertrophied nuclei and chromatin margination in the cuticular epidermis, gill epithelium, antennal gland, haematopoeitic tissue, nervous tissue and connective tissue and cellular necrosis and detachment of intestinal epithelial tissue (Wongteerasupaya et al., 1995).

1.3.1.2 Yellow head disease

Yellow head disease (YHD) is a viral infection of shrimp and prawn, in particular of *P. monodon*, one of the two major species of farmed shrimp. The disease is highly lethal and contagious, killing shrimp quickly (Wongteerasupaya et al., 1995). Outbreaks of this disease have wiped out in a matter of days the entire populations of many shrimp farms that cultivated *P. monodon*, i.e. particularly Southeast Asian farms (Source: http://nis.gsmfc.org/nis_factsheet.php?toc_id=119). In Thailand, the virus was first reported in 1990's. This syndrome occurs in the juvenile to sub-adult stages of shrimp, 5 to 15 grams in size, especially at 50-70 days of grow-out (Lightner, 1996). At the onset of YHD shrimp have been observed consuming feed at an abnormally high rate for several days. Feeding abruptly ceases and within 1 day, a few moribund shrimp appear swimming slowly near the surface at the pond edges. After that, the light yellow coloration is occurred at dorsal

cephalothorax in YHV infected shrimp moreover a generally pale or bleached are also appeared, later, it will die within a few hours.

YHV was first considered to be a baculovirus. But, it was later discovered during purification and characterization that its morphology differed from that of baculoviruses (Boonyaratpalin et al., 1993). Recently, YHV was classified in new taxa family Roniviridae genus *Okavirus* (Walker et al., 2008). YHV is rod-shaped, enveloped virus. The viral genomes consist of single-stranded RNA (ssRNA) of positive sense with a helical nucleocapsid. Viral replication seems to occur only in the cytoplasm without any sign of replication in the intact nuclei of infected cells. A long filamentous of the virus (some over 800 nm in length), perhaps a precursor to the enveloped and rod-shape form is presented in the cytoplasm of many host cells. Viral envelopes appear to be acquired by passage of these provirions through the endoplasmic reticulum of the host cells. Enveloped virions then cluster in cytoplasmic vesicles, sometimes densely packed, and resembling paracrystalline arrays, where they appear to divide into the smaller rod-shaped units (Chantanachookin et al., 1993). Rod-shaped virions and filamentous precursors were found in normal, healthy, captured (wild) broodstock by Flegel et al. (1992). YHV may occur as latent, asymptomatic infections in broodstock shrimp and may possibly transfer from these shrimp to their offspring in larval rearing facilities (Chantanachookin et al., 1993).

YHV infections can be recognized by densely basophilic inclusions, particularly in H&E stained gill sections and rapidly stained whole gills (Flegel et al., 1997), or by staining of hemolymph smears (Nash et al., 1995). The diagnosis of YHV infection could be performed by using immuno-histochemistry, the monoclonal antibody aggregated with a surface glycoprotein and the nucleocapsid protein of virus (Sánchez-Barajas et al., 2009). In addition to conventional RT-PCR (Wongteerasupaya et al., 1997) or *in situ* hybridization (Wongteerasupaya et al., 1996; Tang et al., 1999) was advantage for detection of YHV infection too.

1.3.2 Bacterial disease

In Thailand, vibriosis is the major cause of production loss in penaeid shrimp farm (Nash et al., 1992). This bacterial outbreak causes mortality of the affected shrimps up to 100%, whether they are larvae, post-larvae, juveniles, sub-adults or adults (Lightner, 1983). *Vibrio harveyi* was originally recovered from a dead sandbar shark (*Carcharhinus plumbeus*) with vasculitis, which died at the National Aquarium in Baltimore, USA, in 1982 (Grimes et al., 1984) and is also called *V. carchariae.* Extracellular products (ECP) were assigned to be important determinants of virulence in *V. harveyi.* The study of pathogenicity from *Vibrio*-infected *P. monodon* determined that virulence occurred with both live bacteria and ECP (Liu et al., 1996).

Vibrio harveyi is Gram-negative bacteria; therefore it has a cell wall that consists of two membranes: an outer membrane made up of lipopolysaccharides and an inner cytoplasmic membrane. In between these is a periplasmic space housing a peptidoglycan layer. *V. harveyi* is a facultative anaerobe, meaning it can swap between aerobic respiration of oxygen and fermentation in order to produce ATP. This characteristic helps *V. harveyi* survive in low oxygen concentrations, if fermentable material is present. The cells of the *Vibrionaceae* are non-sporulating rods, usually 0.5-0.8 μm width and 1.4-2.6 μm in length, and they have locomotor organelles called flagella. Vibrios have a group of flagella at one end if the cell (polar flagella) and the flagella are encased in a sheath that is a continuation of the outer membrane of the bacterial cell wall. In contrast, the Photobacterium genus only has unsheathed flagella. The ability to produce light is dependent upon the concentration of the organisms in the substrate (i.e., sea water or special growth medium in the laboratory). The light generating reactions require oxygen, and the final product of luminescence reactions is excited luciferase which can generate light (Showalter et al., 1990). The substrates are reduced flavin mononucleotide ($FMNH_2$), a long chain aldehyde (RCHO; probably tetradecanal), and molecular oxygen which react according to the following overall stoichiometry:

Bioluminescent bacteria produce a specific chemical called an autoinducer (sometimes more than one), which can induce bioluminescence reactions in bacterial

cells when they are in high concentrations. At lower concentrations, specifically when dispersed in the ocean, the cells do not produce light.

V. harveyi is a pathogen of fish and invertebrates, including sharks, seabass, seahorses, lobster, and shrimp. Its pathogenicity depends on the concentration of *V. harveyi* cells at a given time. Diseases caused by *V. harveyi* include eye-lesions, gastro-enteritis, vasculitis, and luminous vibriosis. Luminous vibriosis is a leading cause of death among commercially farmed shrimp and other aquaculture. The infection, by *V. harveyi*, enters through the mouth and forms plaques, then spreads to the innards and the appendages. Loss of limb function and appendage degradation has been documented. This bacteria has been reported to be a factor in loose shell syndrome and white gut disease in *P. monodon* in India (Jayasree et al., 2006). Contamination can spread all the way to egg and larval tanks, thus causing an even bigger problem for shrimp farmers. Luminous vibriosis has been documented in many other crustaceans all of which glow in the dark when infected. Mortality occurs when penaeid shrimp is exposed to a concentration of *V. harveyi* at 102 cells/g of tissue homogrnate (Lightner, 1993).

The past controlling of this disease by using antibiotic in broader shrimp farming was the problem of drug resistance (Karunasagar et al., 1994). The probiotic such as *Pseudomonas* I-2 or *Bacillus subtilis* BT23 have been a choice for solution of Vibriosis in present farming (Chythanya et al., 2002; Vaseeharan et al., 2003).

1.4 Crustacean immune system

Invertebrate animal have the native immune responses called innate immune system. These immune response differences to the adaptive immune system in vertebrate animal that produce the specific molecule, typical immunoglobulins are known as antibody memorizes foreign molecules. Both vertebrate and invertebrate have developed unique modalities to detect and response to microbial surface molecules like lipoplysaccharide (LPS), lipoteichoic acid, peptidoglycan (PGN) and β-glucan (Begum et al., 2000). This assumed that the recognition system was developed at early state of animal evolution (Janeway Jr et al., 1999; Medzhitov et al., 2000).

Crustacean immune responses are involved by cell-mediated and humeral component in the circulatory system. First defense process is initiated by the hemocyte and the plasmatic proteins are recognizing the invading microorganisms. Crustacean hemocyte plays important role in host immune system including phagocytosis, melanization, cytotoxicity and cell-cell communication (Jiravanichpaisal et al., 2006). The hemolymph plasma of crustacean contains many defense molecules including enzyme and protein such as antimicrobial peptide, proteinase inhibitors, hydrolytic enzyme, hemocyanin, phenoloxidase etc.

1.4.1 Blood cell

Crustacean hemocyte could be classified by presenting of cytoplasmic granules to three major types that consist of hyaline, semi-granular and granular cell. Each hemocyte cell type has a difference role for its immune responses such as phagocytosis, encapsulation, cytotoxicity, haemolysis, cell adhesion, and degranulation (Johansson et al., 2000). The phagocytosis and coagulation are the responsibility from the hyaline, smallest hemocyte that without cytoplasmic granules (Söderhäll et al., 1986). Semigranular, the most number of total hemocyte approximately 75 % of all hemocyte, contain small granular (0.4 μm diameter) which exhibit some phagocytic capacity. Semigranular that most sensitive and first to reaction during an immune response, are a function of encapsulation and degranulation. Granular cell (10-20 % of total hemocyte) contains a large number of secretary large granular (0.8 μm diameter). Granular and semi-granular cell can store the cytotoxic reaction and the component for activation of prophenoloxidase system (Smith et al., 1983). L-granules contain at least 24 proteins, a majority of which are clotting factors, a clottable protein, coagulogen, proteinase inhibitors, lectins, and antimicrobial proteins. In contrast, S-granules contain at least 6 proteins with molecular masses of less than 30 kDa, in addition to an antimicrobial peptide tachyplesin and its analogues (Muta et al., 1990; Shigenaga et al., 1993).

1.4.2 Pattern recognition protein

The innate immune system employs germline-encoded pattern recognition receptors to identify pathogen-associated molecular patterns (PAMPs) which are absent in the host but present on the surface of pathogens (Medzhitov et al., 2002).

The best known examples of PAMPs include LPS of gram-negative bacteria, PGN of gram-positive bacteria, the manan of yeast, glucan of fungi and double-stranded RNA of viruses (Hoffmann et al., 1999; Kurata et al., 2006). The process of recognition of invading microorganism is mediated by the hemocyte and by plasmatic protein (Medzhitov et al., 1997).

The carbohydrates are regular components of microbial cell wall. Heamagglutinin or lectins can bind to specific carbohydrates expressed on different cell surfaces due to an occurrence of agglutination reaction. The β–1,3-glucan binding protein (BGBP) were reported in many crustaceans such as freshwater crayfish, *Pacifastacus leniusculus* (Duvic et al., 1990), and three marine shrimp species, *P. californiensis* (Vargas-Albores et al., 1996), *P. chinensis* (Du et al., 2007), *P. vannamei* (Vargas-Albores et al., 1997; Jiménez-Vega et al., 2002) and *P. monodon* (Sritunyalucksana et al., 2002). The BGBP has not been shown to contain glucanase activity although it has glucanase-like motif. After BGBP binding with glucans, it can operate as elicitors of defense responses (Muta, 1995; Seki et al., 1995). LPS-binding protein, a multivalent carbohydrate-binding agglutinin, can increases phagocytic rate (Vargas-Albores, 1995).

1.4.3 The prophenoloxidase (proPO) system and melanization

The prophenoloxidase activating melanization is an important innate immune system in invertebrate. The phenoloxidase (PO) occurs as inactive enzyme which named prophenoloxidase (proPO). PO catalyzes both the o-hydroxylation of monophenol and oxidation of phenol to quinone following non-enzymatically polymerized to melanin (Cerenius et al., 2004). The proPO was activated by proPO-activating enzyme, which is the terminal proteinase of serine proteinase cascade (Figure 1.2). Some arthropod, the proPO activation required one or more serine proteinase homolog, non-catalytic proteinase. This cascade is initiated via recognition of microbial surface molecules by specific binding proteins. The melanization in arthropod is involving in process of wound healing and sclerotization (Sritunyalucksana et al., 2000). This defense reaction results in nodule formation and encapsulation which caused against invading microorganism entering the body. The expression analysis shows that proPO mRNA of *P. monodon* is only expressed in hemocyte (Sritunyalucksana et al., 1999). The enzymes of proPO system are normally

localized in the semigranular and the granular cell of Penaeid shrimps (Vargas-Albores et al., 1993; Perazzolo et al., 1997).

From the blast searching, deduced amino acid sequence of *P. monodon* proPO has highly similarity to crayfish proPO (74%). In horseshoe crab, hemocyanin binding to some clotting factor component can be exhibited *in vitro* PO activity (Nagai et al., 2000). They demonstrated that proPO and hemocyanin are the same evolutionally relationship. PO can bind to the parasite leading to induction of malanization. A second isoform of proPO genes was identified from *P. monodon* (Amparyup et al., 2009A) and *P. vannamei* (Ai et al., 2009) which are shown more similarity to proPO of other penaeids than proPO from crayfish, lobsters, crab, or a freshwater prawn. Two proPOs are mainly expressed in hemocyte (Yeh et al., 2009). The dsRNA of a proPO gene was injected to freshwater crayfish resulting the increasing of *Aeromonas hydrophila* in hemolymph and reduction of survival bacterial infected crayfish (Liu et al., 2007). The same result was observed in proPO knocked down *P. monodon* that was infected with *V. harveyi* (Amparyup et al., 2009A).

Most serine proteinases in proPO cascade of arthropod contain clip domain at N-terminus of zymogen. The terminal SPs which activate proPO are also called proPO activating enzyme (PPAE). In crayfish, PPAE is expressed in the hemocyte granules as an inactive from. After the presenting of microbial in hemolymph, PPAE will be secreted into plasma and converted to an active form (Aspàn et al., 1991; Aspàn et al., 1995). The specific inhibitor of crayfish PPAE, pacifastin, can be inactivated PO activity in crayfish. Recently, a cDNA encoding a PPAE (*Pm*PPAE1) from *P. monodon* was cloned and characterized. RNAi-mediated silencing of *Pm*PPAE1 gene significantly decreased the total PO activity in shrimp and additionally increased the mortality of *V. harveyi* infected shrimp, the latter of which correlated with an increase in the number of viable bacteria in the hemolymph (Charoensapsri et al., 2009). In several insect and crustacean immunities, there is a report that the proPO activation was corresponding to the enhancing of phagocytosis. The crayfish PPAE is involved in processing both proPO and peroxinectin (Lin et al., 2007). The crustacean peroxinectin, an active form of a large family of cell adhesion proteins, is a protein expressing strong cell adhesion, opsonin and degranulation activities (Jiravanichpaisal et al., 2006).

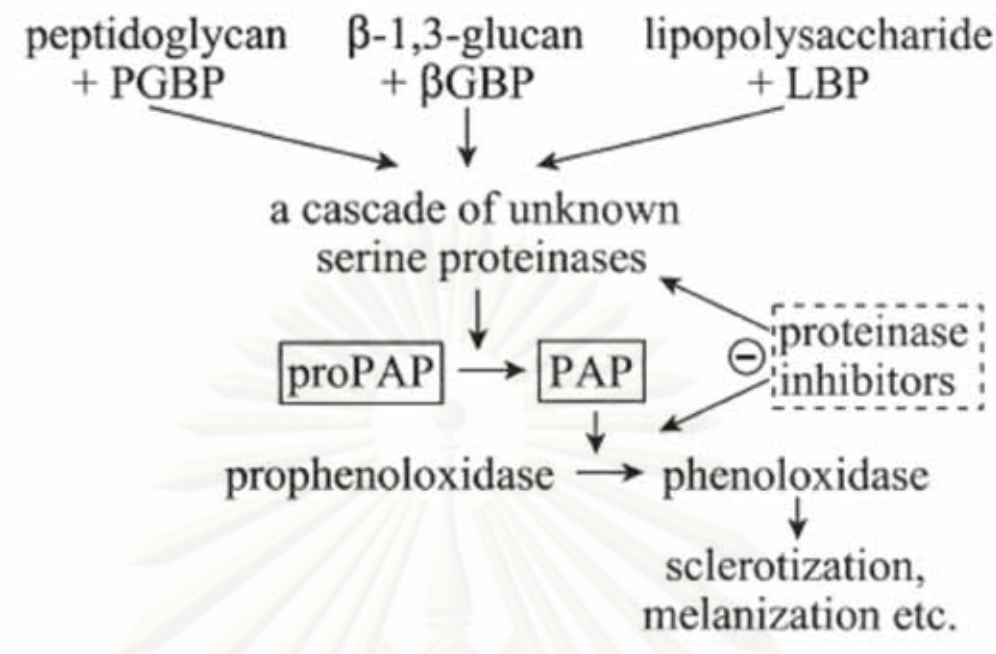


Figure 1.2 The prophenoloxidase activating system in insect hemolymph. Peptidoglycan from Gram-positive bacteria, lipopolysaccharide from Gram-negative bacteria, and β -1,3-glucan from fungi are recognized by specific binding proteins in hemolymph: peptidoglycan-binding protein (PGBP), lipopolysaccharide-binding protein (LBP), and β-1,3 glucanbinding protein (β-GBP), respectively. Formation of recognition complexes somehow triggers a cascade of unknown serine proteinases. At the end of the proposed pathway, proPO activating proteinase (PAP or PPAE) is activated through limited proteolysis. Activated PAP (or PPAE) cleaves prophenoloxidase to generate phenoloxidase. (Jiang et al., 2000)

1.4.4 The clotting system

The blood clotting system or coagulation is the protection system form blood lost after injury. Moreover this system is the first line of defense and an integral part of the overall invertebrate immune system. The blood clotting system in arthropods has two mainly difference mechanisms: crayfish and horseshoe crab. The clotting system in crayfish depends on the direct tranglutaminase (TGase)-mediated cross linking of a specific plasma protein, whereas the process in horseshoe crab is regulated by a proteolytic cascade activated by bacterial elicitors through specific recognition proteins.

In horseshoe crab, four serine proteinase and a clottable protein (coagulogen) are involved microbial polysaccaride-mediated coagulation cascade (Iwanaga et al., 1998). The factor C is autocatalytically activated when bacterial lipopolysaccaride was detected and involve the factor B becoming the activated factor B (Muta et al., 1993). Then the activated factor B will convert the procloting enzyme to the clotting enzyme (Nakamura et al., 1993). In addition the factor G directly activate the proclotting enzyme after the presence of β–1,3-glucan (Iwanaga, 1993). The clotting factor are stored in the granule of hemocyte until the cell are activated, which then the clotting factor will be released into hemolymph by degranulation. After the serine proteinase cascade activation, the coagulogen, a soluble protein, is conveted to coagulin, an insoluble aggregate. The clot formed through the activation of this cascade is effective for immobilizing invading microorganisms (Kawabata et al., 1996).

In crayfish, the blood clot is formed by the clottable proteins (CPs) in plasma that catalyzed by a Ca^{2+} dependent TGase (Hall et al., 1999). The coagulation is processed by the TGase forms the ε-(γ-glutamyl)-lysine crosslinks between glutamine and lysine of the CPs (Kopacek et al., 1993). The releasing of TGase is a result from the hemocytes under foreign particle stimulus or tissue damage. The CPs were found in several crustaceans such as crayfish (Kopacek et al., 1993), *P. monodon* (Yeh et al., 1998), lobster, *Panulirus interruptus* (Doolittle et al., 1990). The CPs are glycoprotein that has physiological functions in the prevention of pathogen infection and the lipid transport (Hall et al., 1995). TGase have been cloned and localized in crayfish (Wang et al., 2001) and further characterized in tiger shrimp (Huang et al., 2004).

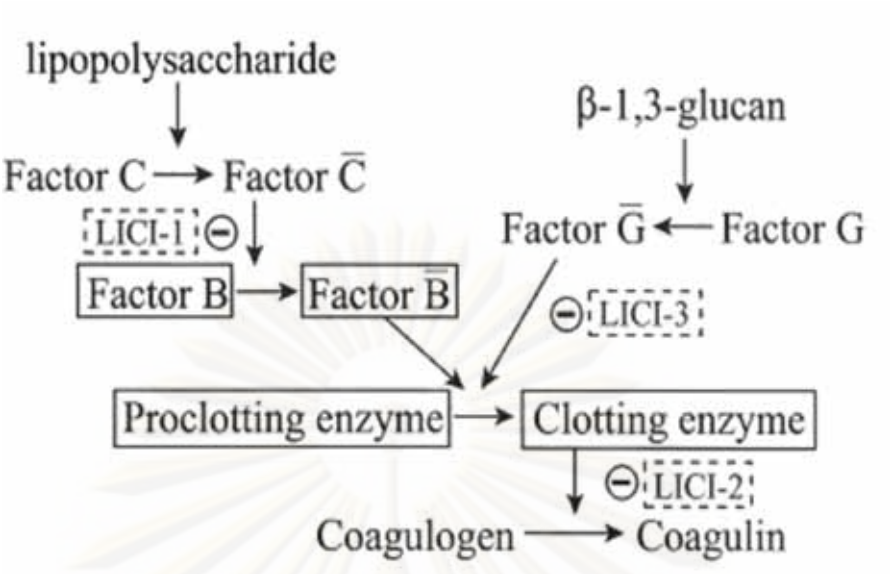


Figure 1.3 The hemolymph clotting system of horseshoe crab. Microbial cell surface molecules can bind and trigger a cascade of serine proteinases that are released from hemocytes upon infection. As a result, coagulogen is converted to coagulin which forms an insoluble gel. These clotting enzymes are subject to negative regulation by specific serine proteinase inhibitors of the serpin superfamily. (Iwanaga et al., 1998)

1.4.5 Antimicrobial peptides (AMPs)

Antimicrobial peptides are a component of the innate immune response that has been evolutionarily conserved. They are found in all different classes of life. Antimicrobial peptides are the small molecules generally less than 150-200 amino acid residues. The most of AMPs are the cationic peptide and have amphipathic structure. The target of cationic AMPs generally is bacterial membrane. The integration of peptide into the cell membrane, anionic phospholipids, resulting aqueous content might leak from target cell lead to cell lysis. The several of antimicrobial peptides were isolated and characterized from crustacean, as mainly anti-lipopolysaccaride factors, penaeidins and crustins.

Anti-lipopolysaccaride factor (ALF) is an AMP that was found in haemocytes of horseshoe crabs, *Limulus polyphemus,* named LALF (Morita et al., 1985), and *Tachypleus tridentatus,* named TALF (Tanaka et al., 1982). This protein is localized in large granule of horseshoe crab hemocyte which was found functionally prevention of Gram-negative bacteria (Muta et al., 1990). ALF can bind and neutralized the endotoxic or lipopolysaccharide (LPS). A moiety of LPS leads to inhibition of the

endotoxin-mediated activation of the coagulation cascade. It can exhibits strong antibacterial activity on the growth of Gram-negative R-type bacteria (Morita et al., 1985). In shrimp, at least 5 isoforms of ALF was identified from *P. monodon* EST database (Supungul et al., 2004). The expression of ALF*Pm*2 and ALF*Pm*3 was rapidly increased in *V. harveyi* challenged shrimp (Tharntada et al., 2008). Moreover, the recombinant ALF*Pm*3 protein exhibits antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria and fungi (Somboonwiwat et al., 2008) and exhibits antiviral activity (Tharntada et al., 2009).

Crustins are antibacterial proteins of 7-14 kDa with a characteristic four-disulphide core-containing whey acidic protein (WAP) domain. Recently, crustin was classified into three types (Type I, Type II and Type III) based mainly on the domain structure between the signal sequence and the WAP domain (Smith et al., 2008). Genomic approaches, such as expressed sequence tag (EST) analysis, have identified several homologues of crustin from a variety of crustacean species including shrimps, crabs, crayfish and lobsters (Smith et al., 2008). In *P. monodon*, the recombinant crustin*Pm*1, crustin*Pm*5, crustin-like*Pm* and SWD*Pm*2 (Type III crustin) were expressed in *E. coli*. The recombinant crustin*Pm*1, crustin*Pm*5 and SWD*Pm*2 were exhibited anti-gram positive bacterial activity (Supungul et al., 2008; Vatanavicharn et al., 2009) whereas crustin-like*Pm* can inhibit the growth of both Gram positive and Gram negative bacteria (Amparyup et al., 2008).

Peneadin belong to a family of AMP which combines a proline-rich amino-terminal domain and a carboxyl-domain containing six cysteines engaged in three disulfide bridges. It was first isolated from the plasma and hemocyte of *P. vannamei*. The fist three were named penaeidin-1, -2 and -3, and their structure and antimicrobial were studied using recombinant protein (Destoumieux et al., 1997). The cDNA clones of penaeidin isoform were also isolated from the haemocytes of *L. vannamei* and *L. setiferus* (Gross et al., 2001) and *P. monodon* (Supungul et al., 2004). Penaeidins are constitutively synthesized and stored in the shrimp hemocytes, localized in granulocyte–cytoplasmic granules, and released in response to appropriate stimuli such as infections (Munìfoz et al., 2002). In black tiger shrimp, penaedin-2 precursor was reported from EST libralies (Supungul et al., 2002). In addition, the synthetic peptide of penaiedin-5 exhibited anti-bacterial activity and anti-fungal activity. The

expression level of penaeidin-5 was induced after *V. harveyi* challenged (Hu et al., 2006).

1.4.6 Proteinase inhibitor

The proteinases of invading pathogens and the endogenous proteinases of the several zymogen cascades, involved in blood clotting, proPO activating system or signaling pathway, have the potential for undesirable destructive action. It allowed they survive to act beyond the limits of their intended target. The inhibitors of proteinases have evolved as important elements in the system of host defenses against pathogens and as regulators of endogenous proteinases.

Some proteinase cascades were regulated by proteinase inhibitors such as serine proteinase inhibitors in the Kazal and Serpin families (Kanost et al., 2001; Kanost et al., 2001; De Gregorio et al., 2002; De Gregorio et al., 2002), α-macroglobulin (Vargas-Albores et al., 1996; Armstrong et al., 1999) .

Like blood clotting, prophenoloxidase activation is normally regulated *in vivo* local reaction with brief duration. Also comparable to blood clotting, the regulation may be partly due to serine proteinase inhibitor in plasma (Kanost et al., 1996). For example, pacifastin and α–macrogrobulin can inhibit crayfish proPO activation (Aspàn et al., 1990). In *M. sexta*, serine proteinase in proPO system can be inhibited by serpin-1J (Jiang et al., 1997). In addition, serpin-6 from *M. sexta* hemolymp inhibit proPO-activating proteinase-3 (PAP-3) (Wang et al., 2004). While serpin-4 and -5 decreased pro-PO activation when added to plasma, but they did not directly inhibit the pro-PO-activating proteases (Tong et al., 2005). The proteinase inhibitors in proPO system may functionally protect host cell damage from toxically by product in this activation system. The serpin mark up a superfamily of protein, most of which function as serine proteinase inhibitor. Serpin contain an exposed reactive site loop, which interacts with the active site of a proteinase, leading to the formation of a vary stable serpin-proteinase complex (Stone et al., 1997). It appears likely to be an acyle enzyme complex that represents a normal intermediate on the substrate pathway of a serine proteinase (Olson et al., 1995). In shrimp, the transcription level of of serpin from *F. chinensis* was up regulated when challenged with bacteria, *Vibrio anguillarum* or *Stephylococcus aureus* , and WSSV (Liu et al., 2009).

Kazal, Kunitz and light chain of pacifastin are the path of low molecular weight serine proteinase inhibitor that may occur as single, small protein or some cases as chains of inhibitor domain (Nakamura et al., 1987; Johansson et al., 1994; Liang et al., 1997). The Kazal-type serine proteinase inhibitors were identified from many shrimp such as *P. chinensis*, *P. vannamei*, *P. monodon* etc. (Jarasrassamee et al., 2005; Jiménez-Vega et al., 2005; Kong et al., 2009). The mRNA level of *L. vannamei* four Kazal domain protein was modified after injected with *Vibrio anguillarum* (Jiménez-Vega et al., 2005). The Kazal-type SPI*Pm*2 exhibited the inhibitory activity against subtilisin. This function may as a defend component against proteinases from pathogenic bacteria (Somprasong et al., 2006). Their recombinant inhibitor was found to possess bacteriostatic activity against the *Bacillus subtilis* (Donpudsa et al., 2009). Besides, pacifastin and α–macroglobulin inhibit crayfish PPO activation (Aspàn et al., 1990). The kuruma shrimp, *Marsupenaeus japonicus*, α_2-macroglobulin was responded to oral administration of peptidoglycan (Rattanachai et al., 2004).

1.4.7 Apoptotic and tumor proteins

The apoptosis is a mechanism of cell suicide in response to verity of stimulus. In muticellular organism, apoptosis is essential for development, tumor suppression, immune function and maintenance of homeostasis. Viruses can directly induce apoptosis of infected cell (O'Brien, 1998). In insect, apoptosis is reported to be extremely powerful in suppressing of virus replication, infectivity and spread, via mechanisms that involve the premature lysis of infected cell (Clem, 2005; Wakiyama et al., 2006). Apoptosis has been detected in several virus target tissue of shrimp such as hemocyte, hematopoietic tissue and lymphoid organ (Khanobdee et al., 2002; Wongprasert et al., 2003; Sahul Hameed et al., 2006; Anantasomboon et al., 2008). The study in *P. monodon*, apoptosis was detected after YHV infection. There is a major cause of dysfunction and death of the host (Khanobdee et al., 2002). In *P. japonicus*, high mortality of WSSV infection was occurred together with a high incident of apoptosis (Wu et al., 2004). This result associated the recent research that apoptosis might be implicated shrimp death owing to viral infection (Flegel et al., 1995).

1.5 Serine proteinases (SPs) and serine proteinase homologues (SPHs)

1.5.1 Mechanism of action of serine proteinases

Serine proteinase (SP) is a group of endopeptidase that cleaved peptide bond in protein (Neurath, 1985) in which one of the amino acid at the active site is serine (Phillips et al., 1992). The SP is belonging to one of four protease families. Generally SP can be classified to 6 clans. Six clans are consisted of clan A to clan F especially clan A contains a families that share a common origin with chymotrypsin such as trypsin, elastase and the enzyme of blood clotting system (Barrett et al., 1995). These enzymes typically are synthesized in inactive forms which require activation by cleavage of a peptide bond near the N-terminus (Neurath, 1989). In chymotrypsin this is between Arg-15 and Ile16; the free, protonated amino group of Ile16 is important for the mechanism. The amino-terminal peptide with residues 1 through 13 stays attached to the rest of the protein through a disulfide bond. In trypsin the activation cuts off an amino-terminal hexapeptide, which does not remain attached (Neurath et al., 1976). Whereas, thrombin, does not have its amino terminal domain attached by a disulfide bond and goes free in the plasma to attack fibrinogen and generate clots (Dunn et al., 1982).

The peptide bond is cleaved by nucleophilic attack of the serine hydroxyl group on the scissile carbonyl bond, forming an acyl enzyme intermediate (Figure 1.4). The carbonyl carbon of this bond is position near the nucleophilic serine. The serine-OH attacks the carbonyl carbon, and the nitrogen of the histidine accepts the hydrogen from the -OH of the serine and a pair of electrons from the double bond of the carbonyl oxygen moves to the oxygen. As a result, a tetrahedral intermediate is generated. The bond joining the nitrogen and the carbon in the peptide bond is now broken. The covalent electrons creating this bond move to attack the hydrogen of the histidine, breaking the connection. The electrons that previously moved from the carbonyl oxygen double bond move back from the negative oxygen to recreate the bond, generating an acyl-enzyme intermediate. Now, water comes in to the reaction. Water replaces the N-terminus of the cleaved peptide, and attacks the carbonyl carbon. Once again, the electrons from the double bond move to the oxygen making it

negative, as the bond between the oxygen of the water and the carbon is formed. This is coordinated by the nitrogen of the histidine. This accepts a proton from the water. Overall, this generates another tetrahedral intermediate. In a final reaction, the bond formed in the first step between the serine and the carbonyl carbon moves to attack the hydrogen that the histidine just acquired. The now electron-deficient carbonyl carbon re-forms the double bond with the oxygen. As a result, the C-terminus of the peptide is now ejected. In trypsin, the catalytic triad is composed of Ser195, His57 and Asp102 (Phillips et al., 1992).

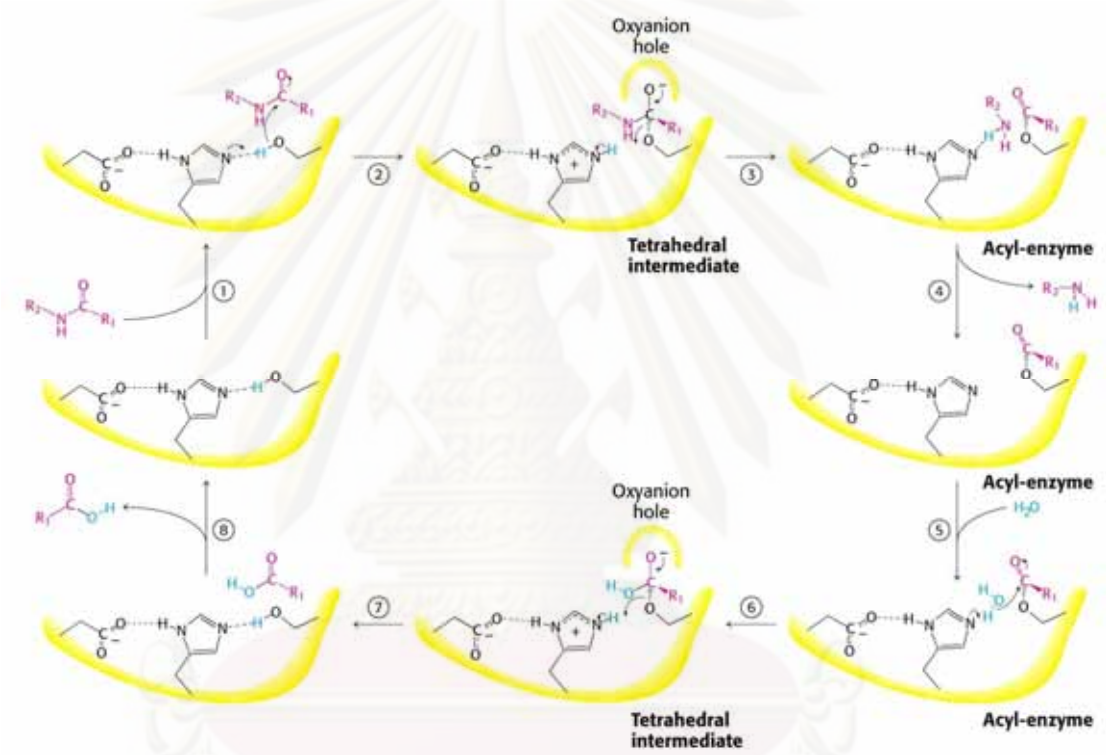


Figure 1.4 A detailed mechanism for the chymotrypsin-like SP reaction. (Source: http://www.bmolchem.wisc.edu/courses/spring503/503-sec1/503-3a.htm)

1.5.2 Clip-domain serine proteinases and clip-domain serine proteinase homologues

The first clip domain SP was initially identified from horseshoe crab, *Tachypleus tridentatus* that have function in clotting system (Muta et al., 1990). The clip domain is a conserved domain that was found in the N-terminus of SP enzyme especially 6 highly conserved cysteine residues. Six cysteine residues forming 3 disulfide bonds have a topology similar to β–defensin in vertebrate and invertebrate (Wang et al., 2001; Ganz, 2004) but the X-ray crystallography demonstrated that the structure appear to be difference (Piao et al., 2005). The clip domain was firstly called by Iwanaga et al. (1998) since the disulfide bridges forming the shape look like a paper clip. The activation cleavage site is presented between catalytic domain and clip domain by two cysteines formed a disulfide bond linking the two domains after activation reaction (Figure 1.5)(Jiang et al., 2000).

There are some groups of clip-SP that share a common feather to regular clip families SP except differ slightly in having a catalytic triad with the amino acid Ser replaced by Gly. These proteins are also called serine proteinase homolog (SPH). Although the SPHs are non-proteolytic activity, due to substitution of Ser to Gly at catalytic triad but it have role in several biological function for instance of prophenoloxidase activation cascade (Kwon et al., 2000), cell adhesion or as an immune molecule (Huang et al., 2000; Lin et al., 2006).

1.5.3 Role of clip-domain family of serine proteinases and serine proteinase homologues in arthropods

Arthropod clip-domain serine proteinases (clip-SPs) and clip domain serine proteinase homologues (clip-SPHs) have been shown to be involved in various biological functions, especially in embryonic development and innate immune responses (Jiang et al., 2000; Gorman et al., 2001; Ross et al., 2003; Jang et al., 2008).

The clotting system of horseshoe crab is activated by microbial cell wall component via a proteolytic cascade. The factor C and Factor G, a serine proteinase zymogen, was activated by gram negative bacteria and fungi invading the horseshoe

crab hemolymph. Consequently, the active clotting enzyme will cleave coagulogen to coagulin, an insoluble aggregate (Kawabata et al., 1996; Iwanaga et al., 1998).

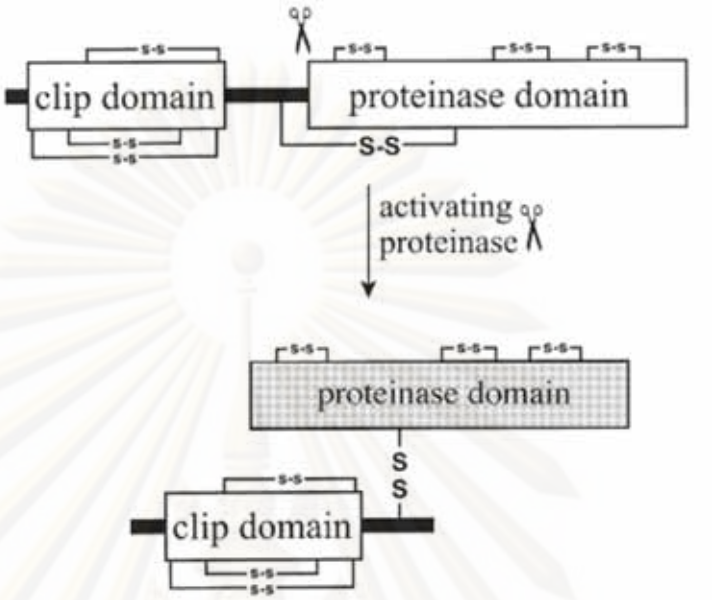


Figure 1.5 Domains organization of clip-domain proteinases. The proteinases contain an amino-terminal clip domain followed by a linker region of variable length and a carboxyl-terminal serine proteinase domain typical of the chymotrypsin family. A disulfide bond connects the linker region to the proteinase domain such that when the proteinase zymogen is activated by a specific proteolysis at the amino-terminus of the proteinase domain, the clip domain and proteinase domain remain covalently attach (Jiang et al., 2000)

In *D. melanogaster*, snake and easter are the clip-domain SPs which controls a development of dorsal-ventral axis in *D. melanogaster* embryo. The snake functionally activated the easter which activates the morphogen spätzle (Dissing et al., 2001). Basically, the spätzle is activated by recognition of gram positive bacteria or fungi(Royet et al., 2005). The proteinase inhibitor called serpin 27A was observed that it can regulate proteolytic activity of ester (Ligoxygakis et al., 2003). This activation system was named Toll signaling pathway. The *Drosophila* Toll pathway is corresponding to immune system in post-embryonic state but understanding of the immune respond in this system was not clear. The activation of proteins corresponding Toll pathway are required for transcription induction of antifungal peptide called Drosomycin (Anderson, 2000). In *Drosophila* embryo, active spätzle is

Toll ligand that is generated by localized proteolytic processes. In the other hand, the pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan and mannans might activate the classical protein ligand via unidentified protease cascade. The novel five SP were identified that have function associated the Toll signaling pathway. Generally four of these SPs are related to Toll signaling pathway that activated by fungi whereas another one is required for signaling in respond to gram positive bacteria (Kambris et al., 2006).

Serine proteinase cascade in prophenoloxidase activating system are well studied in many arthropod. The serine proteinase that convert prophenoloxidase to phenoloxidase are named prophenoloxidase activating enzyme (PPAE) in *B. mori* (Satoh et al., 1999), *P. leneusculus* (Wang et al., 2001) or *H. diomphalia* (Kim et al., 2002), prophenolxidase activating proteinase (PAP) in *M. sexta* (Gupta et al., 2005) or melanization proteinase (MP) in *D. malanogaster* (Tang et al., 2006). Some SP in this cascade would be auto-activated when presenting of microbial call wall component such as *M. sexta* hemolymp proteinase 14 (HP14) (Gorman et al., 2007).

Basically the activation of proPO may require SPH as SP cofactor in many organisms such as *H. diomphalia*, *Tenebrio molitor*, and *M. sexta* (Lin et al., 2006; Wang et al., 2008). However PPAE from *B. moli* does not need SPH to assisting for proPO activation (Satoh et al., 1999). Prophenoloxidase activating factor II (PPAFII), SPH that activated proPO, is processed by PPAFIII (PPAFIII, SP) (Kim et al., 2002). On the other hand the enzyme that activated SPHs in *H. diomphalia* has been unknown (Yu et al., 2003). The SPH was first discovered in 1990 which was shown to stabilize muscle attachment in *D. malanogaster* embryo and was called masquerade (Mas) (Murugasu-Oei et al., 1995). Similar to general SP, SPHs are required the proteolytic activation. In crayfish, the proteolytic cleavage of SPH, from hemocyte, are processed at the three indicated site during binding to bacteria or yeast and could be involved in granulocyte adhesion, pattern recognition and opsonizaton but not corresponded to phophenoloxidase activation (Lee et al., 2001).

1.6 RNA interference (RNAi)

RNA interference (RNAi) or RNA-based gene silencing is a immune system in eukaryotic organism. The double stranded RNA (dsRNA) can directly prevent eukaryotic cell form viral infection (for example HIV-1, RSV, HPV, poliovirus etc.) and also induced sequence-specific inhibition of gene expression (Bagasra et al., 2004). First step, dsRNA is attached with RNAaseIII-like enzyme such as Dicer in *D. malanogaster* (Elbashir et al., 2001; Agrawal et al., 2003; Bernstein et al., 2003). And then this enzyme will cleave the dsRNA into short interfering RNA (siRNA) of 21-23 nucleotides (Hammond et al., 2000). The siRNA will be incorporated with helicase, RecA, exo-, endo-nucleases and other protein forming RNAi-induced silencing complex (RISC). The RISC binds to and claves the target mRNA at the center of the region complement to siRNA. As a result, mRNA is suddenly degraded leading to increasing of gene expression. MicroRNA (miRNA) is a special class of siRNA by endogenous gene is a source (Bartel et al., 2004; Ambros et al., 2007). In mammalians, miRNA is function in regulation of specific expression of immune gene (Chowdhury et al., 2005).

The discovery of RNAi was initiated in plant (Matzke et al., 1989) but the effect of dsRNA leading to gene specific silencing was elucidated in *Caenorhabditis elegans* (Fire et al., 1998). Now post-transcriptional gene silencing mechanism initiated by dsRNA has been discovering in various organism (Mello et al., 2004). The RNAi become to an important tool for functional genomic studied and other applications. Specific dsRNA was used to silencing of known immune related gene especially in insect (Eleftherianos et al., 2006). RNAi mediated gene silencing have been successes in many organism such as *Drosophila* (Misquitta et al., 1999), Zebrafish (Wargelius et al., 1999), *Planaria* (Sánchez Alvarado et al., 1999) and plants (Jensen et al., 2004). In crustacean, RNAi have been became to a technique for understanding of functional immune respond in crayfish (Liu et al., 2007) and *P. monodon* (Charoensapsri et al. 2009).

1.7 Previous studies

Partial cDNA sequence coding a *Pm*ClipSP1 gene was identified from *Penaeus monodon* EST database (http://pmodon.biotec.or.th). Subsequently, the full length cDNA was obtained by RACE-PCR. In addition, the realtime PCR revealed that *Pm*ClipSP1 mRNA was highest expressed in hemocyte and upregulated at 3 hr. after *V. harveyi* infection in shrimp hemocyte (Amparyup et al., 2009B). However, the role of *Pm*ClipSP1, which is involved in the shrimp immunity, is yet to be clarified.

1.8 Objectives

The objective of this thesis is to identify and characterize the function of *Pm*ClipSP1 in shrimp immunity. The recombinant proteins of *Pm*ClipSP1 (mature protein and SP domain) were expressed in *E. coli* system and the biological function of *Pm*ClipSP1 were analyzed. Furthermore, the potential participation of *Pm*ClipSP1 in the proPO system and shrimp immunity was also elucidated by dsRNA-mediated RNA interference (RNAi). This study provides a basic knowledge of a clip domain serine proteinase leading to an understanding of shrimp immunity.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

-20 ^{o}C Freezer (Whirlpool), -80 ^{o}C Freezer (ThermoForma)

Costar® 96-well plate (Corning Incorporation)

Amicon Ultra-4 concentrators (Millipore).

Automatic micropipette: P10, P20, P100, P200, and P1000 (Gilson Medical Electrical S.A.)

Balance: Satorius 1702 (Scientific Promotion Co.)

Gel documentation (SYNGENE)

Gene Pulser (Bio-RAD)

Incubator (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

Insulin syringes U 100 **(**Becton, Dickinson and Company**)**

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets (NuAire, Inc.)

Microcentrifuge tubes 0.5 ml and 1.5 ml (Bio-RAD Laboratories)

Microtiter plate reader (Beckman Coulter AD200)

PCR Mastercycler (Eppendorf AG)

pH meter Model # SA720 (Orion)

Power supply: Power PAC 3000 (Bio-RAD Laboratories)

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen)

Spectrophotometer (eppendorf)

Sterring hot plate (Fisher Scientific)

Syringe (NIPRO)

Needle 21GX1”(NIPRO)

Touch mixer Model # 232 (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

2.2 Chemicals, Reagents and Biological substance

100 mM dATP, dCTP, dGTP, and dTTP (Fermentas)

2-Mercaptoethanol, C_2H_6OS (Fluka)

5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (Fermentas)

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

Absolute methanol, CH3OH (Scharlau)

Acrylamide (Plus one)

Agarose (Sekem)

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

Ammonium persulfate, $(NH_4)_2S_20_8$ (USB)

Amplicillin (BioBasic)

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (MERCK)

Bovine serum albumin (Fluka)

Bromophenol blue (MERCK)

Calcium chloride ($CaCl_2$) (MERCK)

Chloramphenicol (Sigma)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

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

Dithiothreitol (Pharmacia)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA)(Fluka)

Formaldehyde, CH_2O (BDH)

Formamide deionized (Sigma)

GeneRuler™ 100bp DNA ladder & GeneRuler™ 1kb DNA ladder (Fermentas)

Glacial acetic acid , CH_3COOH (J.T. Baker)

Glucose, $C_6H_{12}O_6$ (Ajax chemicals)

Glycerol, $C_3H_8O_3$ (Scharlau)

Glycine, NH_2CH_2COOH (Scharlau)

Hydrochloric acid (HCl) (MERCK)

Imidazole (Fluka)

Isopropanol, C_3H_7OH (MERCK)

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

Laminarin from Laminaria (Sigma)

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

Magnesium chloride ($MgCl_2$) (MERCK)

Methanol, CH_3OH (MERCK)

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

N, N', methylenebisacrylamide (Fluka)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Paraformaldehyde (Sigma)

pET22b(+) vector (Novagen)

Phenol, saturated (MERCK)

Prestained protein molecular weight marker (Fermentas)

RNA markers (Promega)

Skim milk powder (Mission)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium cacodylate trihydrate (CAC), (CH3)2AsO2Na · 3H2O, (Sigma)

Sodium chloride, NaCl (BDH)

Sodium dihydrogen orthophosphate, $NaH_2PO_4.H_20$ (Carlo Erba)

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

Sodium hydroxide, NaOH (Eka Nobel)

Triethanolamine (Unilab)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH2OH)_3$ (USB)

Tri reagent (Molecular Research Center)

Tryptic soy broth (Difco)

Urea (Fluka, Switzerland)

Xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$ (Sigma)

2.3 Kits and enzymes

ImProm-II™ Reverse Transcription system kit (Promega)

Mini Quick Spin RNA Columns (Roche Applied Science)

NucleoSpin® Extract II Kits (MACHEREY-NAGEL)

QIAprep® Miniprep kits (QIAGEN)

pGEM®-T Easy Vector Systems (Promega)

T & A Cloning vector Kit (RBC Bioscience)

T7 RiboMAX(TM) Express RNAi System (Promega)

Taq DNA polymerase (Fermentus)

Advantage® 2 Polymerase Mix (Clontech)

*Eco*RI (Biolabs)

*Hin*dIII (Biolabs)

*Nde*I (Biolabs)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

T7 RNA polymerase (Roche)

T4 DNA ligase

Trypsin (Sigma)

α-Chymotrypsin (Sigma)

2.4 Microorganisms

Escherichia coli strain Rosetta (DE3)

Escherichia coli strain JM109

Vibrio harveyi 639

2.5 Software

BlastX (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)

Clustal X (Thompson, 1997)

GENETYX (Software Development Inc.)

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

PHYLIP (Felsenstein, 1993)

SECentral (Scientific & Educational Software)

SignalP (http://www.cbs.dtu.dk/services/SignalP/)

SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1)

2.6 DNA sequence analysis

The full length cDNA sequence was edited and translated using the GENETYX software program (Software Development Inc.). This sequence was further compared with database from the GenBank (http://www.ncbi.nlm.nih.gov) using the BlastX program (Altschul et al., 1997). The significant probabilities and identity were considered from E-values < 10^{-4} and the match included > 10 amino acid residues for BlastX. Putative motifs and domains were investigated using SMART program. Related sequences that had been searched from GenBank, were aligned using Clustal X program (Thompson et al., 1997). The potential cleavage site of the signal peptide and putative N-Glycosylation sites were predicted by SignalP software (http://cbs. dtu.dk/services/SignalP/) and NetNglyc software (http://cbs.dtu.dk/services/NetNG lyc), respectively. Aligned sequences were bootstrapped 1000 times using Seqboot. Boostrapped neighbour-joining trees were constructed using Neighbour and Consense. All phylogenetic reconstruction programs are routine in PHYLIP (Felsenstein, 1993). Trees were appropriately illustrated using TreeView (http:// taxonomy.zoology.gla.ac.uk/rod.html).

2.7 Construction of expression plasmid for recombinant *Pm*ClipSP1 production in *E. coli*

2.7.1 Amplification of the mature sequence of the *Pm*ClipSP1 and the proteinase domain of *Pm*ClipSP1

DNA fragments coding the mature sequence and the proteinase domain of the *Pm*ClipSP1 from *P. monodon* cDNA were amplified using specific primers. A pair of primers 22NdeISP1-F and 22HidIIISP1-R (Table 2.1) was used for the mature sequence of the *Pm*ClipSP1 amplification and a pair of primers ExSPSP1-F and 22HidIIISP1-R (Table 2.1) was used for the proteinase domain of *Pm*ClipSP1 amplification. The PCR reaction was performed using Advantage 2 polymerase mix. The amplification reactions were preformed in 25 µl total volume containing 2 µl cDNA sample, 1x PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1 µM each primer and 1 units Advantage 2 *Taq* DNA polymerase (Clontech) following thermal cycle conditions were: pre-denaturing at 94 ^{o}C for 10 min, 5 cycles of denaturing step

94 ^{o}C for 1 min, annealing step 55 ^{o}C 1 min, extension step 72 ^{o}C for 1 min, 30 cycles of denaturing step 94 ^{o}C for 1 min, annealing step 62 ^{o}C 1 min, extension step 72 ^{o}C for 1 min and the final extension was carried out 72 ^{o}C for 10 min

Table 2.1 Primer sequence using for cloning strategies and dsRNA synthesis

Primer name	Sequence	Tm (^{o}C)
	For gene cloning and protein expression	
22NdeISP1-F	5' GGAATTCCATATGCATCATCATCATCATCAT CAGGGTGCAGATTGTGTACGCAGTCAGT 3'	78
22HidIIISP1-R	5' CCCAAGCTTTTATGGCTTTAAGTTCTGCTCA ATCCATGTC 3'	73
ExSPSP1-F	5' GAATTCCATATGCATCATCATCATCATCATA GAATTGTGGGTGGAGAAGTAGCC3'	79
	For dsRNA synthesis	
SP1i-F	5' CGTGGTTGCGTGGCGTGGTTAG 3'	70
T7SP1i-R	5' CCTATAGTGAGTCGTATTAGGATCCGCCTG TTGAGTCTGATGAGTGC 3'	76
SP1i-R	5' GCCTGTTGAGTCTGATGAGTGC 3'	65
T7SP1i-F	5' GGATCCTAATACGACTCACTATAGGCGTGG TTGCGTGGCGTGGTTAG 3'	79
GFP-F	5'ATGGTGAGCAAGGGCGAGGA 3'	68
GFPT7-R	5'TAATACGACTCACTATAGGTTACTTGTACAG CTCGTCCA 3'	71
GFP-R	5' TTACTTGTACAGCTCGTCCA 3'	60
GFPT7-F	5' TAATACGACTCACTATAGGATGGTGAGCAA GGGCGAGGA 3'	75
	For RT-PCR	
PmPPAE1-F	5'TGGGGCGAAGGCAGGGCACAAGGCGCAG3'	81
PmPPAE1-R	5'CTCTTCTTCAAGCTCACCACTTCTATCT3'	65
PmPPAE2rt-F	5'GCGGCGGTCACGCTCCTTGTTC3'	72
PmPPAE2rt-R	5'ACTCTCGGGGGCACGCTTGTTG3'	71
PmSP2rt-F	5'GGCGTTGGTCTTCACTGCTCTC3'	67
PmSP2rt-R	5'CAGAACTGCCTTCCAAGGATAG3'	62
PmSPH1rt-F	5'TACGTACTCATTGATATCAGGTTTGG3'	62
PmSPH1rt-R	5'GCCTCGTTATCCTTGAATCCAGTGA3'	66
PmSPH2rt-F	5'CCGTGAACCAGCGATGTCCTTA3'	66
PmSPH2rt-R	5'GCCACACTCTCCGCCTGCTCCG3'	73
PmSPH3rt-F	5'GCTCTTGGTGCTGCCGCTGTTG3'	71
PmSPH3rt-R	5'CACCGTCCACGCACAGGTAATA3'	66

2.7.2 Agarose gel electrophoresis

The PCR products were analyzed on 1.2% (w/v) agarose gel in 1x TBE (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) which were prepared by melt slurry in microwave oven until it completely dissolved. After solution cool down at 55-60 ^{o}C, then the gel was pored into the tray that was applied with the well comb. The PCR products were mixed into the 6X loading dye and loading into each well. The GeneRuler^{TM} 100 bp or 1 kb DNA Ladder plus (Fermentas) were used as standards DNA markers.

Electrophoresis was executed in 1X TBE buffer until dye moved about ¾ of gel length. After that, the gel was stained in a 2.5 µg/ml ethidium bromide (EtBr) solution for 1.5 min and destained in water for 15 min. DNA fragments were detected by visualization in UV transilluminator and photographed.

2.7.3 Purification of PCR product from agarose gel

The expected PCR bands were spited form the gel and purified by using NucleoSpin® Extract II Kits (MACHEREY-NAGEL) following manufacturer protocol. The gel fragment was cut and weighed. Three volume of NT buffer was added with gel slice and incubated at 60 ^{o}C until gel completely dissolved. The solutions were filled in NucleoSpin® column and centrifuged at 12,000x g for 1 min to remove the supernatant. The columns were washed with 500 µl of NT2 buffer and centrifuged as described above. The column was centrifuged again to remove remained ethanol from NT2 buffer. The DNA was eluted with 15 µl of elution NE buffer and stood at room temperature for 1 min before centrifugation. Eluted DNA was stored at -20 ^{o}C.

2.7.4 Cloning of DNA fragment into pGEM-T Easy vector or T&A cloning vector

The purified PCR product of the complete sequence of *Pm*ClipSP1 was ligated into pGEM-T Easy® (Promega) vector and the purified PCR product of the proteinase domain of *Pm*ClipSP1 was ligated into T&A cloning vector (RBC bioscience). For the ligation to pGEM-T Easy, the reaction was composed of 5µl of 2x Rapid ligation buffer, 1µl of pGEM-T Easy Vector (50 ng), proper amount of PCR product, 1µl of T4 DNA ligase (3 Weiss units/µl), and deionized water to a final volume of 10 µl.

Moreover the ligation to T&A cloning vector (RBC bioscience) was achieved follow by the reaction with 5 µl of 1x Rapid A and B ligation buffer, 2 µl of T&A Cloning Vector (50 ng), proper amount of PCR product, 1 µl of T4 DNA ligase (3 Weiss units/µl), and deionized water to a final volume of 10 µl. The reactions were mixed by pipetting, briefly spun and incubated overnight at 4 ℃.

The appropriate amount of insert in the ligation reaction was calculated following equation:

$$\text{ng of insert} = \frac{[\text{ng of vector} \times \text{kb size of insert}]}{\text{Kb size of vector}} \times \text{insert:vector molar ratio}$$

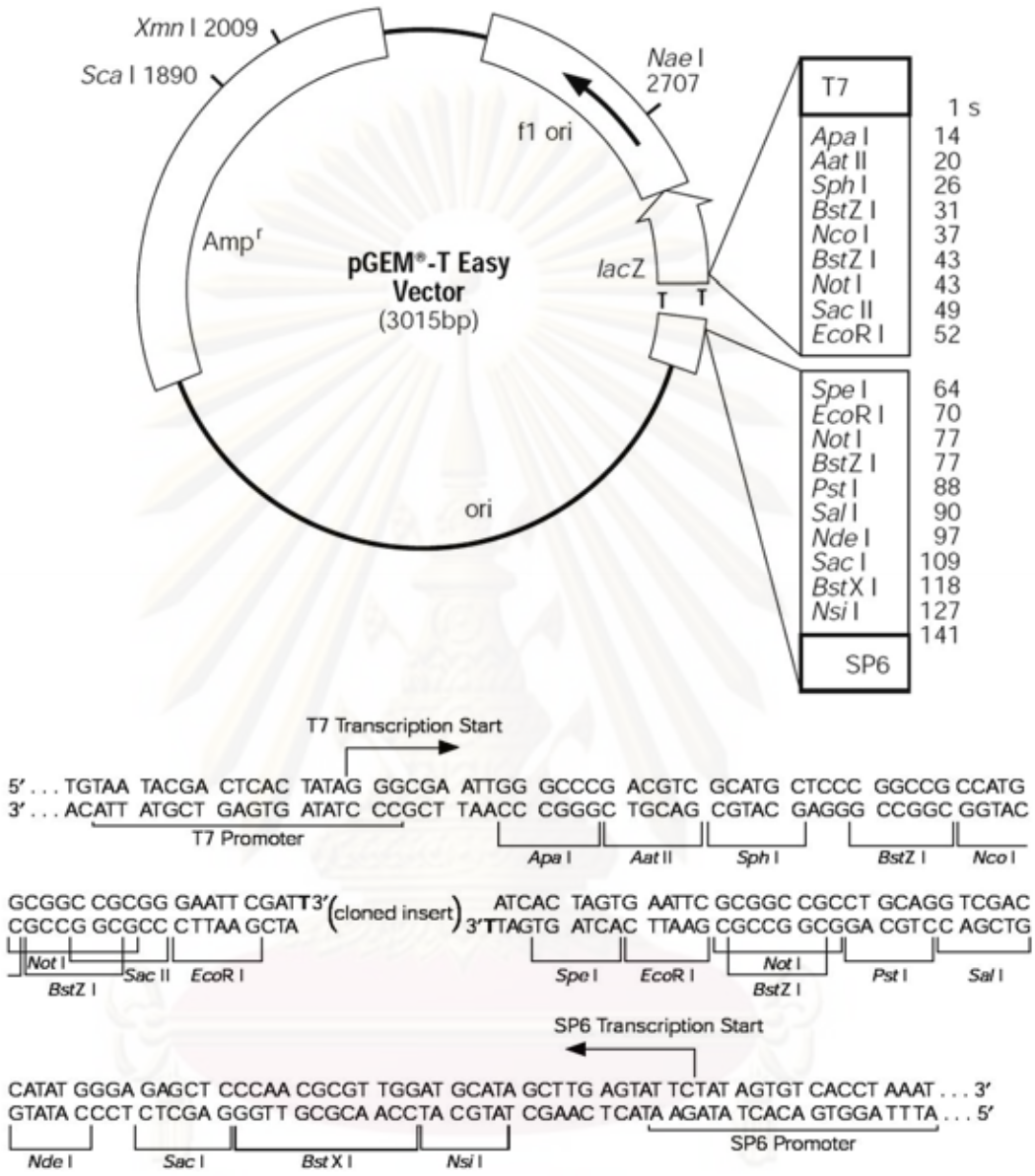
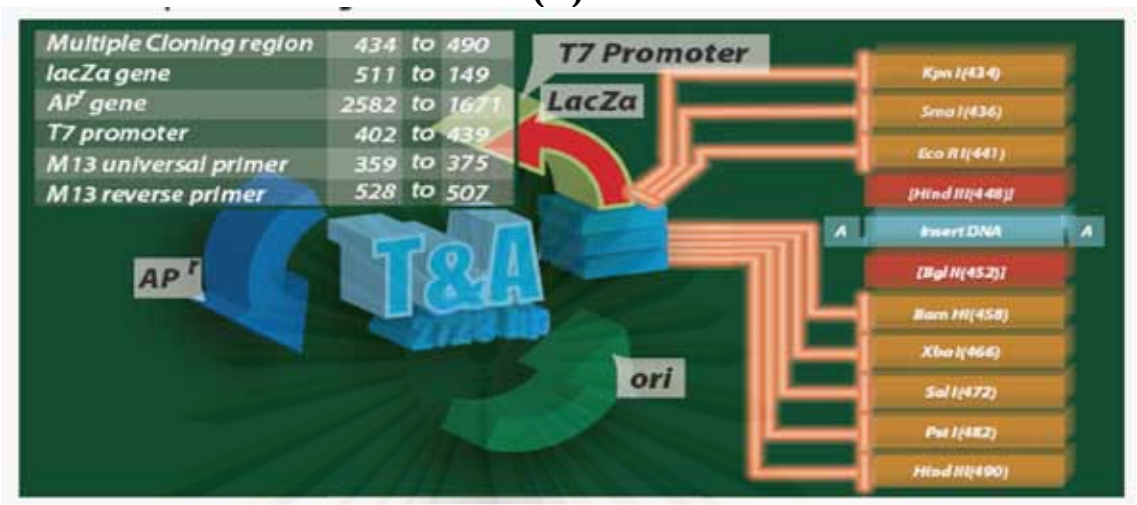


Figure 2.1 pGEM®-T easy vector map (A) and multiple cloning site sequences (B) (Promega)

(A)



(B)

Figure 2.2 T&A Cloning vector map (A) and multiple cloning site sequences (B) (RBC) (Source: T&A Cloning vector kit User Manaul)

2.7.5 Competent cells preparation

E. coli strain JM 109 from stock glycerol was streked in LB agar (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 1% (w/v) NaCl and 1.5 % (w/v) agar C) and incubated at 37 °C until single colonies were appeared. The starter culture was prepared by picked single colony into LB broth (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl). It was incubated overnight in shaker at 37 °C. The starter culture was diluted 100 fold in 250 ml LB broth and incubated in shaker at 37 °C for approximately 2 hr until OD_{600} of the cells reached 0.5-0.7. And then, cells were chilled on ice for 10 min and harvested by centrifugation at 5,000Xg

for 10 min at 4 ^{o}C. The supernatant was removed and the cell pellet was washed twice in 10 mM and 100 mM ice cool $CaCl_2$ at half volume and one twentieth to one fifth volume of initial cell culture respectively. The competent cell was stocked in 15% glycerol and stored in -80 °C until used.

2.7.6 Transformation by heat shock

A hundred microliters of competent cell was mixed with ten microliters of ligate, that have been prepared. The mixture of competent cell and ligate were chilled on ice for 10 min and heated at 45 °C for 45-50 s after that the mixture was chilled on ice immediately for 5 min and 1ml LB broth was added. The transfomant was incubated at 37 °C for one hour. After incubation, transformants were spread on LB agar plate containing 100 µg/ml of ampicillin, 20 µg/ml of X-gal and 30 µg/ml of IPTG and then incubated at 37 ^{o}C for overnight. After incubation the white colonies were selected for screening of insert in plasmid by colony PCR.

2.7.7 Screening and selection of transformant

White colonies were randomly selected for screening the insert by colony PCR. The colony PCR was performed in 25 µl reaction that consisted of 1x PCR buffer, 0.2 mM of dNTP mix, 1.5 mM $MgCl_2$, 1 µM of forward and reverse primers, and 1 units of *Taq* DNA polymerase (Fermentus). For the cloning in pGEM-T Easy vector, T7 and SP1-R primers were used to analyzed insert sizes, whereas M13 forward and M13 reverse primers were used for T&A cloning. The single colony was picked into the reaction by using sterile pipette tip. The thermocycles were 94 °C for 1 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min and a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

2.7.8 Plasmid DNA extraction using QIAprep® Miniprep kit

The plasmid was isolated from the positive clones by a QIAprep™ Miniprep kits (QIAGEN) as described in Qiagen's handbook. The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane under high salt condition. Firstly, bacterial cells were harvested by centrifugation and resuspend in 250 µl P1 buffer containing RNase A. Next, the 250 µl P2 buffer was added and mixed thoroughly by inverting the tube 4–6 times for cell

lysis. The cell lysate was neutralized by adding 350 µl N3 buffer. After maximum speed centrifugation for 10 min, the supernatant containing the plasmid was applied to column by pipetting. The column was centrifuged for 30–60 s, and then the flow-through was discarded. The QIAprep spin column was washed twice by adding 0.5 ml Buffer PB and 0.75 ml Buffer PE, respectively, and then centrifuged to remove residual ethanol from PE Buffer. Finally, the QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl EB buffer (10 mM Tris-HCl, pH 8.5) to the center of each column. After incubation at room temperature for 1 min, the eluted fraction was collected by centrifugation for 1 min.

2.7.9 Verification of recombinant plasmids

The correct sequence of insert in recombinant plasmid was determined by restriction enzyme digestion using *Nde*I and *Hin*dIII. Conditions for enzymatic digestion of recombinant plasmid composed of 3 µl of extracted recombinant plasmid, 1x reaction buffer (10mM NaCl, 5 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, pH 7.9: New England Biolabs), 0.5 unit of *Hin*dIII and *Nde*I (New England Biolabs) and in a 10 µl reaction volume. The digested plasmid was analyzed by 1.0% agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (100 bp or 1kb ladder marker). The recombinant plasmid was sequenced by an automatic DNA sequencer at the Macrogen Inc (Korea).

2.7.10 pET-22b(+) expression vector preparation

pET-22b(+) (Novagen) was used as an *E. coli* expression vector consisting of T7 promoter, His Tag coding sequence, T7 Tag coding sequence, multiple cloning sites, *lacI* coding sequence, pBR322 origin and Amp^{rest} coding sequence. *E. coli* from stock glycerol containing pET-22b(+) was cultured in 10 ml LB medium 37 °C with orbital shaking at 250 rpm an overnight. The plasmid was extracted by QIAprep® Miniprep kits (QIAGEN). The extracted pET-28b(+) was cut with *Hin*dIII and *Nde*I and then purified from gel by NucleoSpin® Extract II Kits.

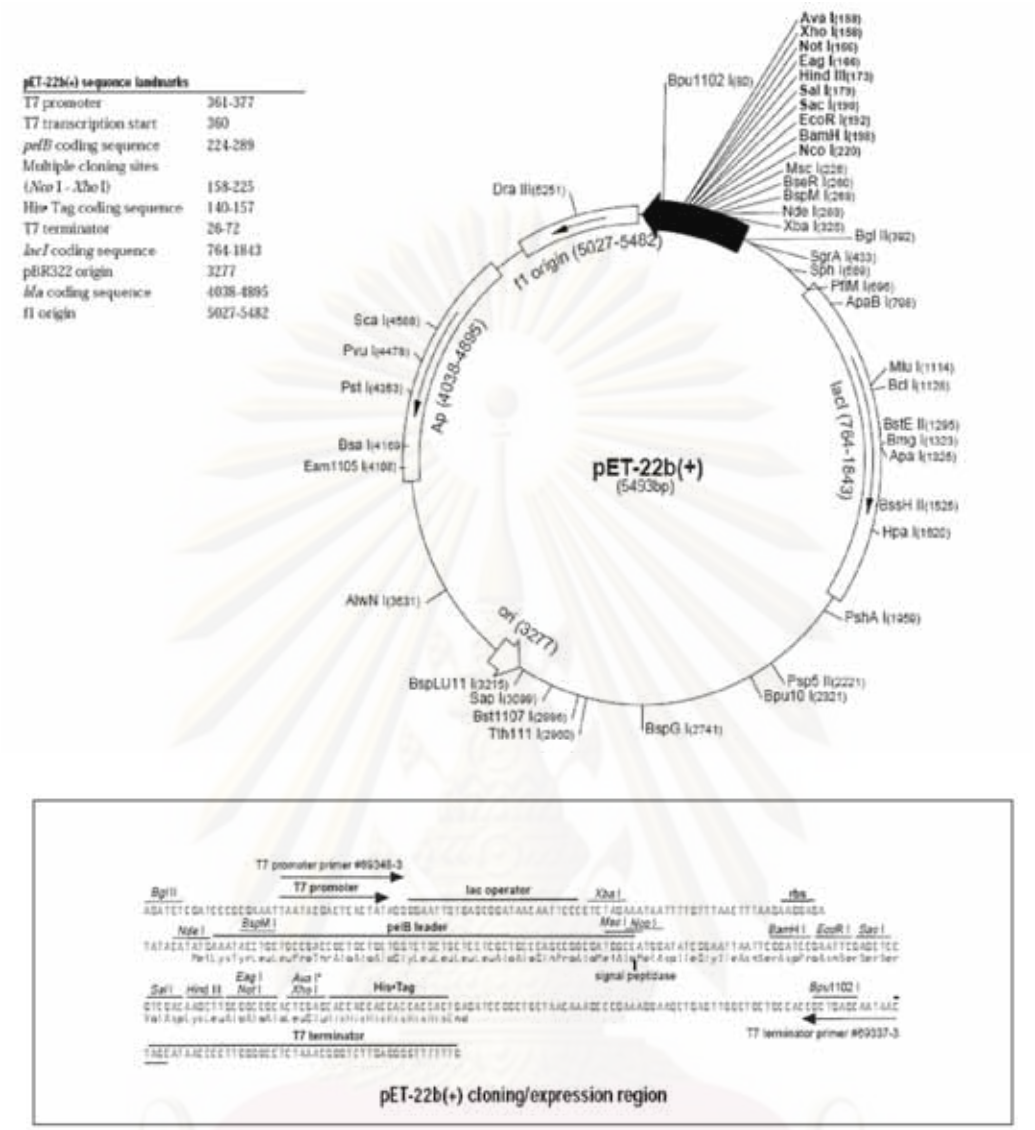


Figure 2.3 pET-22b(+) vector map and sequences in and around the multiple cloning sites (Novagen)

2.7.11 Ligation and transformation into *E. coli* strain Rosetta (DE3)

The correct sequence inserts that has been digested from recombinant pGEM-T Easy or T&A cloning vector by restriction enzymes, were purified following the method described above. The expression vectors were constructed by ligation between pET-22b(+) and interested gene that both were previously digested with *Hin*dIII and *Nde*I. The ligation reactions were assembled using 2 µl of 10x ligation buffer, 2 µl of T4 ligase (New England Biolab), 2 µl of interested gene and 8 µl pET-22b(+) vector which molar ratio of insert:vector was 3:1. The total volume was adjusted to 20 µl with sterile water. The ligation reaction was incubated at 4 °C for

overnight. Ten microliters of ligation was transformed into *E. coli* strain Rosetta (DE3).

2.7.12 Screening and determination of *E. coli* strain Rosetta (DE3) transformation

Transformant was screened in LB agar containing 100 mg/ml ampicillin and examined by colony PCR. The positive appeared clones were selected for extraction of the recombinant plasmid by using QIAprep® Miniprep kits (QIAGEN). The recombinant plasmids were detected by restriction enzyme digestion with *Hin*dIII and *Nde*I. The digested plasmid was detected by 1.0% agarose gel electrophoresis. One kilo base pair marker was used as standard for comparison with size of digested DNA.

2.7.13 Over expression of *Pm*ClipSP1-mature protein and *Pm*ClipSP1 -SP domain in *E. coli* system

The exact sequence clone was selected and cultured in LB medium containing 100 mg/ml ampicillin at 37 °C with orbital shaking at 250 rpm an overnight. The bacterial culture was diluted 100 fold LB broth and incubated for about 2 hr until OD_{600} of the cultures reached 0.6. The expression was induced by adding 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) into the cell culture broth. The cells were collected at 1 hr, 2 hr, 3 hr, 4 hr and 5 hr after induction. The cells were centrifuged at 8000 rpm for 2 min and discarded supernatant. The cell pellet was resuspended in 1X SDS loading buffer and boiled for 15 min before determine by sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7.14 Purification of recombinant *Pm*ClipSP1-mature protein and *Pm*ClipSP1 -SP domain

The cell culture was harvested at 4 hr after induction following previous method. The cells were washed twice and resuspended in 20 mM phosphate buffer pH 7.4. The resuspended cells were treated with deoxyribonuclease (DNase) before sonication. After sonication, the call lysate was centrifuged at 8000 rpm for 10 min. The aqueous fraction was kept for examining expression protein by SDS-PAGE and the precipitate was dissolved with lysis buffer (20 mM phosphate buffer pH 7.4 containing 8 M urea and 20 mM imidazole) shaking at room temperature overnight.

This fraction called inclusion body was purified under denaturing conditions in 8 M urea using nickel affinity chromatography (GE Healthcare). Ni-NTA agarose was mixed with the inclusion body and shaked at room temperature for 1 hr. The mixture was filled into column collecting flow through. The column was washed twice with the wash buffer (20 mM phosphate buffer pH 7.4 containing 8 M urea and 20 mM imidazole). After washing, the purified protein was eluted with elution buffer (20 mM phosphate buffer pH 7.4 containing 8 M urea and 500 mM imidazole). Each fraction was determined recombinant protein by SDS-PAGE. The purified protein was dialyzed in 20 mM Tris–HCl, pH 8.0 to allowing refold protein. The dialysis buffer was changed twice every 10 hr and each step was performed at 4 °C. The refolding proteins were concentrated by ultrafiltration through 30 kDa cut off Amicon Ultra-4 concentrators (Millipore). The purified recombinant *Pm*ClipSP1 -SP domain was send to the commercial service of the AMS Clinical Services Center, Chiang Mai University, for production of a specific polyclonal antibody.

2.7.15 Analysis of recombinant protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was prepared according to the method of Laemmli method (1970). The separating gel was prepared as 12% acrylamide gel by mixing of 4.16 ml solution A (30% w/v acrylamid, 0.8% w/v bis-acrylamide), 2.5 ml solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS) 60 µl of 10% ammonium persulfate (APS), 6 µl N,N,N,N'-tetramethylenebisacrylamide (TEMED) and 3.34 ml water. Before loading of solution into the slab gel (10 × 10 × 0.75 cm) system, the mixture was vigorous shaked until solution was homogeneous and the gels were placed in a vertical position at room temperature for complete polymerization. The 5% stacking gel was prepared by mixing 0.67 ml solution A, 1 ml solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS) 30 µl of 10% APS, 5 µl TEMED and 2.3 ml water. The solution was slotted on the separating gel surface and the well comb was buried in the top of stacking gel. The protein samples were mixed with 2X SDS loading buffer and boiled for 15 min before loading. The electrophoresis was performed at constant current of 20 mA per slab in 1x Tris-glycine electrophoresis buffer pH 8.3 (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS) at room temperature until the protein marker was distinctively separated. After that, the gel was stained in coomassie blue staining solution (0.25% (w/v)

coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) glacial acetic acid) for 30 min and de-stained by soaking in de-staining solution (30% (v/v) methanol and 10% (v/v) glacial acetic acid).

2.7.16 Determination of protein concentration

The concentration of protein was determined by Bradford method (1976) and bovine serum albumin (Fluka) using as the standard (Appendix C). This method is based on the binding of coomassie brilliant blue G250 dye to proteins. When the dye binds to proteins, the red form of dye is converted to the blue color. One hundred microliters of diluted sample solution was mixed with Bradford working buffer and left for 2 min before the absorbance at 595 nm was measured. The Bradford working buffer (100 ml) was a mixture of 6 ml Bradford stock solution (350 g Coomassie blue G250, 100 ml 95% ethanol and 200 ml 85% phosphoric acid), 3 ml 95% ethanol, 6 ml 85% phosphoric acid and 85 ml distilled water.

2.7.17 Phenoloxidase activity assay

The hemocyte lysate supernatant (HLS) was prepared by collecting the hemolymph from the ventral sinus using a 1 ml syringe with a 26 gauge-needle containing SAC buffer (Shrimp anti-coagulant buffer, 1% Triton X-100, 0.5% sodium dodecyl sulfate, and 2.5 mM EDTA in PBS) as two volume of hemolymph. The hemocyte was harvested by centrifuged at 500Xg for 5 min and washed once with SAC buffer again. Latter, the cell pellet was homogenized in CAC buffer (Calcium cacodylate buffer, 0.01 M sodium cacodylate, 0.45 M NaCl, 100 mM $CaCl_2$, 26 mM $MgCl_2$, pH 7.0) and centrifuged at 25000Xg for 20 min in 4 °C. The remained solution is hemocyte lysate supernatant (HLS) that used for phenoloxidase activity assay. The protein concentration of the HLS was determined by the Bradford method.

To determine property of PO system activation of recombinant protein, PO activity was examined according to the method described by Söderhäll and Smith (1983). A hundred microliters of HLS and five micrograms of recombinant protein were incubated with fifty microliters of larminalin (1 mg/ml) and fifty microliters of lipopolysaccaride (1 mg/ml) at 25 °C for 1 hr. After incubation 50 µl of L-3,4-dihydroxyphenylalanine (L-DOPA, 3 mg/ml) was added and incubated at 20 °C for 15 min. For negative control, CAC buffer was substituted with the recombinant

protein. PO activity would be detected by spectophotometry at OD_{490} by Microliter plate reader (Beckman Coulter AD200). Specific PO activity was defined as the increase in OD_{490}/min/mg protein of HLS (Leonard et al., 1985).

2.7.18 Protease activity assay

In the measurement of amidase activity, *N*-benzoyl–Phe–Val–Arg–*p*-nitroanilide (Sigma), *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide (Sigma) and *N*-benzoyl-D,L-arginine 4-nitroanilide hydrochloride (Sigma) was used as a substrate of trypsin, chymotrypsin and trypsin respectively. The blend of substrate and recombinant SP1-SPdomain (0.02, 0.2, 2.0 and 20.0 µM) were incubated at 30 °C for 15 min. Reaction was stopped by added 50 % acetic acid 50 µl and final volume was adjusted by adding water until 500 µl. The optical density at 410 nm of the chromogenic substrate was measured using a spectrophotometer. A specific enzyme was used as the positive control by substituted with the recombinant protein and the negative control added nothing.

2.7.19 Western blot analysis

Hemolymph was obtained using ice cool SAC buffer. Hemocyte was separated by centrifugation at 500Xg for 10 min at 4 °C. The plasma was suddenly harvested. The hemocyte pellet was washed twice and resuspended with 150 mM NaCl containing 2 mM EDTA. After homogenization, the cells were centrifuged at 25,000Xg for 20 min at 4 °C. The aqueous phase solution is hemocyte lysate supernatant. Protein concentration was determined by Bradford method. Twenty micrograms of total protein from HLS and plasma were loaded to 12% SDS-PAGE under denaturing condition. After SDS-PAGE completely running, the proteins were blotted by using electro-transfer with Trans-Blot SD® (Bio-Rad) at 110 mA for 1 hr. Six filter papers (Whatman®) and a nitrocellulose membrane were simultaneously soaked on transfer buffer (25 mM Tris, 190 mM glycine and 20% methanol) while SDS-PAGE was drenched in de-ionize water. Three sheets of filter paper were placed on Trans-Blot SD® platform followed by the membrane, the gel and three filter papers respectively. After electro-transfer complete, the membrane was washed twice in TBST buffer for 10 min per time then it was blocked with 2% bovine serum albumin (BSA) in TBST buffer for over night. After three washing in TBST, the membrane

was incubated in anti-rabbit SP1 antibody (1: 2000 diluted in TBST) for 1 hr and washed twice in TBST for 15 min each. The washed membrane was incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG (1: 5000 dilutions in TBST) for an hour at room temperature. After that, it was washed with TBST and deionized water each three times. The colour development was allowed in 15 ml of NBT/BCIP (Fermentas) and this development was stopped by adding 20 mM EDTA.

2.8 RNA interference

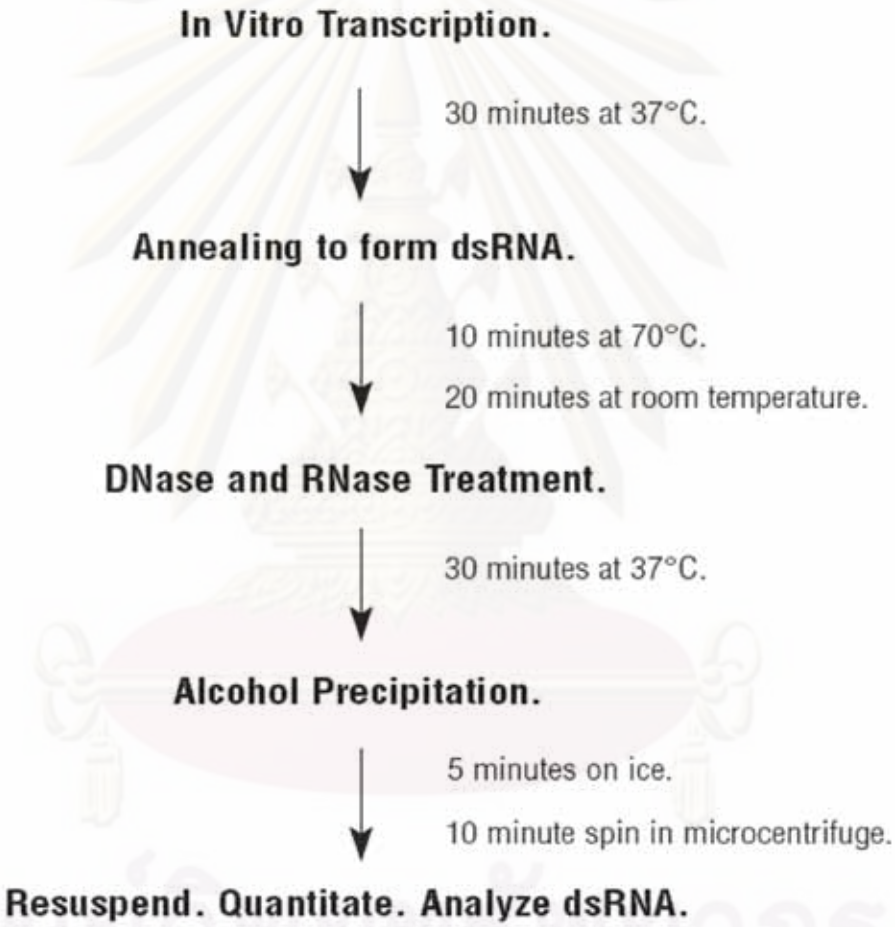


Figure 2.4 Outline of procedure for the production and purification of dsRNA using the T7 RiboMAX Express RNAi System. (Source: T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega))

2.8.1 Construction of SP1i-T&A vector

For the amplification of template which has a size of 600 bp approximately, PCR products were amplified with specific RNAi primers of *Pm*ClipSP1 gene (Table 2.1). The PCR reaction mix contained 2 µl cDNA, 1x PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1 µM SP1i-F and SP1i-R primer and 1 units *Taq* DNA polymerase (Fermentus) The amplification reaction was as follows: 94 °C for 3 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension at 72 °C for 10 min. Then the PCR product was purified by using NucleoSpin® Extract II Kits (MACHEREY-NAGEL). After that the purified product was ligated with T&A cloning vector (RBC Bioscience) and the ligation was transformed into *E. coli* strain JM109. Positive colonies were tested for the insert by colony PCR and digestion by *Hind*III/*Bgl*II. The plasmids were extracted using QIAprep® Miniprep kits (QIAGEN) and sequenced at Macrogen Inc (Korea).

2.8.2 dsRNA synthesis

To generate dsRNA, two specific DNA templates were amplified having T7 promoter sequence at 5'end of sense and anti-sense strand. Amplification strategies using two PCR reactions that are consisted of 50 µl total volume containing 2 µl SPi-T&A vector diluted 1: 50, 1x PCR buffer, 0.25 mM $MgCl_2$, 0.2 µM each dNTP, 0.8 µM each primer and 2 units *Taq* DNA polymerase (Fermentus). The pair of T7SP1i-F and SP1i-R using as sense strand whereas SP1i-F and T7SP1i-R using as anti-sense strand. Thermocycling condition were as follows: a predenaturing at 94 °C for 14 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. The green fluorescent protein (GFP), exogenous gene, was used to produce a dsRNA control. PCR template was amplified as described above. Two pairs of GFP primers containing T7 promoter sequence at 5' end and primer without T7 promoter sequence, GFPT7-F, GFP-R, GFP-F and GFPT7-R, were required for amplification together with pEGFP-1 vector (Clontech) which was used as PCR template. The individual PCR product was determined by agarose gel electrophoresis and purified by NucleoSpin® Extract II Kits (MACHEREY-NAGEL).

The ssRNA was synthesized by *in vitro* transcription using the T7 RiboMAX^{TM} Express Large Scale RNA Production Systems (Promega) following the manufacturing protocol. The 20 µl of T7 components comprised 10 µl at the components of RiboMAX express T7 2X buffer, 1 to 8 µl of linear DNA template (~1 µg total), 2 µl of enzyme mix-T7 Express and the final volume was adjusted by nuclease-free water. The components were mixed gently and incubated at 37 °C for 30 min or over. To anneal RNA strand, equal volume of the complementary ssRNAs were mixed. The mixture was thereupon heated at 70 °C for 10 min and slowly cooled down to room temperature (~20 min). The dsRNA solution was incubated with RQ1 RNase-Free DNase at 37 °C for 10 min, at ratio reaction: enzyme = 20:1. For the purification of dsRNA, the solution was added with 0.1 volume of 3M sodium acetate (pH 5.2) and 1 volume of isopropanol or 2.5 volumes of 95% ethanol. The reaction was mixed and placed on ice for 5 min, spun at top speed in a microcentrifuge for 10 min. A white pellet was washed with 0.5 ml of cold 70% ethanol, air-dried and resuspended in nuclease-free water. The dsRNA was examined by agarose gel electrophoresis and the concentration was determined by spectrophotometer at OD_{260}. The dsRNA was stored at -20 °C or -70 °C.

2.8.3 Injection of dsRNA into shrimp

Juvenile shrimps (2±0.2 g. fresh weigh) were injected with 5 µg of SP1 dsRNA in 25 µl injection buffer (150 mM NaCl) using insulin syringes U100, via third abdominal segment area. While GFP dsRNA or only injection buffer were changed with SP1 dsRNA for injection in control group. The RNA treated shrimps were repeat injected with dsRNA in related volume together with injection of 10 µg of the lipopolysaccharide (LPS) from *Escherichia coli* 0111:B5 (Sigma) and laminarin from laminaria (Sigma) at 24 hr after the first dsRNA injection. Total RNA from hemocyte was isolated for determination of affective gene silencing at 48 hr after the second injection.

2.8.4 RNA isolation

Hemolymph was collected from the ventral-sinus cavity of individual shrimp using the 1 ml syringe with a 26 gauge-needle containing one-forth volume of the pre-cooled anticoagulant (10% sodium citrate). Hemocytes were immediately harvested

by centrifugation at 500Xg for 10 min at 4 °C to discard the plasma from hemocytes. The hemocyte pallet was homogenized in 1 ml Tri reagent (Molecular Research Center). The homogenate was stored at room temperature and then adding 0.2 ml of chloroform. The mixture was vigorously shaken for 15 seconds and incubated at room temperature for 3 min before centrifugation at 12,000Xg for 15 min at 4 °C. The colorless upper aqueous phase was transferred to a new 1.5 ml microcentrifuged tube. Total RNA was precipitated by incubation with 500 µl of precool isopropanol at -20 °C for 15 min and centrifuged at 12,000Xg for 10 min at 4 °C. After the supernatant was removed, RNA pellet was washed with 1 ml of 75 % ethanol. The RNA pellet was air dried about 30 min. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC) – treated water.

The concentration of total RNA was determined by measuring the OD at 260 nm and estimated in µg/ml using the following equation,

$$[RNA] = OD_{260} \times \text{dilution factor} \times 40^*$$

*A 1 OD unit at 260 nm corresponds to approximately 40 µg/ml of RNA (Sambrook et al., 1989)

2.8.5 DNase treatments of total RNA samples

The obtained total RNA was further treated with RQ1 RNase-free DNase (Promega, 1 units/5 µg of the total RNA) at 37 °C for 30 min to remove the contaminating chromosomal DNA. Then, the RNA pellets were purified by phenol/chloroform extraction following by ethanol precipitation. Briefly, the reaction volume was adjusted to 40 µl with DEPC-treated water, 250 µl of Trizol reagent were added and vortex for 10 sec. Two hundred microliters of chloroform was then added and vigorously shaken for 15 sec. The resulting mixture was stored at room temperature for 2 to 5 min and centrifuged at 12,000Xg for 15 min at 4 °C. The RNA in upper phase was precipitated by isopropanol and washed by 70% (v/v) ethanol. After that, RNA pellet was briefly air-dried and dissolved with an appropriate amount of DEPC- treated water. The concentration of DNA-free total RNA was determined as described in 2.4

2.8.6 First-strand cDNA synthesis

The first stranded cDNA was synthesized using an ImProm-II™ Reverse Transcription system kit (Promega). Total RNA (160 ng) was combined with 0.5 µg of oligo (dT_{15}) primer and appropriate DECP-treated water in final volume of 0.5 µl. The reaction was heated at 70 °C for 5 min and immediately placed on ice for 5 min. After that, the reaction was mixed with 4 µl of 5x reaction buffer, 2.6 µl of 25 mM $MgCl_2$, 1 µl of dNTP Mix (10 mM each), 20 units of ribonuclease inhibitor and 1 µl of ImProm-II reverse transcriptase. The reverse transcriptase (RT) reaction was performed after the following reagents were sequentially added into the mixture and incubated at 25 °C for 5 min and at 42 °C for 60 min. At last, the reaction was incubated at 70 °C for 15 min. A cDNA sample was stored at -20 °C until ready for use.

2.8.7 Determination of gene silencing by RT-PCR

The expression of *Pm*ClipSP1 gene from SP1 silencing shrimp hemocyte was determined by RT-PCR. The PCR reaction was consisted of 25 µl total volume containing 2.0 µl cDNA (1:10 dilution), 1x PCR buffer, 0.25 mM $MgCl_2$, 0.2 µM each dNTP, 0.2 µM SP1i-F and SP1i-R primer and 2 units *Taq* DNA polymerase (Fermentus). The amplification reaction was performed following 94 °C for 1 min, 25 cycles or 30 cycle of 94 °C for 30 s, 55 °C (or 54 or 57 °C following Tm of primer) for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 5 min. The expression of *Pm*ClipSP1, other SPs and SPHs gene was normalized to the expression of elongation factor-1 gene (EF-1) for each sample. That is, primer EF-1F and EF-1R were used for amplification of EF-1 gene. All PCR products were analysed on 1.5% agarose gel. The gel images were photographed by CCD camera and analysed using GeneTools™ (SynGene).

2.8.8 Hemolymph PO activity assay

The injection of dsRNA was performed following the method as described above. The shrimp hemolymph was collected at 24 hr after second RNA injection. Concentration of the total hemolymph protein form the individual shrimp was determined following Bradford method.

Two milligrams hemolymph protein was dissolved in 10mM Tris-HCl pH 8.0 at final volume 435 µl and added 65 µl of 3,4-dihydrophenyl alanine (L-dopa, 3mg./ml.). The solution was incubated at 30 ^{o}C for 15 min and then added 500 µl of 10% acetic acid for stopping reaction. The amount of dopachrom produced was measured by using spectrophotometer at OD_{490}. One unit of PO activity was defined as 0.001 absorbance/mg total protein/minute.

2.8.9 *V. harveyi* 639 preparation for bacterial challenge

Ten microliters of *V. harveyi* 639 from glycerol stock was inoculated on four milliliters of tryptic soy broth (TSB) medium and incubated at 30 ^{o}C overnight using as a starter culture. Forty microliters of the starter culture was inoculated in four mililitrers of TSB medium and incubated at 30 ^{o}C for 1 hr 45 min or until OD_{600} as 0.59-0.6. After that it was diluted 10 fold in phosphate buffer saline (PBS) pH 7.4, final concentration of cell culture as 10^7 CFU/ml.

2.8.10 Bacterial count in *Pm*ClipSP1 knocked down shrimp

The injection of RNA was carried out as previous method but for the bacterial challenged, a shrimp was injected with *V. harveyi* 639 (2 x 10^5 CFU/shrimp) substituting an injection of LPS. Hemolymph was collected at 6 hr after challenge with out using anticoagulant. Hemolymph was serial diluted, 1:2 1:4 1:8 and 1:16 in phosphate buffer saline (PBS) pH 7.4 and 10 µl of diluted hemolymph was dot onto LB agar plate. And 1 µl, 5 µl and 10 µl undiluted hemolymph was also doted onto agar. The culture plates were incubated at 30^{o}C over night. Bacterial colony forming units (CFUs) was counted from the individual shrimp. Three shrimps were used for each experiment.

2.8.11 Cumulative mortality assay of *V. harveyi* in *Pm*ClipSP1 knocked down shrimp

The injection of RNA and the challenge of *V. harveyi* were performed as described above. The fatal shrimps should be suddenly picked out from culture tank. The mortality would be recorded at 3 hr after challenge and observed for 5 days. Ten shrimps were used per one experiment and each assay was repeated three times.

2.8.12 Statistical analysis

The significance of the difference between the two sample groups was determined using a two-tailed, independent sample t-test. The significance of the difference between 3 or more group samples was determined by one-way ANOVA followed by Duncan's new multiple range test. Statistical package, SPSS-PC$^+$ (SPSS Inc) was used for statistical analyzes. A P value of less than 0.05 was considered statistically significant. Values were expressed as means±S.D.

CHAPTER III

RESULTS

3.1 Sequence analysis of the *Pm*ClipSP1 from *P. monodon*

A search from *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon et al., 2006) identified four clip-domain serine proteinases (Clip-SPs). In this study, we selected a clip-SP, namely *Pm*ClipSP1, for further characterization. The complete cDNA sequence of *P. monodon* serine proteinase (*Pm*ClipSP1; FJ620688) gene was obtained from the previous study (Amparyup et al., 2009B). A full-length sequence of a *Pm*ClipSP1 cDNA of *P. monodon* consisted of 1,509 bp, containing 55 bp in the 5'-untranslated region (UTR), 1,101 bp in an open reading frame (ORF) and 353 bp in 3'-UTR with a polyadenylate signal (AATAAA), at positions 1,343-1,348, and poly (A) tail (Figure 3.1).

The ORF encoded a polypeptide of 366 amino acid residues. Analysis of the SignalP program indicated the presence of a cleavage site between amino acids 25 and 26 (Ser-Gln). The calculated molecular mass of the mature protein was 36.48 kDa with a predicted isoelectric point (pI) of 5.56. Two putative *N*-glycosylation sites, NFS (aa position 219) and NKS (aa position 228) sites were found suggesting that it is a glycoprotein (Appendix A).

Using the SMART program analysis, the six conserved cysteines of the clip domain at N-terminal region and the three conserved catalytic sites (His151, Asp216 and Ser314) of a serine proteinase domain at C-terminal region were annotated as being present in the mature protein of *Pm*ClipSP1 (Figure 3.2). The domain organization of *Pm*ClipSP1 is shown in Figure 3.2.

```
GAGTTCTCAACCCACGCCAGCATTAGAAACCATGTTGCTTATTCACGGGATCAACATGAA     60
                                                      M  N

TATTAAACGTGGTTGCGTGGCGTGGTTAGTACCCGCAGTTCTACTGGTGGTGGCGCAGCA    120
  I  K  R  G  C  V  A  W  L  V  P  A  V  L  L  V  V  A  Q  Q

GGTAACCAGCCAGGGTGCAGATTGTGTACGCAGTCAGTGTATCTCAATTCGAGAATGTCC    180
  V  T  S  Q  G  A  D  C  V  R  S  Q  C  I  S  I  R  E  C  P

AGCTCTGCTAAAACTTTTACAGGATCCTACACGAATCAATATCAGGAAGCTACAAGATGC    240
  A  L  L  K  L  L  Q  D  P  T  R  I  N  I  R  K  L  Q  D  A

CACCTGCTATGTCAGGAACCGGGAACCTATGGTATGCTGTCCATCTATAACTACAACTGA    300
  T  C  Y  V  R  N  R  E  P  M  V  C  C  P  S  I  T  T  T  E

AACACCGACGATTCCCACAAAGTCTCTCCTCCCAGAAAATTGTGGGCACAGTGCTCACTT    360
  T  P  T  I  P  T  K  S  L  L  P  E  N  C  G  H  S  A  H  L

GAACAGAATTGTGGGTGGAGAAGTAGCCCCACTTGATGCATACCCATGGAAAGCTGTTCT    420
  N  R  I  V  G  G  E  V  A  P  L  D  A  Y  P  W  K  A  V  L

AGGATATAAAGATAAAGGATTAGCTGCCATTGAATTTCTCTGCGGGGGTTCAGTCATTAA    480
  G  Y  K  D  K  G  L  A  A  I  E  F  L  C  G  G  S  V  I  N

CGAGAGATATGTTCTTACTGCTGCTCATTGTGTAGACCCTGGTACACTTGGCACACGAAG    540
  E  R  Y  V  L  T  A  A  H  C  V  D  P  G  T  L  G  T  R  R

ATTGGAAGTAGTTCGACTGGGTGAATGGGACCTCACCACCACTGAAGACTGTGAGAGCAC    600
  L  E  V  V  R  L  G  E  W  D  L  T  T  T  E  D  C  E  S  T

AAATAGTGGAGGGGTATTCTGTGCTCCTCCAGTTCAAGATTTCGAGGCTGAGGAAATTAT    660
  N  S  G  G  V  F  C  A  P  P  V  Q  D  F  E  A  E  E  I  I

CGGTCATCCCTCATACAACACTCGTGTGAGATTCTCCGATGACATTGCACTCATCAGACT    720
  G  H  P  S  Y  N  T  R  V  R  F  S  D  D  I  A  L  I  R  L

CAACAGGCCCATTAACTTCCAGGAATCAGCAGGATTTGTGTTGCCTGTGTGCCTGCCTCC    780
  N  R  P  I  N  F  Q  E  S  A  G  F  V  L  P  V  C  L  P  P

ATCTAACTTCTCCCCTCGTACAGCAGCTGGTAACAAATCAGCAATTGCAGCTGGATGGGG    840
  S  N  F  S  P  R  T  A  A  G  N  K  S  A  I  A  A  G  W  G

CTTCACTGAAACTGGCTCTGCAAGTAACAAAATTAAGCATGTAAAGCTGCCATTGGTTGA    900
  F  T  E  T  G  S  A  S  N  K  I  K  H  V  K  L  P  L  V  D

CAGTACTGAGTGTAGTCAGGTGTACAAAGGCAGTACAGTCAGTGAACAACTCTGTGCCGG    960
  S  T  E  C  S  Q  V  Y  K  G  S  T  V  S  E  Q  L  C  A  G

TGGCAATGCTGGTGAAGACTCGTGCGGTGGAGACTCTGGTGGTCCCTTGGTACTTGCCGG   1020
  G  N  A  G  E  D  S  C  G  G  D  S  G  G  P  L  V  L  A  G

TACTTTTGGTCCTCCCTACCAGCAGATTGGCATTGTTTCCTACGGTCCTGTCAGCTGTGG   1080
  T  F  G  P  P  Y  Q  Q  I  G  I  V  S  Y  G  P  V  S  C  G

CCAGCAGGGGGTACCTGGTATCTACACATCTGTAAGCAGCTACAGGACATGGATTGAGCA   1140
  Q  Q  G  V  P  G  I  Y  T  S  V  S  S  Y  R  T  W  I  E  Q

GAACTTAAAGCCATAAATGAGTTCTCAGGCAACTGAACTTATTTCAACAGTATTGAGAGA   1200
  N  L  K  P  *

ATAGTAATGAAAGTAATCTGACAGGAAATAGTGATGATAGTATGTTCTACTTCAAAAAAA   1260
TGATGAATATTTTTGTTTGGTTTGGTTTTGTATGATTTTTTGTCTACATTTTCTTAATAC   1320
TGTAGCTGGTATCCACTCAGGAAATAAAAAGTGGTCAAAAGAAATAATTATCAGTTTCATC   1380
AATGTCACAAGTAGATTTTTGAAAATTGAGTCTCGAAAGATTAGGAAAACTGAACGTAAT   1440
TCTGCTTTAACAGTTGATATAATGCTATTACCTCTTTGATACATTCCTGTAAAAAAAAAA   1500
AAAAAAAAA                                                      1510
```

Figure 3.1 The full-length nucleotide and deduced amino acid sequences of the *Pm*ClipSP1 from the black tiger shrimp. The deduced amino acid sequence is shown below the nucleotide sequence. The putative polyadenylation signal is in bold and italicized. The putative signal peptide sequence is bolded and underlined. The N-terminal clip domain is in dot line box with six cysteine residues (open circle) and the C-terminal SP-like domain is in solid line box. The catalytic triad (H, D and S) were marked as the stars. The arrows and diamonds indicate the putative activation cleavage sites and putative N-linked glycosylation sites, respectively. The nucleotide sequence coding start and stop codon are highlighted.

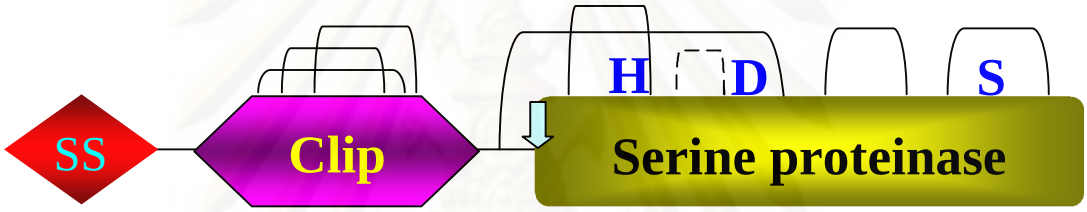


Figure 3.2 Domain organization of *Pm*ClipSP1 protein. Signal sequence (SS), clip domain and SP domain are show as diamond, hexagon and rectangle respectively. The arrows point the putative activation cleavage sites. The typically disulfide linkages are indicated by solid lines and the additional disulfide bond in SP domain is indicated by a dot line. The catalytic triad (H, D and S) are presented in the SP domain.

3.2 Sequence comparison of *Pm*ClipSP1

Searching for sequence similarities of known proteins by BlastX revealed that the amino acid sequence of the *P. monodon Pm*ClipSP1 is similar to a serine proteinase (ABC33918) of *P. chinensis* (58% sequence similarity), the serine protease 14D (ACN38198) from *Anopheles gambiae* (57%), prophenoloxidase activating factor (PPAF) I (BAA34642) from *Holotrichia diomphalia* (54%), the melanization protease 1 (NP_649450) of *Drosophila melanogaster* (52%) and PPAE3 (AAX18637) of *Manduca sexta* (51%) (Table 3.1).

Multiple sequence alignment of the deduced *Pm*ClipSP1 amino acid sequence with those of other clip-SPs in arthropods revealed the six conserved clip domain

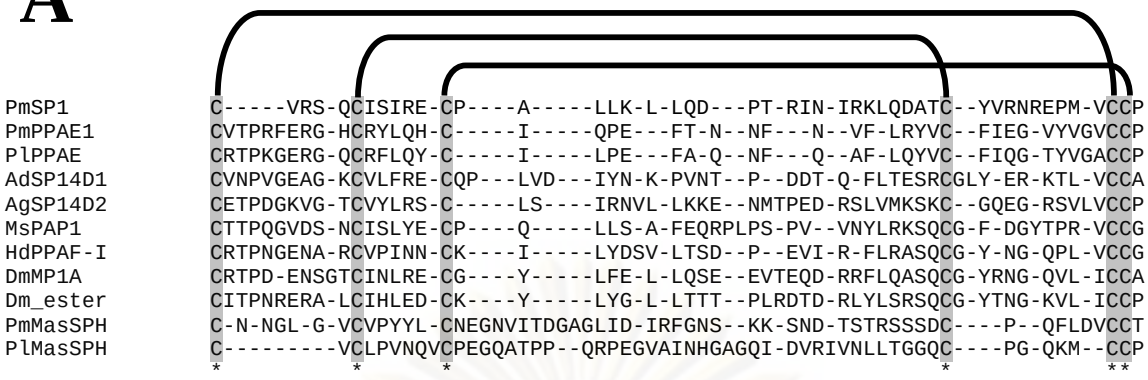
cysteines at the N-terminus (Fig. 3.3A) as well as, at the C-terminus, the three conserved catalytic sites (His151, Asp216 and Ser314) and the substrate-binding pocket (Asp308, Gly337 and Gly348) of a typical trypsin-like serine proteinase domain (Fig. 3.3B), indicating that this protein likely belongs to the family of clip domain serine proteinases.

Moreover, the eight conserved cysteine residues in the SP domain that participate in the formation of four disulfide bonds are found in *Ag*SP14D2, *Ms*PAP1, *Hd*PPAF-I, *Dm*MP1 and *Dm*Ester while *Pm*PPAE1, *Pl*PPAE, *Ag*SP14D, *Pm*MasSPH and *Lv*MasSPH showed only six conserved cysteine residues (Figure 3.3B).

Table 3.1 The BLASTX results and percentages of similarity of *Pm*ClipSP1 sequence to other clip-SPs in GenBank database.

Gene	**Closest species**	**% similarity**	**Accession No.**
Serine proteinase	*Penaeus chinensis*	58%	ABC33918
Serine proteinase 14D	*Anopheles gambiae*	57%	ACN38198
PPAF1	*Holotrichia diomphalia*	54%	BAA34642
Melanization protease-1	*Drosophila melanogaster*	52%	NP_649450
PAP3	*Manduca sexta*	51%	AAX18637

A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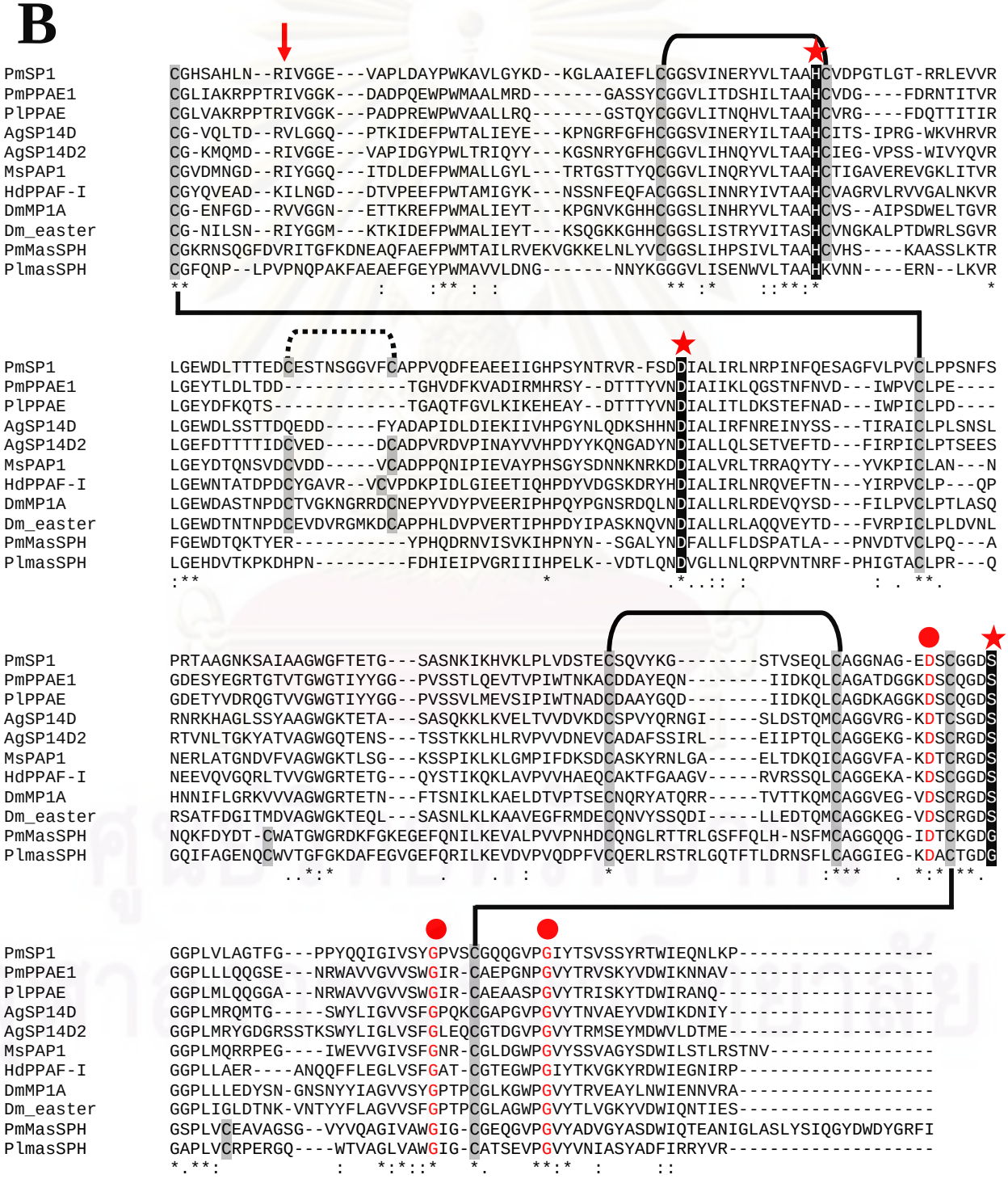
PmPPAE1 CVTPRFERG-HCRYLQH-C-----I-----QPE---FT-N--NF---N--VF-LRYVC--FIEG-VYVGVCCP
PlPPAE CRTPKGERG-QCRFLQY-C-----I-----LPE---FA-Q--NF---Q--AF-LQYVC--FIQG-TYVGACCP
AdSP14D1 CVNPVGEAG-KCVLFRE-CQP---LVD---IYN-K-PVNT--P--DDT-Q-FLTESRCGLY-ER-KTL-VCCA
AgSP14D2 CETPDGKVG-TCVYLRS-C-----LS----IRNVL-LKKE--NMTPED-RSLVMKSKC--GQEG-RSVLVCCP
MsPAP1 CTTPQGVDS-NCISLYE-CP----Q-----LLS-A-FEQRPLPS-PV--VNYLRKSQCG-F-DGYTPR-VCCG
HdPPAF-I CRTPNGENA-RCVPINN-CK----I-----LYDSV-LTSD--P--EVI-R-FLRASQCG-Y-NG-QPL-VCCG
DmMP1A CRTPD-ENSGTCINLRE-CG----Y-----LFE-L-LQSE--EVTEQD-RRFLQASQCG-YRNG-QVL-ICCA
Dm_ester CITPNRERA-LCIHLED-CK----Y-----LYG-L-LTTT--PLRDTD-RLYLSRSQCG-YTNG-KVL-ICCP
PmMasSPH C-N-NGL-G-VCVPYYL-CNEGNVITDGAGLID-IRFGNS--KK-SND-TSTRSSSDC----P--QFLDVCCT
PlMasSPH C---------VCLPVNQVCPEGQATPP--QRPEGVAINHGAGQI-DVRIVNLLTGGQC----PG-QKM--CCP

B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PlPPAE CGLVAKRPPTRIVGGK---PADPREWPWVAALLRQ-------GSTQYCGGVLITNQHVLTAAHCVRG----FDQTTITIR
AgSP14D CG-VQLTD--RVLGGQ---PTKIDEFPWTALIEYE---KPNGRFGFHCGGSVINERYILTAAHCITS-IPRG-WKVHRVR
AgSP14D2 CG-KMQMD--RIVGGE---VAPIDGYPWLTRIQYY---KGSNRYGFHCGGVLIHNQYVLTAAHCIEG-VPSS-WIVYQVR
MsPAP1 CGVDMNGD--RIYGGQ---ITDLDEFPWMALLGYL---TRTGSTTYQCGGVLINQRYVLTAAHCTIGAVEREVGKLITVR
HdPPAF-I CGYQVEAD--KILNGD---DTVPEEFPWTAMIGYK---NSSNFEQFACGGSLINNRYIVTAAHCVAGRVLRVVGALNKVR
DmMP1A CG-ENFGD--RVVGGN---ETTKREFPWMALIEYT---KPGNVKGHHCGGSLINHRYVLTAAHCVS--AIPSDWELTGVR
Dm_easter CG-NILSN--RIYGGM---KTKIDEFPWMALIEYT---KSQGKKGHHCGGSLISTRYVITASHCVNGKALPTDWRLSGVR
PmMasSPH CGKRNSQGFDVRITGFKDNEAQFAEFPWMTAILRVEKVGKKELNLYVCGGSLIHPSIVLTAAHCVHS----KAASSLKTR
PlmasSPH CGFQNP--LPVPNQPAKFAEAEFGEYPWMAVVLDNG-------NNYKGGGVLISENWVLTAAHKVNN----ERN--LKVR
PmSP1 LGEWDLTTTEDCESTNSGGVFCAPPVQDFEAEEIIGHPSYNTRVR-FSDDIALIRLNRPINFQESAGFVLPVCLPPSNFS
PmPPAE1 LGEYTLDLTDD-----------TGHVDFKVADIRMHRSY--DTTTYVNDIAIIKLQGSTNFNVD---IWPVCLPE----
PlPPAE LGEYDFKQTS------------TGAQTFGVLKIKEHEAY--DTTTYVNDIALITLDKSTEFNAD---IWPICLPD----
AgSP14D LGEWDLSSTTDQEDD-----FYADAPIDLDIEKIIVHPGYNLQDKSHHNDIALIRFNREINYSS---TIRAICLPLSNSL
AgSP14D2 LGEFDTTTTIDCVED-----DCADPVRDVPINAYVVHPDYYKQNGADYNDIALLQLSETVEFTD---FIRPICLPTSEES
MsPAP1 LGEYDTQNSVDCVDD-----VCADPPQNIPIEVAYPHSGYSDNNKNRKDDIALVRLTRRAQYTY---YVKPICLAN---N
HdPPAF-I LGEWNTATDPDCYGAVR--VCVPDKPIDLGIEETIQHPDYVDGSKDRYHDIALIRLNRQVEFTN---YIRPVCLP---QP
DmMP1A LGEWDASTNPDCTVGKNGRRDCNEPYVDYPVEERIPHPQYPGNSRDQLNDIALLRLRDEVQYSD---FILPVCLPTLASQ
Dm_easter LGEWDTNTNPDCEVDVRGMKDCAPPHLDVPVERTIPHPDYIPASKNQVNDIALLRLAQQVEYTD---FVRPICLPLDVNL
PmMasSPH FGEWDTQKTYER-----------YPHQDRNVISVKIHPNYN--SGALYNDFALLFLDSPATLA---PNVDTVCLPQ---A
PlmasSPH LGEHDVTKPKDHPN---------FDHIEIPVGRIIIHPELK--VDTLQNDVGLLNLQRPVNTNRF-PHIGTACLPR---Q
PmSP1 PRTAAGNKSAIAAGWGFTETG---SASNKIKHVKLPLVDSTECSQVYKG--------STVSEQLCAGGNAG-EDSCGGDS
PmPPAE1 GDESYEGRTGTVTGWGTIYYGG--PVSSTLQEVTVPIWTNKACDDAYEQN--------IIDKQLCAGATDGGKDSCQGDS
PlPPAE GDETYVDRQGTVVGWGTIYYGG--PVSSVLMEVSIPIWTNADCDAAYGQD--------IIDKQLCAGDKAGGKDSCQGDS
AgSP14D RNRKHAGLSSYAAGWGKTETA---SASQKKLKVELTVVDVKDCSPVYQRNGI-----SLDSTQMCAGGVRG-KDTCSGDS
AgSP14D2 RTVNLTGKYATVAGWGQTENS---TSSTKKLHLRVPVVDNEVCADAFSSIRL-----EIIPTQLCAGGEKG-KDSCRGDS
MsPAP1 NERLATGNDVFVAGWGKTLSG---KSSPIKLKLGMPIFDKSDCASKYRNLGA-----ELTDKQICAGGVFA-KDTCRGDS
HdPPAF-I NEEVQVGQRLTVVGWGRTETG---QYSTIKQKLAVPVVHAEQCAKTFGAAGV-----RVRSSQLCAGGEKA-KDSCGGDS
DmMP1A HNNIFLGRKVVVAGWGRTETN---FTSNIKLKAELDTVPTSECNQRYATQRR-----TVTTKQMCAGGVEG-VDSCRGDS
Dm_easter RSATFDGITMDVAGWGKTEQL---SASNLKLKAAVEGFRMDECQNVYSSQDI-----LLEDTQMCAGGKEG-VDSCRGDS
PmMasSPH NQKFDYDT-CWATGWGRDKFGKEGEFQNILKEVALPVVPNHDCQNGLRTTRLGSFFQLH-NSFMCAGGQQG-IDTCKGDG
PlmasSPH GQIFAGENQCWVTGFGKDAFEGVGEFQRILKEVDVPVQDPFVCQERLRSTRLGQTFTLDRNSFLCAGGIEG-KDACTGDG
PmSP1 GGPLVLAGTFG---PPYQQIGIVSYGPVSCGQQGVPGIYTSVSSYRTWIEQNLKP----------
PmPPAE1 GGPLLLQQGSE---NRWAVVGVVSWGIR-CAEPGNPGVYTRVSKYVDWIKNNAV----------
PlPPAE GGPLMLQQGGA---NRWAVVGVVSWGIR-CAEAASPGVYTRISKYTDWIRANQ----------
AgSP14D GGPLMRQMTG-----SWYLIGVVSFGPQKCGAPGVPGVYTNVAEYVDWIKDNIY----------
AgSP14D2 GGPLMRYGDGRSSTKSWYLIGLVSFGLEQCGTDGVPGVYTRMSEYMDWVLDTME----------
MsPAP1 GGPLMQRRPEG----IWEVVGIVSFGNR-CGLDGWPGVYSSVAGYSDWILSTLRSTNV----------
HdPPAF-I GGPLLAER----ANQQFFLEGLVSFGAT-CGTEGWPGIYTKVGKYRDWIEGNIRP----------
DmMP1A GGPLLLEDYSN-GNSNYYIAGVVSYGPTPCGLKGWPGVYTRVEAYLNWIENNVRA----------
Dm_easter GGPLIGLDTNK-VNTYYFLAGVVSFGPTPCGLAGWPGVYTLVGKYVDWIQNTIES----------
PmMasSPH GSPLVCEAVAGSG--VYVQAGIVAWGIG-CGEQGVPGVYADVGYASDWIQTEANIGLASLYSIQGYDWDYGRFI
PlmasSPH GAPLVCRPERGQ----WTVAGLVAWGIG-CATSEVPGVYVNIASYADFIRRYVR----------

Figure 3.3 Multiple alignment of amino acid sequence of clip domain (A) and SP domain (B) of clip-SPs and SPHs. The amino acid sequence of *P. monodon* Clip SP1 (PmClipSP1, FJ620688) was aligned with those of *P. monodon* PPAE (PmPPAE1, FJ595215) and MasSPH (PmMasSPH, ABE03741); *P. leniusculus* PPAE (PlPPAE, CAB63112) and MasSPH (PlMasSPH, AAX18636); *A. gambie* SP14D (AgSP14D, AAB62929) and SP14D2 (AgSP14D2, AAD38335); *M. sexta* PAP1 (MsPAP1, CAL25132); *H. diomphalia* PPAF-I (HdPPAF-I, BAA34642); *D. melanogaster* MP1A (DmMP1A, NP_649450) and ester (Dm ester, NP_524362). (A) The six conserved cysteines in the clip domain are highlighted and linked by solid line. Gaps (-) were introduced to maximize the alignmant of the clip domain cysteines. (B) Activation cleavage site and the amino acid residues forming the substrate specificity pocket were demonstrated by arrow head and dots respectively. The amino acid residues coresponding to the catalytic triad were presented as dark highligh and stars The disulfide linkages are shown by solid lines and the additional disufide bond in SP domain is represented by dot line.

3.3 Phylogenetic analysis

To determine the relationship of the *Pm*ClipSP1 protein to other arthropod clip-SP proteins, a phylogenetic tree was constructed using the NJ distance based method to compare the amino acid sequences of the SP domain (Figure 3.4). According to NJ analysis, arthropod clip-SPs and clip-SPHs can be classified into two major groups: (1) clip-SPs and (2) clip-SPHs. The group of clip-SPs can be classified into four subgroups. The first subgroup is a group of insect PPAEs (*Hd*PPAFI, *Ms*PAP1, *Ms*PAP2, *Ms*PAP3, *Bm*PPAE) and insect clip-SPs (*Ag*SP14D, *Ag*SP14D2, *Dm*MP1, *Dm*SP7, *Dm*SPE, *Dm*Ea, *Bm*proBAEEase, and *Tm*44kDa). The second subgroup contained crustacean PPAEs (*Pl*PPA and *Pm*PPAE1) and horse shoe crab clip-SPs (*Tt*PCE and *Tt*CB). The third subgroup composed of *Tm*41 (*Tenebrio molitor* 41 kDa zymogen) and *Dm*Snk (*Drosophila melanogaster* snake) and the last subgroup is a group of shrimp clip-SPs or clip-SPH (*Pm*ClipSP1, *Pm*ClipSP2, *Pm*PPAE2, *Fc*SPH) and an insect PPAF (*Hd*PPAFIII).

Although *Pm*ClipSP1 clusters together with some of the shrimp clip-SPs (*Pm*ClipSP2 and *Pm*PPAE2) and clip-SPH (*Fc*SPH), their functions are unknown,

except for that of PPAF-III from *H. diomphalia*, which is a clip-SP that activates PPAF-II, a cofactor for the serine proteinase PPAF-I, by cleavage (Kwon et al., 2000). Nevertheless, the phylogenetic tree clearly indicates that the *Pm*ClipSP1 was more closely related to clip-SPs than clip-SPHs (Figure 3.4).

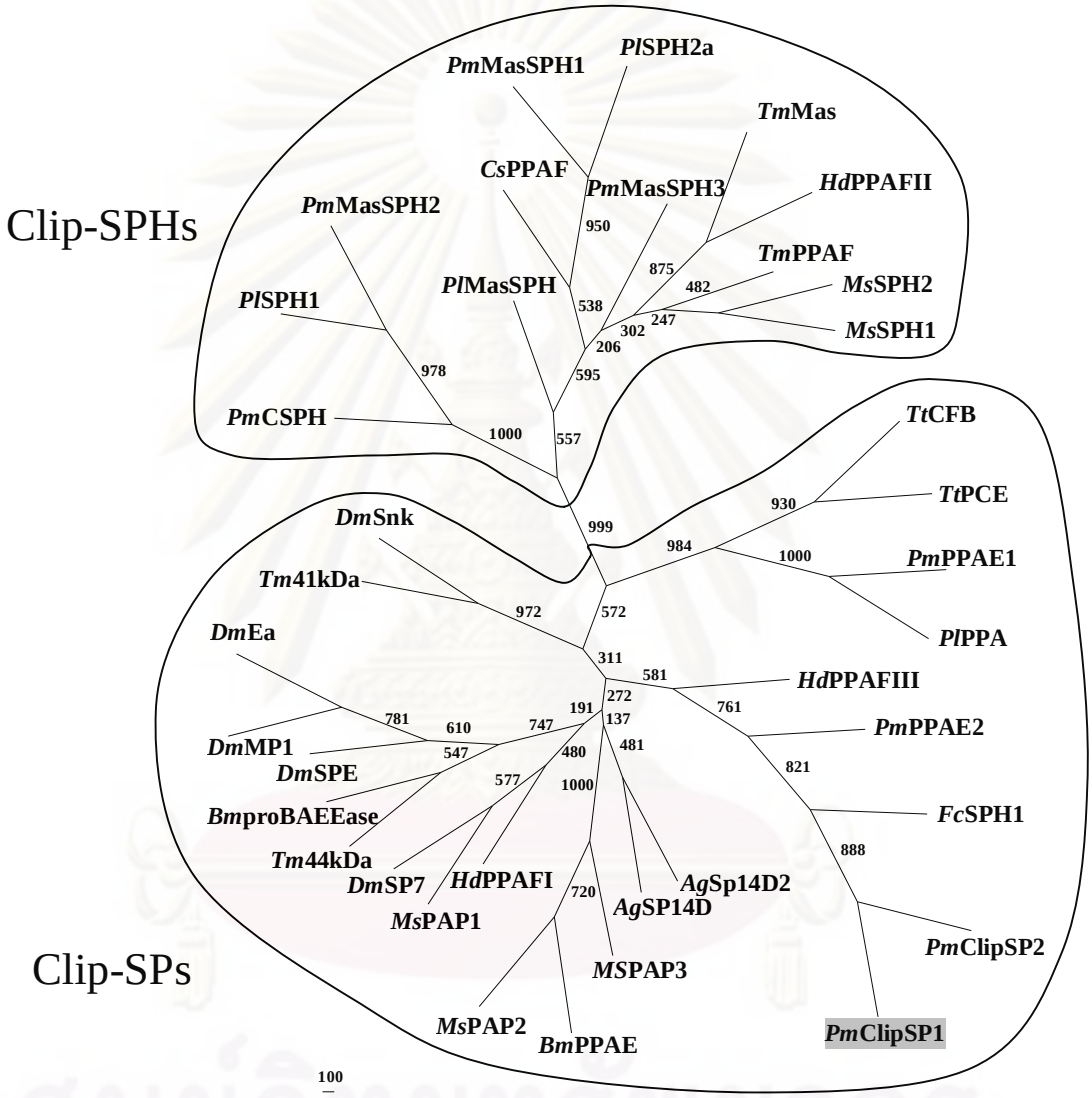

Figure 3.4 Bootstrapped unrooted neighbour-joining tree of the serine proteinase domain of clip-SPs and clip-SPHs from arthropods: *Penaeus monodon* clip-SP1 (*Pm*ClipSP1; ACP19562), clip-SP2 (*Pm*ClipSP2; ACP19561), PPAE1 (*Pm*PPAE1; ACP19558), PPAE2 (*Pm*PPAE2; ACP19559)**,** Mas-like SPH1 (*Pm*MasSPH1; ABE03741), Mas-like SPH2 (*Pm*MasSPH2; ACP19560), Mas-like SPH3 (*Pm*MasSPH3; ACP19563), Mas-like protein (*Pm*CSPH; AY600627); *Penaeus chinensis* SPH (*Fc*SPH1; DQ318859); *Pacifastacus leniusculus* PPA (*Pl*PPA; CAB63112), Mas-like protein (*Pl*Mas; Y11145), SPH1 (*Pl*SPH1; AY861652), SPH2a (*Pl*SPH2a; EU552456); *Callinectes sapidus* PPAF (*Cs*PPAF; AY555734); *Anopheles gambiae* serine protease 14D (*Ag*SP14D; FJ653845), serine protease 14D2 (*Ag*Sp14D2; AF117749); *Drosophila melanogaster* melanization protease 1 (*Dm*MP1; NM_141193), Spätzle-Processing enzyme (*Dm*SPE; NM_142911), snake (*Dm*Snk; NM_079614), easter (*Dm*Ea; NM_079638), serine protease 7 (*Dm*SP7; NM_141477); *Bombyx mori* PPAE (*Bm*PPAE; NM_001043367), SP zymogen (*Bm*proBAEEase; NM_001043379); *Holotrichia diomphalia* PPAFI (*Hd*PPAFI; AB013088), PPAFII (*Hd*PPAFII; AJ400903), PPAFIII (*Hd*PPAFIII; AB079666); *Manduca sexta* PAPI (*Ms*PAP1; AY789465), PAP2 (*Ms*PAP2; AY077643), (*Ms*PAP3; AY188445), SPH1 (*Ms*SPH1; AF518767), SPH2 (*Ms*SPH2; AF518768); *Tenebrio molitor* PPAF (*Tm*PPAF; AJ400904), Mas-like SPH (*Tm*MasSPH; AB084067), 41 kDa zymogen (*Tm*41kDa; AB363979), 44 kDa zymogen (*Tm*44kDa; AB363980); *Tachypleus tridentatus* proclotting enzyme (*Tt*PCE; M58366) and coagulation factor B (*Tt*CFB; D14701). Bootstrap values indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

3.4 Recombinant expression of a serine proteinase (SP) domain of *Pm*ClipSP1 in the *E. coli* system

To further characterize the function of *Pm*ClipSP1, a mature protein and a SP-like domain of this gene were cloned and expressed in *E. coli* expression system using pET22b(+) as an expression vector**.**

3.4.1 Amplification of a mature protein and a SP-like domain of the *Pm*ClipSP1

DNA fragments encoding a mature protein (m*Pm*ClipSP1) or a SP-like domain (SP-*Pm*ClipSP1) of *Pm*ClipSP1 protein with hexa His-tag sequences at the N-terminus were amplified using primers *Hin*dIIISP1-F and *Nde*ISP1-R for m*Pm*ClipSP1 or primers ExSPSP1-F and 22HidIIISP1-R for SP-*Pm*ClipSP1. After amplification, a single band of each protein was observed on agarose gel electrophoresis. The size of m*Pm*ClipSP1 and SP-*Pm*ClipSP1 was approximately 1,050 bp (Figure 3.5A) and 800 bp (Figure 3.5B), respectively, which corresponded to the expected size of m*Pm*ClipSP1 (1067 bp) and SP-*Pm*ClipSP1 (810 bp) of *Pm*ClipSP1 (Appendix B). The amplified products were then cloned and sequenced.

3.4.2 Construction of the recombinant plasmid pET-22b(+)-SP1

After DNA sequence analyses, the m*Pm*ClipSP1 and SP-*Pm*ClipSP1 fragments of *Pm*ClipSP1 were sub-cloned into an expression plasmid pET22b(+) at the *Hind*III and *Nde*I sites fused with six His encoded nucleotides at the N terminus and transformed into *E. coli* JM109 (Appendix B). The recombinant plasmid was extracted and verified by restriction enzyme (*Hin*dIII and *Nde*I) digestion. The recombinant plasmid screening was demonstrated in Figure 3.6.

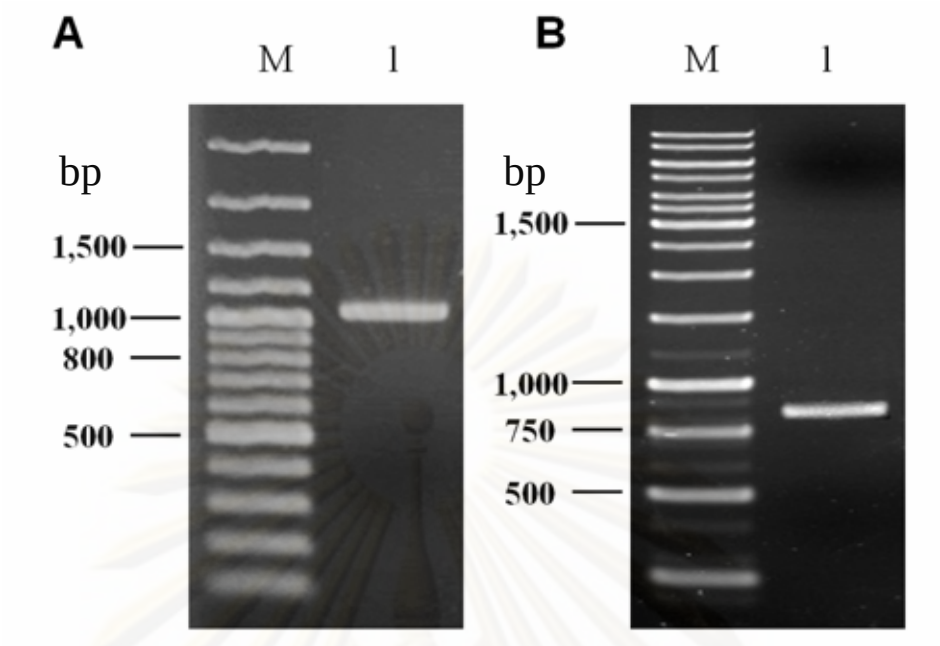

Figure 3.5 Amplification of DNA fragments encoding a mature protein (m*Pm*Clip SP1, A: lane 1) and a serine proteinse domain (SP-*Pm*ClipSP1, B: lane 1). Lane M is a GeneRuler™ 100 bp DNA ladder marker (A) or GeneRuler™ 1 kb DNA ladder marker (B) (Fermentas).

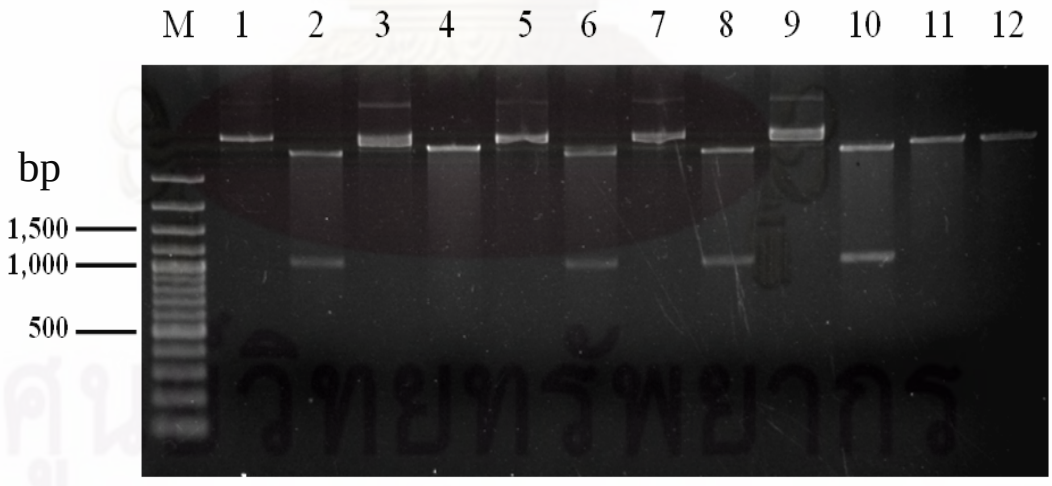

Figure 3.6 Screening and detection of the recombinant plasmid of the mature *Pm*ClipSP1 (m*Pm*ClipSP1) in pET-22b(+) digesting with *Hin*dIII and *Nde*I on 1.2% agarose gel electrophoresis. Lane M is GeneRuler™ 1kb DNA ladder (Fermentas). The odd numbers represent the uncut recombinant plasmid and even numbers indicate the m*Pm*ClipSP1 recombinant plasmid cutting by *Hin*dIII and *Nde*I.

3.4.3 Over-expression of the *Pm*ClipSP1 protein in the *E. coli* Rosetta (DE3) strain

After selection of the corrected clone, the resulting plasmids were then transformed into *E. coli* Rosetta (DE3) pLysS cells for protein expression. The single colony of transformed cells was grown at 37 °C in LB medium containing ampicillin until they reached an OD_{600} of 0.6. Expression was then induced by the addition of 1 mM IPTG. The cultured cells were harvested at 1, 2, 3, 4 and 5 hr after IPTG addition. After induction, the Coomassie brilliant blue staining of the gels revealed the induction of an approximately 37 kDa protein (Figure 3.7) for m*Pm*ClipSP1 and an approximately 28 kDa protein (Figure 3.8) for SP-*Pm*ClipSP1. The protein was detected after 1 h of IPTG induction and gradually increased following induction period. However, the highest expressions of recombinant proteins were detected at 4 hr after induction (Figures 3.7 and 3.8). After sonication, both the supernatant and pellet fractions were analyzed by 12 % SDS-PAGE, which showed that target proteins with an expected molecular weight around 37 kDa and 28 kDa proteins for m*Pm*ClipSP1 and SP-*Pm*ClipSP1, respectively, were only expressed in the insoluble fraction (inclusion bodies) not in the soluble fraction (Figure 3.9). Therefore, this condition was applied for a large-scale expression of the recombinant m*Pm*ClipSP1 and SP-*Pm*ClipSP1.

3.4.4 A single-step purification of the recombinant proteins

The recombinant (r) m*Pm*ClipSP1 and SP-*Pm*ClipSP1 proteins of *Pm*ClipSP1 were purified from inclusion bodies using Ni-NTA affinity chromatography. The purified proteins were refolded by dialysis step for removing urea. The purity of the recombinant proteins was analyzed using 12% SDS-PAGE. The results showed that a major protein band of each recombinant protein with apparent molecular weight of 37 kDa (Figure 3.10A) and 28 kDa, (Figure 3.10B), respectively, which is close to the calculated molecular mass of the rm*Pm*ClipSP1 (37.44 kDa) and rSP-*Pm*ClipSP1 (28.79 kDa) proteins, was achieved after purification process.

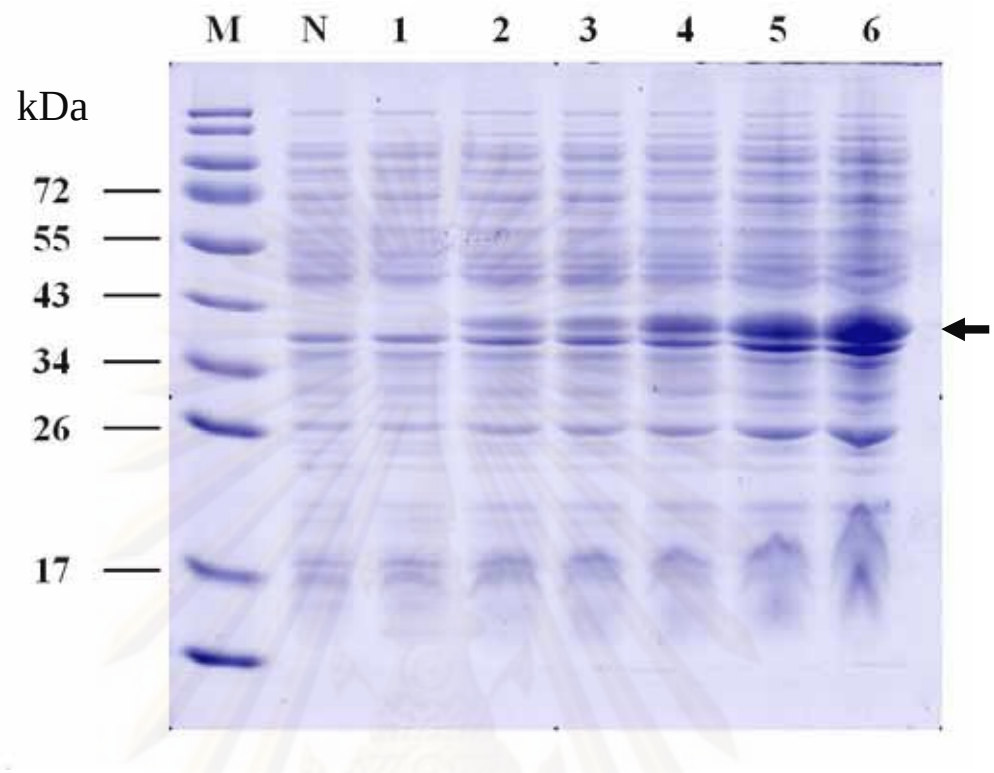

Figure 3.7 Expression of the recombinant protein of m*Pm*ClipSP1 after IPTG induction at 0, 1, 2, 3, 4 and 5 hr respectively (lanes 1 to 6). Lane N is protein expression of non-insert pET-22b(+) transformant. Lane M indicates the PageRuler™ pre-stained protein molecular weight marker (Fermentas). An arrow indicates the expected protein (~37 kDa).

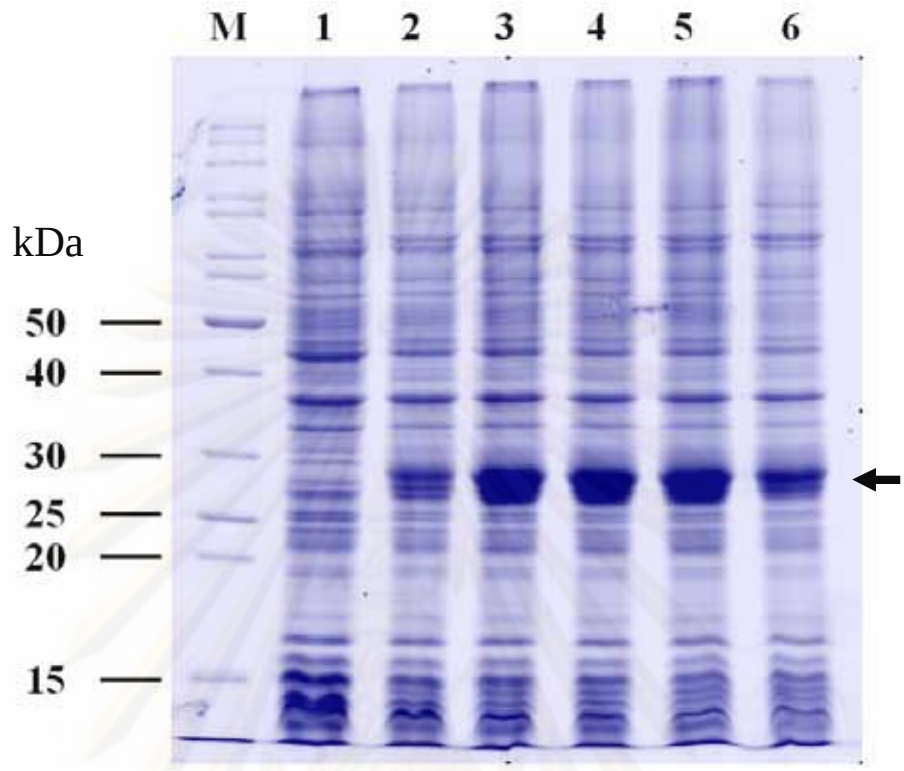

Figure 3.8 Expression of the recombinant protein of SP-*Pm*ClipSP1 after IPTG induction at 0, 1, 2, 3, 4 and 5 hr respectively (lanes 1 to 6). Lane M indicates the PageRuler™ unstained protein ladder (Fermentas). An arrow indicates the expected protein (~28 kDa).

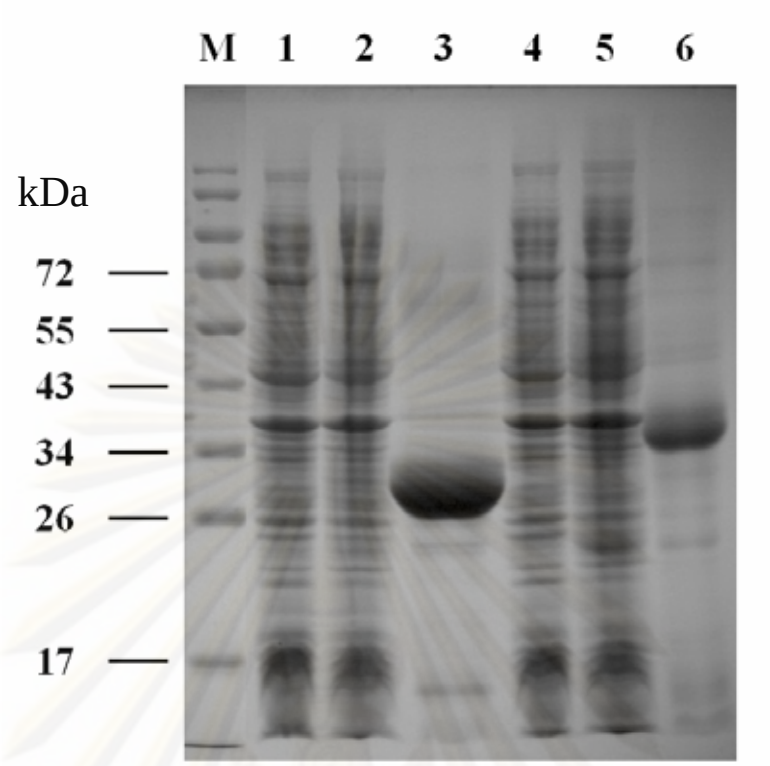

Figure 3.9 Fractional analysis of the expressed proteins of m*Pm*ClipSP1 and SP-*Pm*ClipSP1 showing recombinant protein was mainly expressed as inclusion body form (lanes 3 and 6, respectively) but not found in soluble fraction (lanes 2 and 5). Total protein before induction is shown in lanes 1 and 4. Lane M is PageRuler™ prestrained protein ladder (Fermentus) in 12 % acrylamide gel electrophoresis.

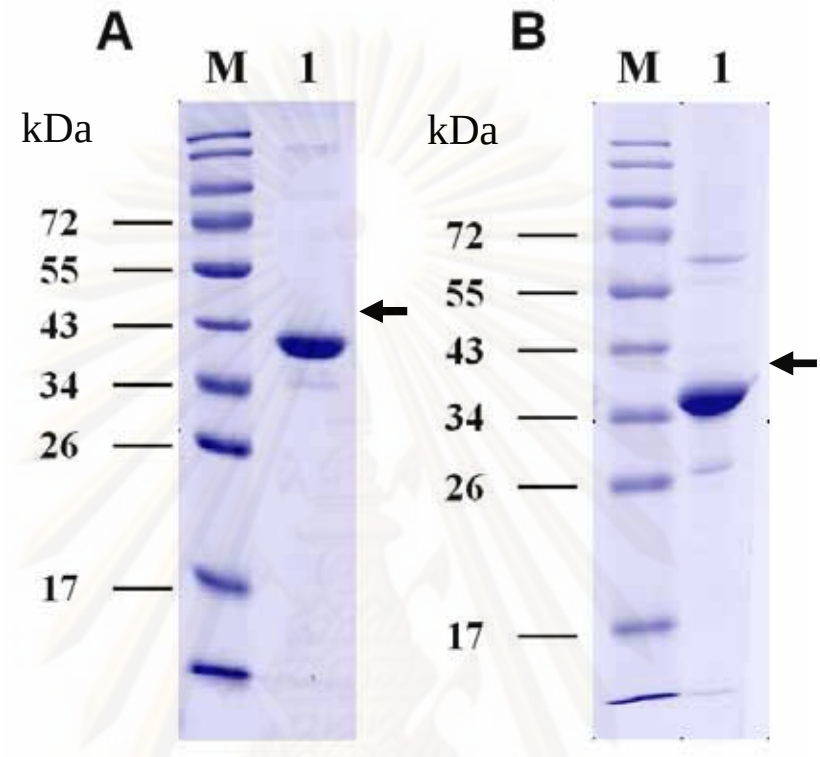

Figure 3.10 Purification and refolding of recombinant proteins of m*Pm*ClipSP1 (A: lane 1) and SP-*Pm*ClipSP1 (B: lane 1) were determined by 12 % acrylamide gel electrophoresis. Lane M is PageRuler™ prestrained protein ladder (Fermentus). Arrows indicate the expected proteins of ~37 kDa for m*Pm*ClipSP1 and ~28 kDa for SP-*Pm*ClipSP1).

3.4.5 Immunoblotting analysis

After purification, the rSP-*Pm*ClipSP1 was used to generate rabbit polyclonal antibodies by a commercial service. To confirm the binding interaction of antibody and rSP-*Pm*ClipSP1, the immunoblotting with anti-rSP-*Pm*ClipSP1 antibody was performed. The purified protein of rSP-*Pm*ClipSP1 was electrophoresed on SDS-PAGE, subsequently blotted to nitrocellulose membrane, then hybridized with rabbit anti-rSP-*Pm*ClipSP1 antibody and the second antibody conjugated with alkaline phosphatase. Finally, the rSP-*Pm*ClipSP1 was detected with colorimetric method. Immunoblotting analysis indicated that the polyclonal rabbit antisera reacted with the major protein band (~28 kDa) of rSP-*Pm*ClipSP1 protein (Figure 3.11A and B), which is consistent with the expected value (28.79 kDa).

To detect endogenous *Pm*ClipSP1 protein expression in hemocytes of healthy shrimp, 20 μg each of the HLS and plasma proteins was subjected to reducing 12%SDS-PAGE and then transferred onto a nitrocellulose membrane. Western blot analysis showed that one protein band (~39 kDa), which corresponded to *Pm*ClipSP1 with a predicted molecular weight of 39.16 kDa, was detected in hemocytes but not in the cell-free plasma. However, a band of 50 kDa and bands between 70 to 90 kDa were observed. A band of 50 kDa were probably the cross reactivity of *Pm*ClipSP1 antibody with a *Pm*PPAE1 that previously detected in hemocytes with a molecular weight of 50 kDa (Charoensapsri et al., 2009). For other protein bands between 70 to 90 kDa, these bands were probably the nonspecific binding of *Pm*ClipSP1 antibody with the high concentration of the hemocyanin protein (Figure 3.11C and D).

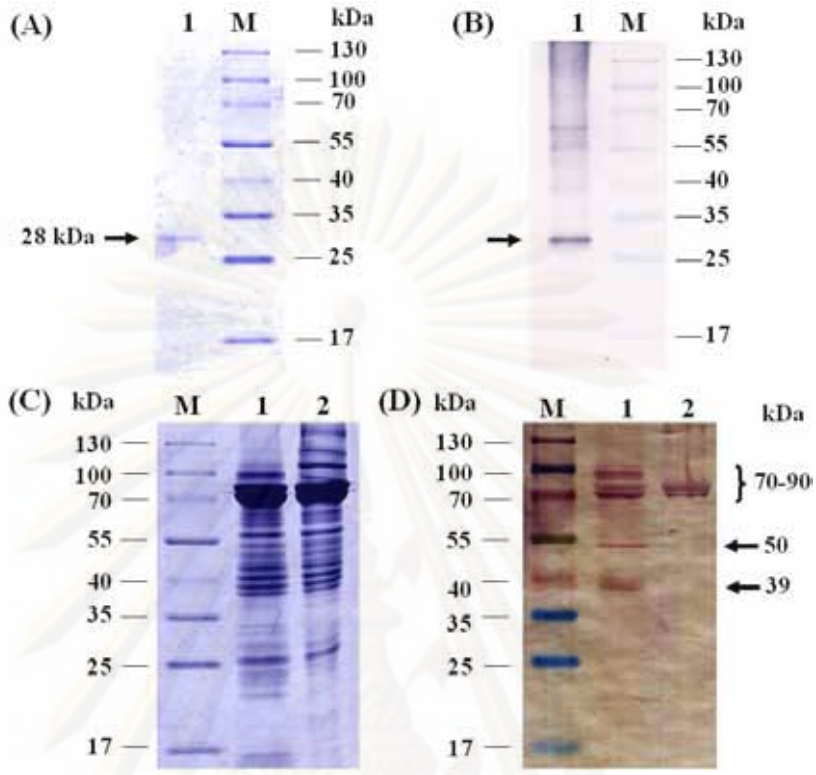

Figure 3.11 SDS-PAGE (A and C) and western blot (B and D) analysis of recombinant SP-*Pm*ClipSP1 (A and B: lane1), hemocyte protein (C and D: lane1) and cell free plasma protein (C and D: lane2). Lane M is Spectra™ Multicolor Broad Range Protein Ladder (Fermentas). Twenty μg of hemocyte protein (C and D) and cell free plasma protein were separated on 12% reducing SDS-PAGE and detected by coomasie blue or transfer to Nitrocellulose membrane and probed with rabbit anti-*Pm*ClipSP1 /SP domain antibody. Arrows indicate the expected proteins.

3.5 Functional characterization of the recombinant *Pm*ClipSP1

The purified rm*Pm*ClipSP1 and rSP-*Pm*ClipSP1 were assayed for the biological activity including the proteinase activity and the activation of PO activity.

3.5.1 Proteinase activity assay

The function of *Pm*ClipSP1 as a proteinase was investigated using purified rm*Pm*ClipSP1 and rSP-*Pm*ClipSP1 proteins. The proteinase activity on the hydrolysis of synthetic chromogenic substrates for serine proteinases trypsin (N-benzoyl–Phe–Val–Arg–*p*-nitroanilide or N-benzoyl-D,L-arginine 4- nitroanilide hydrochloride) and chymotrypsin (N-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide) were examined. The enzymatic activity was monitored as the release of *p*-nitroaniline at A_{405} nm. The results showed that no proteinase activity was detected in all assays using both rm*Pm*ClipSP1 and rSP-*Pm*ClipSP1 proteins of *Pm*ClipSP1, in contrast to trypsin which was used as a positive control (Table 3.2).

Table 3.2 The proteinase activity assays of the recombinant proteins. Trypsin was adopted as positive control.

Proteinase	A_{410}
Blank	0
Trypsin (0.02 μM)(control)	0.4800
mPmClipSP1 (0.02, 0.2, 2.0 μM)	0
SP-PmClipSP1 (0.02, 0.2, 2.0, 20.0 μM)	0

3.6.2 Assay for activation of phenoloxidase activity

Generally, the family of clip domain serine proteinases is synthesized as zymogens and is activated by a specific proteolytic cleavage. The proPO cascade is activated upon recognition of microbial cell wall components, such as peptidoglycan (PGN), β-1,3-glucan or lipopolysaccharide (LPS), and leads to the limited proteolysis of proPO to the active PO which catalyzes the formation of melanin. In this study, purified rm*Pm*ClipSP1 was tested for the involvement in the prophenoloxidase activation system. The PO activity was determined by measurement of the absorbance

at 490 nm using L-DOPA as a substrate and specific PO activity was defined as an increase in A_{490}/min/mg protein of HLS. The results revealed that the incubation of purified rm*Pm*ClipSP1 with HLS, LPS and laminarin (β-1,3-glucan) did not enhance PO activity, whilst the addition of the trypsin showed an increase in the PO activity compared to control (Figure 3.12).

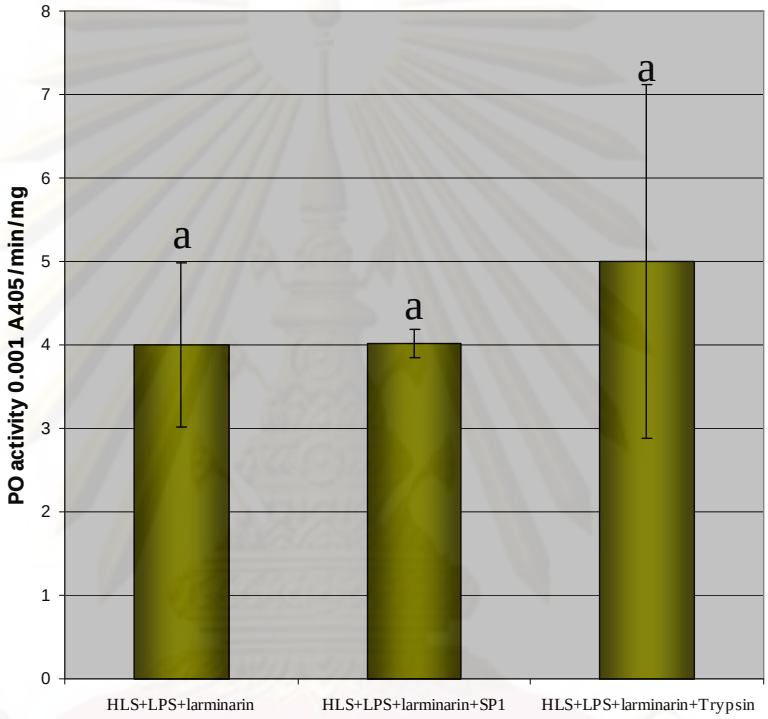

Figure 3.12 Phenoloxidase (PO) activities in the hemocyte lysate supernatant of shrimp. The hemocyte lysate supernatant was pre-incubated with larminarin (β-1,3-glucan) and LPS before incubation with the recombinant m*Pm*ClipSP1. The PO activity was determined by measurement of the absorbance at 490 nm using L-DOPA as a substrate and specific PO activity was defined as an increase in A_{490}/min/mg protein of HLS. All assays were performed in triplicate.

3.6 Gene silencing of the *Pm*ClipSP1 transcript by RNA interference

To characterize the potential role of *Pm*ClipSP1 in shrimp innate immunity, gene silencing of *Pm*ClipSP1 was performed using double stranded RNA (dsRNA) mediated RNA interference (RNAi), with the efficiency of gene knockdown determined at the transcript level by semi-quantitative RT-PCR. The *Pm*ClipSP1 silenced shrimps were then assayed for total PO activity, bacterial clearance and susceptibility to challenge by injection with the pathogenic bacterium, *Vibrio harveyi*.

3.6.1 Generation of dsRNA

The DNA fragment of 660 bp was amplified by PCR from the recombinant plasmid containing the *Pm*ClipSP1 gene using the gene-specific primers SP1i-F and SP1i-R for *Pm*ClipSP1 (Table 2.1). The primers for the dsRNA synthesis consist of the same primer sequences but flanked at the 5'end by a T7 promoter recognition sites. Two separate PCR reactions were set up, one with T7SP1i-F and SP1i-R (Table 2.1) for the sense strand template, the other with SP1i-F and T7SP1i-R (Table 2.1) for the anti-sense strand template. For an exogenous gene as a negative control, a 720-bp fragment of the green fluorescent protein (GFP) was amplified with the pEGFP-1 vector as template using GFPT7-F and GFP-R (Table 2.1) for the sense strand template, and GFP-F and GFPT7-R (Table 2.1) for the anti-sense strand template. After electrophoresis, the results revealed the expected amplicons of 685 bp for *Pm*ClipSP1 and 739 bp for GFP were obtained (Figure 3.13). These fragments were cloned and sequenced. The PCR products were purified and used to construct the dsRNA with a T7 RNA polymerase using T7 RiboMAX™ Express Large Scale RNA Production Systems. The ssRNAs of sense and antisense strands of PmClipSP1 and GFP were synthesized and their concentration were determined before annealing step (Appendix C). Following an annealing of ssRNA, a major band of *Pm*ClipSP1 and GFP dsRNAs was observed after agarose gel electrophoresis analysis (Figure 3.14).

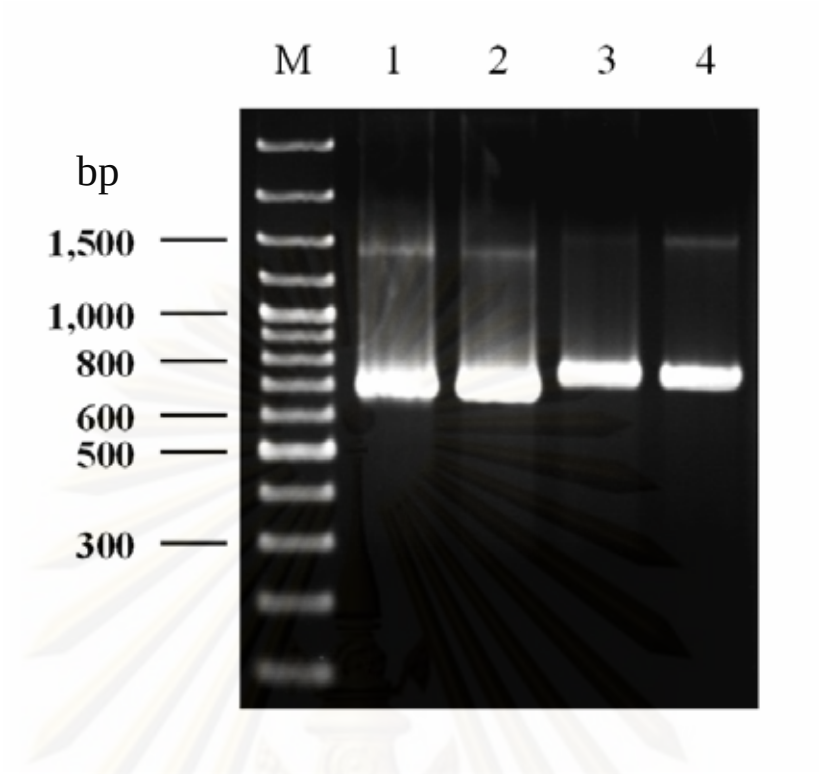

Figure 3.13 PCR products of 685 bp amplified from *Pm*ClipSP1 sense (lane1) and antisense (lane2) strand templates and 739 bp from GFP sense (lane3) and antisense (lane4) strand templates analyzed by 1.2 % agarose gelelctrophoresis. Lane M is GeneRuler™ 100 bp DNA ladder marker (Fermentas).

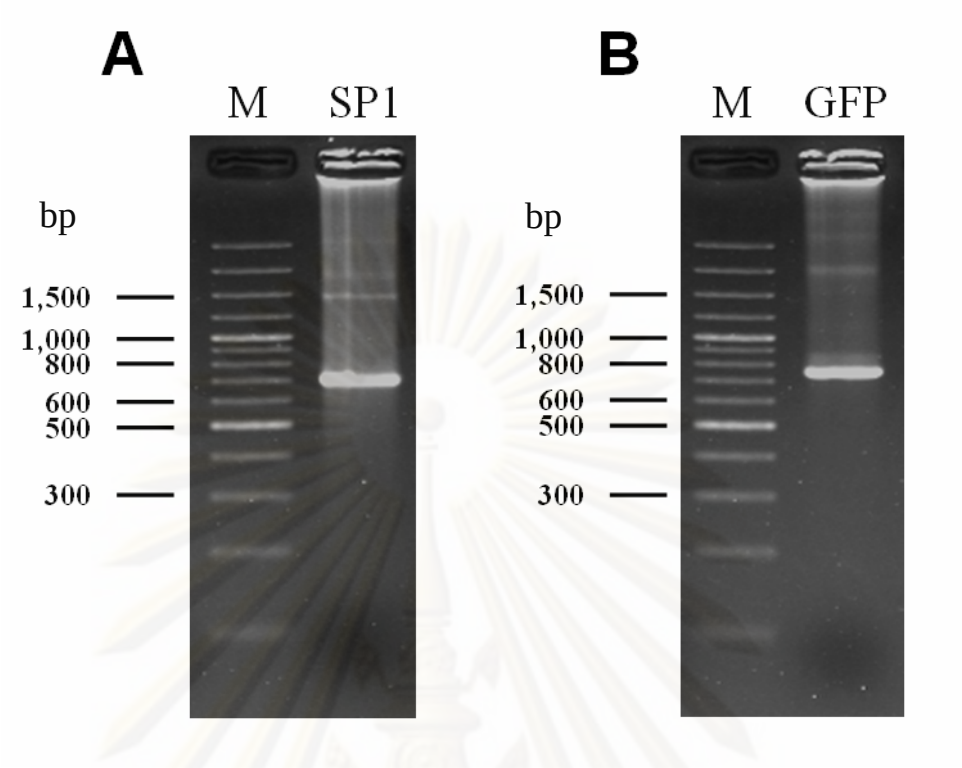

Figure 3.14 Analysis of the dsRNA of *Pm*ClipSP1 (A) and GFP (B) by 1.2% agarose gel strained with ethidium bromide. Lane M is GeneRuler™ 100 bp DNA ladder marker (Fermentas).

3.6.2 Gene silencing of *Pm*ClipSP1

To determine the efficiency of dsRNA mediated knockdown of *Pm*ClipSP1 transcript levels, shrimp (~2 µg) were injected with 5 µg of dsRNA specific for the *Pm*ClipSP1 gene and the level of *Pm*ClipSP1 expression was determined 48 hr after dsRNA injection. For the control groups, GFP dsRNA and 150 mM NaCl were injected into the shrimp. The hemolymph from each group of shrimp (two individuals per group), composed of *Pm*ClipSP1 dsRNA-, GFP dsRNA-, and NaCl-injected shrimp, were randomly collected and subjected to RT-PCR analysis. Elongation factor-1α gene (EF-1α) was used as internal control to monitor the amount of cDNA template and amplification efficiency between samples. The results of RT-PCR analysis showed that the transcriptional level of *Pm*ClipSP1 was decreased in *Pm*ClipSP1 knockdown shrimp, whereas injection of control GFP dsRNA or NaCl buffer had no discernable effect on the *Pm*ClipSP1 transcript levels (Figure 3.15).

The specificity of gene knockdown was further verified by RT-PCR using gene-specific primers for the other known clip-domain serine proteinases of *P. monodon*. The RT-PCR results (Figure 3.15) demonstrated that injection of *Pm*ClipSP1 dsRNA did not detectably suppress transcription of the other clip-SPs (*Pm*PPAE1; FJ595215, *Pm*PPAE2; FJ620685, and *Pm*ClipSP2; FJ620687) and clip-SPHs (*Pm*MasSPH1; DQ455050, *Pm*MasSPH2; FJ620686 and *Pm*MasSPH3; FJ620689), which supports the likely specificity of *Pm*ClipSP1 RNAi knockdown.

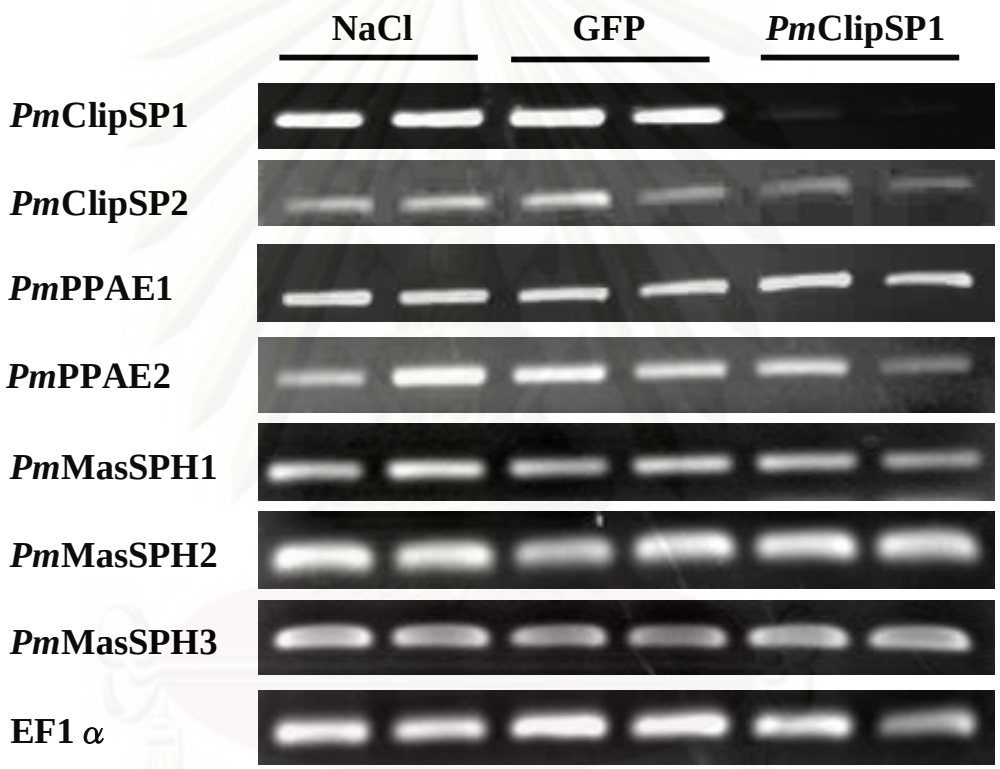

Figure 3.15 Gene-specific silencing of *Pm*ClipSP1 transcript levels in *P. monodon* hemocytes. The effect of *Pm*ClipSP1dsRNA injection on the transcript expression levels of *Pm*ClipSP1, and other shrimp clip-SPs (*Pm*PPAE1, *Pm*PPAE2 and *Pm*ClipSP2) and clip-SPHs (*Pm*MasSPH1, *Pm*MasSPH2 and *Pm*MasSPH3) in *Pm*ClipSP1 dsRNA-, GFP dsRNA- or saline- injected shrimp was examined by RT-PCR using gene-specific primers. Each lane represents cDNA from an individual shrimp. EF1-α was used as a control housekeeping gene to standardize the amount of cDNA template in each reaction.

3.6.3 Hemolymph PO activity of *Pm*ClipSP1 silencing shrimps

To study the effect of *Pm*ClipSP1 RNAi-mediated deficiency on the proPO activating system, the total PO activity in the *Pm*ClipSP1 knockdown shrimp was determined. At 48 hr after the second dsRNA injection, shrimp hemocytes were collected, and the PO activity was determined. The PO activity was measured as the increased absorbance at 490 nm with time. PO activity was recorded as ΔA_{490}/mg total protein/min against control samples that used distilled water instead of shrimp hemolymph. The experiments were repeated three times (Appendix B). The results indicated that no significant decrease in the total PO activity ($P < 0.05$) was detected in the *Pm*ClipSP1 knockdown shrimp when compared to control groups with either GFP dsRNA or NaCl injected shrimp (Figure 3.16). This suggests that *Pm*ClipSP1 was not directly involved in the regulation of the proPO system in shrimp.

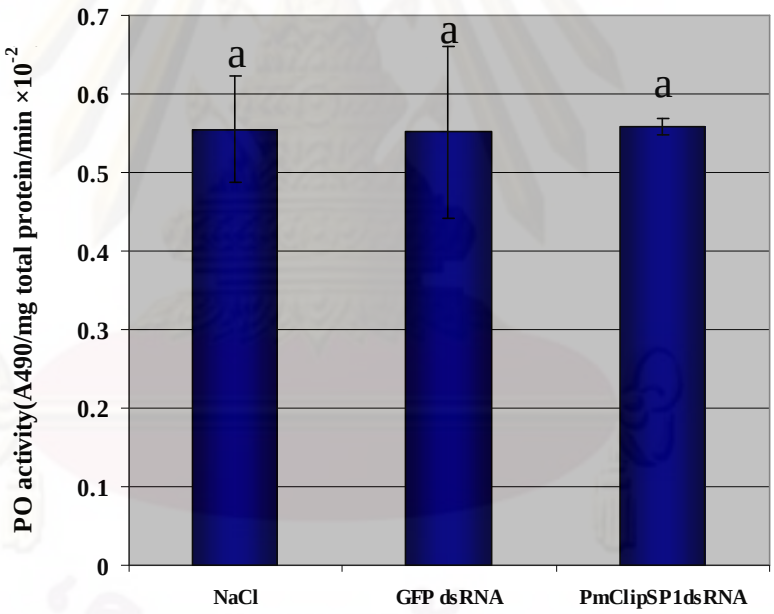

Figure 3.16 Total hemolymph phenoloxidase (PO) activities in *Pm*ClipSP1 knocked down shrimp. Hemolymph was collected 48 hr after the second dsRNA injection. Shrimp injected with either GFP dsRNA in saline (150 mM NaCl), or with only saline, were used as control groups. The total PO enzymatic activity was measured using L-dopa and was defined as ΔA490/mg protein/min. Experiments were repeated three times and the data is shown as the mean ±standard deviation. Means with the same lower case letters (above each bar) are not significantly different at the $p < 0.05$ level.

3.6.4 Cumulative mortality of *V. harveyi* challenge *Pm*ClipSP1 silencing shrimp

To further assess the potential role of *Pm*ClipSP1 in the shrimp defense against bacterial infection, the *Pm*ClipSP1-knockdown shrimp were systemically challenged with *V. harveyi* (2×10^5 CFUs), and the mortality rate was recorded for a period of five days after challenge. Figure 3.17 shows the cumulative mortality for shrimps in each treatment group.

The *Pm*ClipSP1 silenced shrimp had a cumulative mortality of 82 % within the first 24 hr (day 1) post-bacterial infection and reached 86 % by day 3. The mortality remained at this level (86 % mortality rate) over the remainder of the five day assay period. In contrast, in the GFP-dsRNA injected and saline only injected control shrimp, only ~20% and ~27% cumulative mortality was observed at one and two days post-infection and thereafter remained at this level over the five day assay period. Thus, the mortality was induced within the first two days and was significantly higher in the *Pm*ClipSP1 dsRNA mediated *Pm*ClipSP1 knockdown shrimp.

3.6.5 Bacteria clearance

To investigate the role of *Pm*ClipSP1 in the bacterial clearance during *V. harveyi* infection, knockdown of *Pm*ClipSP1 was examined. Shrimp pre-injected with dsRNA of *Pm*ClipSP1 or GFP (as a control), were infected with *V. harveyi* ($2x10^5$ CFUs). At 6 hr post-injection of bacterial suspension, bacterial count in the hemolymph from silenced shrimp was carried out. The result showed that the silencing of *Pm*ClipSP1 significantly increased the number of bacterial colony, 2.4-fold, in silenced shrimp, as compared to control shrimp (Figure 3.18). Thus, the combined results of the cumulative mortality rate and the viable bacterial clearance experiments suggest an important role for *Pm*ClipSP1 in the *P. monodon* shrimp defense against *V. harveyi* infection.

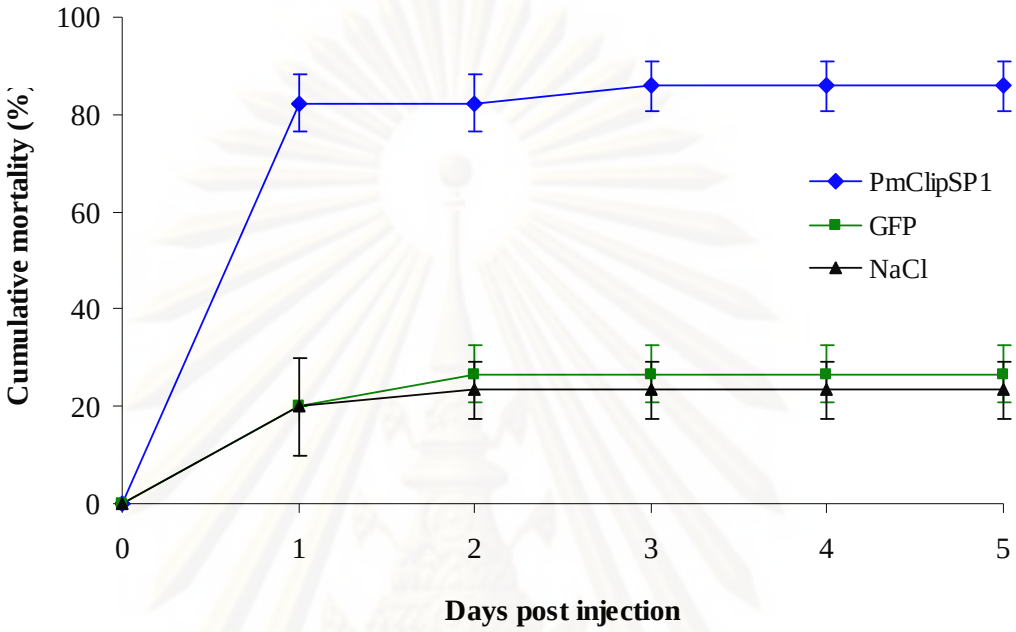

Figure 3.17 Cumulative mortality of *Pm*ClipSP1 silencing shrimp challenged with *Vibrio harveyi*. Shrimp were injected twice with dsRNA specific *Pm*ClipSP1 gene following challenge by *Vibrio harveyi*. The mortality was recorded twice a day for 5 days. This experiment was repeated three times. The statistical data were analyzed by ANOVA and DUNCAN.

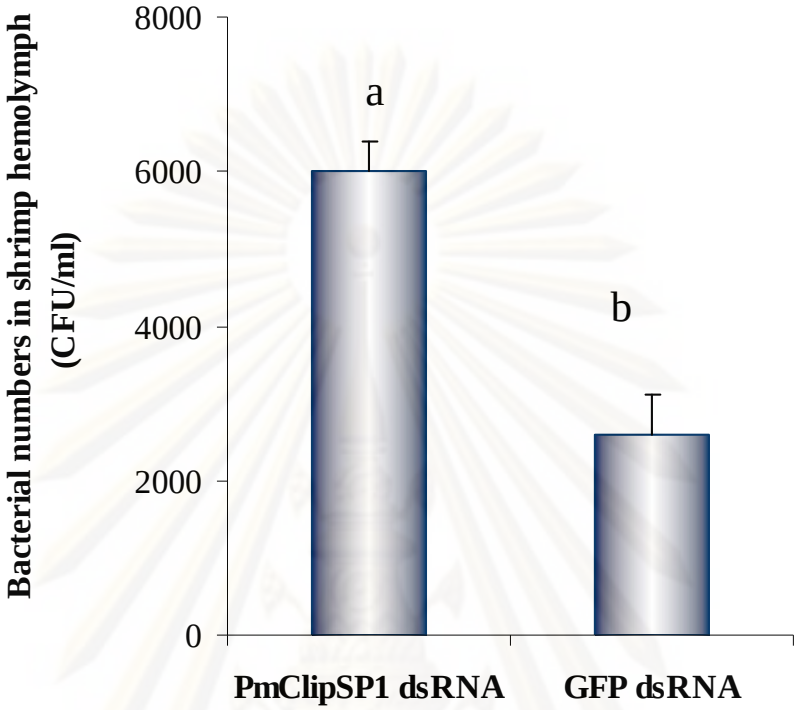

Figure 3.18 Bacterial number in *Pm*ClipSP1 silencing shrimp. Shrimp were injected with dsRNA specific *Pm*SP1 or GFP gene and then injected with *Vibrio harveyi* 639 (2×10^5 CFUs). The bacteria forming unite in shrimp hemolymph were determined as CFU/ml of hemolymp at 6 hr after bacteria challenge. This experiment was repeated three times. The data was analyzed by ANOVA and DUNCAN. Different of CFUs/ml from each group were considered significant at $p< 0.05$

CHAPTER IV
DISCUSSIONS

4.1 Characterization of a clip domain serine proteinase (*Pm*ClipSP1) from black tiger shrimp *Penaeus monodon*

Shrimp viral and bacterial diseases have seriously impacted the sustainability and economic success of the shrimp aquaculture industry worldwide. Prevention and control of diseases are now the priority for the durability of shrimp industry (Bachère et al., 1995). A major problem for the control and prevention of shrimp diseases is the lack of enough knowledge of shrimp immunity. In order to effectively solve the disease problems and increase sustainability of shrimp farming, the study of immune-related genes and their products that would lead to better understanding the immune system is necessary (Bachère, 2000).

Shrimp have a non-specific innate immunity which is composed of diverse processes and molecules to defend themselves against invading pathogens. The immune system of crustaceans consists of both cellular and humoral defenses including encapsulation, phagocytosis, a prophenoloxidase (proPO)-activating system for melanization, a clotting process and specific and general antimicrobial actions (Smith et al., 1992; Söderhäll et al., 1992).

In invertebrates, extracellular SP cascades involving the clip domain SP (clip-SP) family play important roles in signaling cascades in both embryonic development and defense responses, such as in hemolymph coagulation in the horseshoe crab, *Tachypleus tridentatus* (Muta et al., 1990), antimicrobial peptide synthesis in *Drosophila* (Jang et al., 2008), and the activation of proPO in insects and crustaceans (Jiang et al., 2000; Jang et al., 2008; Charoensapsri et al., 2009).

Several clip-SPs and clip-SPHs have been reported in insects. In *Drosophila melanogaster*, 24 genes of SPs and 13 genes of SPHs containing clip was identified form total 147 SPs and 57 SPHs (Ross et al., 2003). In *M. sexta*, 10 hemolymph serine proteinases have a clip domain whereas 4 hemolymph serine proteinases have two clip domains (Jiang et al., 2005). The *A. gambiae* revealed 306 SP-related gene in

malaria mosquito, 41 of which encode protein that contain at lest one clip domain (Holt et al., 2002; Zdobnov et al., 2002).

From the *P. monodon* EST database (http://pmonodon.biotec.or.th) (Tassanakajon et al., 2006), four clip-SPs (*Pm*PPAE1; FJ595215:(Charoensapsri et al., 2009), *Pm*PPAE2; FJ62068; *Pm*ClipSP1; FJ620688 and *Pm*ClipSP2; FJ620687) and three clip-SP homologues (*Pm*MasSPH1; DQ455050: (Amparyup et al., 2007), *Pm*MasSPH2; FJ620686 and *Pm*MasSPH3; FJ620689) were obtained (Charoensapsri et al., 2009). In this study, a clip-SP (*Pm*ClipSP1), similar to the serine protease 14D (AAB62929) of *Anopheles gambiae* (58% similarity), is one of the interested gene because the *Pm*ClipSP1 transcript significantly increased upon *Vibrio harveyi* infection (Amparyup et al., 2009B) indicating its potential role in shrimp immune responses.

To characterize the function of *Pm*ClipSP1 on shrimp immunity, a full-length cDNA encoding the *Pm*ClipSP1 from *P. monodon* was analyzed. The *Pm*ClipSP1 contains a putative signal peptide followed by a clip-domain at the N-terminus and a serine proteinase domain at the C-terminus. This domain organization has been described as the characteristic of arthropod clip-SPs (Jiang et al., 2000; Jang et al., 2008).

By SMART (Simple Modular Architecture Research Tool) program analysis, *Pm*ClipSP1 was synthesized as zymogen and required proteolytic cleavage at position between Ser25 and Gln26 to activated, similar to the activation of other serine proteinases such as clip-SPs as well as trypsinogen and chymotrysinogen (Jiang et al., 2000). The clip-serine proteinase is a group of serine proteinase that consist of serine proteinase domain and one or more clip domain at N-terminus and was found so far only in arthropod insects, crustaceans and horseshoe crab (Jiang et al., 2000).

The first clip domain was discovered in proclotting enzyme from horseshoe crab, *Tachypleus tridentatus* (Muta et al., 1990). It was found six strictly conserve cysteine residues forming three disulfide bonds. The clip domain was named because three disulfide bonds forming the shape look like a paper clip (Iwanaga et al., 1998). The double mutation of cystein in clip domain of *D. melanogaster* ester leads to abnormal in embryonic rescues (Tian et al., 2008). It can be concluded that the

conserve cysteins have an important role in the fuction of serine proteinase. Now the function of clip domain could not had been identified however it perhaps responsible for mediating specific protein-protein interactions or for regulating cascades of SP activities (Jang et al., 2008). There is a report that the recombinant clip domain of crayfish PPAE could be inhibited the growth of gram-positive bacteria such as *Micrococcus luteus* M111 and *Bacillus megaterium* Bm11(Wang et al., 2001). Thus, clip domain may possibly have more than only one function. The number of clip domain are varied in different clip-SPs or clip-SPHs for example 2 domains in PPAE of *Bombyx mori* (Satoh et al., 1999) or PAP-2 of *Manduca sexta* (Yu et al., 2003), 3 domains in MasSPH3 of *P. monodon* (FJ620689), 5 domains in MasSPH of *H. diomphalia* (Kim et al., 2002), 7 domains in Mas-like SP of *P. leniusculus* (Lee et al., 2001).

From the deduced amino acid sequence analysis, the catalytic domain of *Pm*ClipSP1 was presented the three conserved amino acid residues. They were called catalytic triad that consisted of His151, Asp216 and Ser314. These conserve residues are the essential feature for successful proteolytic mechanism (Phillips et al., 1992). Moreover, there are some proteinases that showed a slight difference from average serine protease with the amino acid Ser was replaced by another amino acid (such as Gly) leading to lacking protease activity. As such, these proteins are called serine proteinase homolog (SPH) (Jiang et al., 2000). The data obtained in this study showed that *Pm*ClipSP1 belonging to the serine proteinase family because it contain the catalytic triad. From the sequence alignment analysis, eight conserve cystein residues forming four disulfide bridges was observed in SP domain of *Pm*ClipSP1 similar to *A. gambie* SP14D2, *D. melanogaster* ester, *H. diomphalia* PPAF-I, *D. melanogaster* MP1A and *M. sexta* PAP1. On the other hand, SP domain of *P. monodon* PPAE1, *A. gambie* SP14D1 and *P. leniusculus* PPAE was observed only six conserved cystein residues. This data suggested that a *Pm*ClipSP1 is a trypsin-like serine protease which contains eight conserved cysteine residues (Cys136-Cys152, Cys179-Cys189, Cys268-Cys300 and Cys310-Cys341), believed to form four disulfide bonds (Hartley, 1964). Moreover, a pair of cysteine (Cys96-Cys239) between clip domain and SP domain was found. The inter-domain disulfide bridge probably connected the clip domain and SP domain together after protein cleavage activation (Piao et al., 2005).

4.2 Recombinant protein expression of a clip domain serine proteinase (*Pm*ClipSP1) from black tiger shrimp *P. monodon*

In the study of functional protein, the *E. coli* expression system is the first choice for the production of recombinant protein (Baneyx, 1999) because of its advantages such easy to construct, low cost, large quantity of protein and high success rates (Cabrita et al., 2004). However, it has some disadvantage for expression of eukaryote protein such as the inability to perform many post-translation modifications and the expression of protein often form as inclusion body (Swartz, 2001). According to the using of *E. coli* for expression of recombinant protein, it has more factors effecting to a yield of expression protein such as plasmid copy number, upstream element, temperature and codon usage (Baneyx, 1999; Swartz, 2001; Jonasson et al., 2002). For solution of this problem, pET22b(+) and *E. coli* strain Rosetta (DE3) were selected as an expression vector and an expression host respectively. The *E. coli* strain Rosseta (DE3) supplied rare codon usage, suitable for the expression of eukaryote protein that is associated with codon rarely.

Both the recombinant mature protein and the SP domain of *Pm*ClipSP1 were mainly expressed as inclusion body. Inclusion bodies are a dense amorphous aggregate of misfold protein found in the cytoplasmid and periplasmid space (Singh et al., 2005). High level expression of nonnative protein and highly hydrophobic protein result in accumulating them as insoluble aggregates *in vivo* as inclusion body (Mitraki et al., 1991). Inclusion body proteins are lacking of biological activity and required solubilization, refolding and purification to recover functional protein activation (Rudolph et al., 1996; Singh et al., 2005). So the recombinant *Pm*ClipSP1 proteins were solubilized in 8 M urea and purified by using Ni-NTA affinity chromatography. The purified proteins were obtained although some contaminated cellular components were observed. The renaturation of purified proteins were performed by dialysis in 20 mM Tris-HCl buffer pH 8.0 to remove urea. According to this step, we expected that the recombinant proteins would be correctly refolded and exhibited the function of protease activity and/or PO activity. Unfortunately, the recombinant *Pm*ClipSP1 both the mature protein and the SP domain protein were not exhibited proteolytic activity similar to the recombinant chymotrypsin-like serine protease from the Chinese shrimp, *Penaeus chinensis*, which also showed no proteinase activity (Shi et al.,

2008). The lack of proteinase activity may be due to misfolding form the renaturation processes. The successful of renaturing protein depends on protein concentration, component of the refolding buffer, disulfide bond formation, method of refolding and purity of recombinant protein (Cabrita et al., 2004). Another useful method of reducing the formation of inclusion bodies containing heterologous proteins is to lower the temperature of growth from 37 ℃ to 30 ℃ (Schein, 1989).

So far, there had a little report correlated with successful of the recombinant eukaryote protease enzyme expressing in *E. coli* system. Recombinant human micro-plasminogen over-expressing in *E. coli* could be exhibited urokinase activation (Ma et al., 2007). In arthropod, yeast expression system and baculovirus-infected insect cell were preferentilly adopted for example the baculovirus-infected insect cells expressing recombinant clip-SP, proPAP and proPAP-2 of *M. sexta* (Wang et al., 2001; Ji et al., 2003). In some case, the studies of functional proteinase activity have been done by purification its native protein from specific tissues such as HP14 from *M. sexta* hemolymp, PPAE from larval cuticles of *Bombyx mori* (Satoh et al., 1999) and PPAF from hemolymph of coleopteran, *H. diomphalia* larvae (Lee et al., 1998). The above-mentioned methods could reduce misfolding of eukaryote proteins. Yeast and baculovirus-infected insect cell are often used to produce recombinant proteins that are not successfully expressed in *E. coli* because protein folding problem and requirement for glycosylation or posttranslational modification in insect cells system. However, these systems are more complex with higher cost and lower yield of protein product than *E. coli* system (Demain et al., 2009).

4.3 Gene silencing of a clip domain serine proteinase (*Pm*ClipSP1) from black tiger shrimp *P. monodon* by RNA interference

RNA interference (RNAi) induced by long dsRNA has been used to study the function of immune relate genes in several arthropods (Reynolds et al., 2008). The specific knocking down of *Pm*ClipSP1 was succeeded without the depletion of other related SP genes. Functional analysis using in *Pm*ClipSP1-specific RNA interference revealed that *Pm*ClipSP1 is not involved in the activation of proPO mediated melanization. This result did not support the data searching from Blast and phylogenic analysis. However, Charoensapsri et al. (2009) recently identified and characterized a

clip-SP (named *Pm*PPAE1) in *P. monodon*, and found that *Pm*PPAE1 is required for the shrimp proPO system (Charoensapsri et al., 2009). Consequently, dsRNA-mediated RNAi was used to determine the involvement of *Pm*ClipSP1 in immune defense against *V. harveyi* infection. The suppression of the *Pm*ClipSP1 gene led to a significant increase in the number of viable bacteria in the hemolymph and in the mortality rate of shrimp systemically infected with *V. harveyi*. These findings suggest that *Pm*ClipSP1 plays a role in the antibacterial defense mechanism of *P. monodon* shrimp. These results were in accordance with the recent studied of *Pm*ClipSP1 which reported that the transcription of *Pm*ClipSP1 was increased after 3 hr. of bacterial challenge with *V. harveyi*. Furthermore the transcription of *Pm*ClipSP1 was highest expressed in shrimp hemocyte (Amparyup et al. 2009B). In insect and crustacean, the immune related SPs with clip domain were mostly expressed in hemocyte and/or fat body for example clip domain SP in scallop, *Chlamys farreri* (Zhu et al., 2008), the Masquerade-like SPH of crayfish, *P. leniusculus* (Kopacek et al., 1993), factor D-like SP from *Denmacentor variabilis* (Simser et al., 2004) etc.

Several studies report the discovery of SPs and SPHs that responded to bacterial or virus infection but their physiological function has not been identified. In *Drosophila*, a serine proteinase involved in immune responses could be identified from bacterial-challenged hemocyte (De Morais Guedes et al., 2005). Although only one trypsin-like serine proteinase was responded to bacterial infection in *Fenneropenaeus chinensis* but the four hepatopancreas trypsin-like serine proteinases were up-regulated in WSSV infected shrimp (Shi et al., 2009). A *Pm*MasSPH1 of *P. monodon* was up-regulated after *V. harveyi* infection (Amparyup et al., 2007) while this gene was down-regulated in YHV infection. (Sriphaijit et al., 2007). Moreover, the recent study revealed that *Pm*MasSPH1 is a multifunctional immune molecule involved in hemocyte adhesion, bacterial binding, bacterial clearance and antimicrobial activity (Jitvaropas et al., 2009). On the contrast, the transcription level of hemocyte SP from *L. vannamei* did not changed after *Vibrio alginolyticus* inoculation (Jiménez-Vega et al., 2005). Moreover, the inhibition of *M. sexta* SP by using benzamidine leads to reduction of some antimicrobial protein (Kanost et al., 1999). These data suggested that SPs are involved in antibacterial mechanism of arthropod species.

The role of serine proteinase cascade in immunity are widely study in arthropods, the serine proteinases with clip domain are involved in several defense mechanisms especially the activation of signaling pathway leading to synthesis of antimicrobial peptides (Kurata et al., 2006), blood clotting (Davie et al., 1991) and prophenoloxidase mediated melanization (Cerenius et al., 2008). For example, the *persephone* gene coding blood serine proteinase could induce Toll pathway that was responded to fungi infection in *D. melanogaster* (Ligoxygakis et al., 2002). In *Drosophila*, the Toll pathway is an important pathway for anti-gram-positive bacteria, fungi and virus by an induction of synthesis of antimicrobial peptides (Lemaitre et al., 1996; Rutschmann et al., 2002; Zambon et al., 2005) while Gram-negative bacteria is affected by the activation of the immunodeficiency (Imd) pathway (Gottar et al., 2002; Ramet et al., 2002). Two SPs, known as easter and snake, were required for activation of this pathway during a protease cascade. From our recent study, five novel SPs were found that may activate the Toll pathway. Although the Toll like receptor was found in many shrimps, but the upstream processes of Toll pathway was poor investigated. Recent studies showed the Toll like receptor from *P. monodon* and *P. vannmei* were not responsed to viral infection whereas Toll receptor from *F. chinesis* was reduced after WSSV infection (Arts et al., 2007; Labreuche et al., 2009). However *P. chinesis* Toll was up regulated after injection with *Vibrio anguillarum* for 24 hr (Yang et al., 2008). These data were different from those of *Drosophila* in which Toll pathway was responded to virus and some gram-positive bacteria. Which may imply that Toll pathway in shrimp may be different from the insect.

The clotting system in arthropods has two different mechanisms. In horseshoe crab blood clotting can be activated by the proteolytic cascade. This activation cascade bring about coagulogen molecule are subsequently cleaved and formed insoluble gel (Kawabata et al., 1996). On other hand, the polymerization of clotting proteins (CP) are derived by releasing of the tranglutaminase (TGase) in the crayfish clotting system (Kopacek et al., 1993; Hall et al., 1999). While the clotting system and the proPO system in horseshoe crab are closely related, *Tachypleus* clotting enzyme can transform hemocyanin to phenoloxidase, and the conversion reaches a plateau at 1:1 stoichiometry without proteolytic cleavage (Nagai et al., 2000). It is possible that coagulating system may associate with melanization or other immune responses such as encapsulation (Jiang et al., 2000). While scolexin, a serine

proteinase like protein, may play an important role in *M. sexta* coagulation system (Finnerty et al., 1999). These suggest that SP in arthropod hemolymph may take path in more than one pathway. This is the fact that an extracellular signal transduction network that mediate various immune responses are served in the complex system of serine proteinase cascade (Kanost et al., 1999). However, the silencing of *Pm*ClipPm1 transcript levels did not detectably affect the hemolymph clotting in *P. monodon* shrimp (data not shown).

In summary, this research demonstrates that *Pm*ClipSP1 of *P. monodon* is unlikely to be directly involved in the proPO-activating system, but that it is important for the shrimp defense mechanism against infection with the pathogenic Gram-negative bacteria *V. harveyi* by an unknown mechanism, which is yet to be determined.

CHAPTER V

CONCLUSION

1. A full length cDNA of *Pm*ClipSP1 gene contains an open reading frame (ORF) of 1,101 bp encoding a predicted protein of 366 amino acids including a putative signal peptide of 25 amino acids. The calculated molecular mass of the mature protein was 36.48 kDa with a predicted isoelectric point (pI) of 5.56.

2. Sequence analysis revealed that the deduced amino acid sequence of *Pm*ClipSP1 is composed of N-terminal clip domain with six conserved cysteine residues forming three disulfide bonds and C-terminal serine proteinase domain containing conserved His-Asp-Ser catalytic triad.

3. Sequence comparison of known proteins from NCBI database using BLASTX program revealed that the deduced amino acid of *Pm*ClipSP1 had a similarity of 58%, 57%, 54%, 52% and 51% to those of *Penaeus chinensis* SP, *Anopheles gambiae* SP14D, *Holotrichia diomphalia* PPAFI, *Drosophila melanogaster* MP1 and *Manduca sexta* PAP3 respectively.

4. The recombinant proteins of both a mature protein and a serine proteinase domain protein of *Pm*ClipSP1 gene were over-expressed in *E. coli* and successfully purified by Ni-NTA column. Both recombinant proteins lack a proteolytic activity and the activation of phenoloxidase (PO) activity. The immunobloting analysis showed the *Pm*ClipSP1 that was observed only in hemocyte but not in cell-free plasma of *P. monodon*.

5. RNA interference-mediated suppression of *Pm*ClipSP1, performed by injection of double-stranded RNA (dsRNA) corresponding to the *Pm*ClipSP1 gene into shrimp, resulted in a significant reduction of *Pm*ClipSP1 but not other clip-SPs and related gene transcript levels of *P. monodon*, suggesting gene-specific knockdown.

6. Silencing of *Pm*ClipSP1 gene indicated that no significant decrease in the total PO activity was detected in the *Pm*ClipSP1 knockdown shrimp when compared to control groups with either GFP dsRNA or NaCl injected shrimp. This result suggested that *Pm*ClipSP1 was not directly involved in the regulation of the proPO system in shrimp.

7. Suppression of the *Pm*ClipSP1 gene led to a significant increase in the number of viable bacteria in the hemolymph (~2.4 fold) and in the mortality rate (59%) of shrimp systemically infected with *Vibrio harveyi*.

8. Our data demonstrates that *Pm*ClipSP1 of *P. monodon* is unlikely to be directly involved in the proPO-activating system, but that it is important for the shrimp defense mechanism against infection with the pathogenic Gram-negative bacteria, *V harveyi*.

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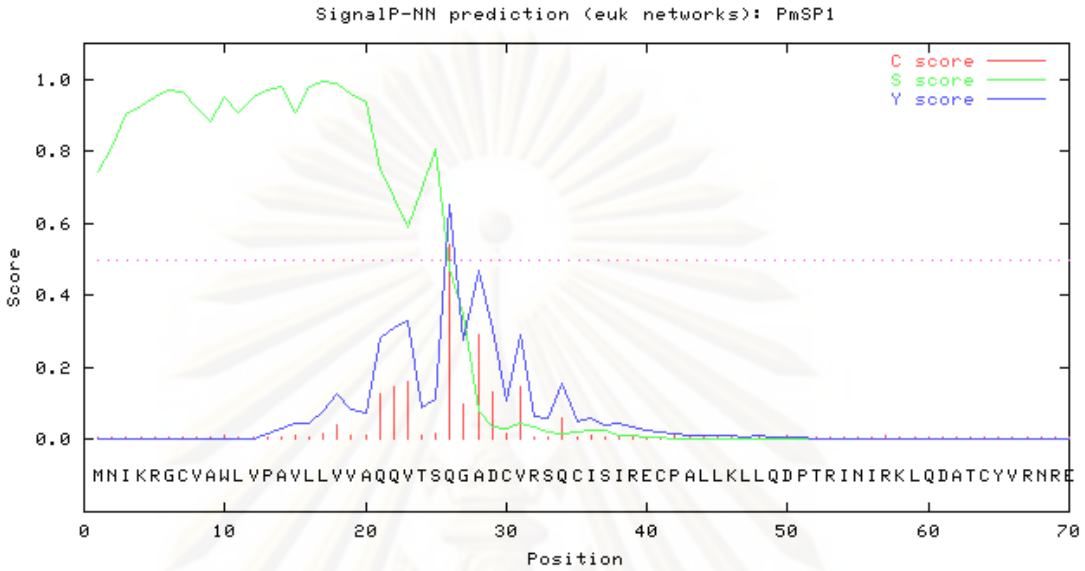
APPENDICES

APPENDIX A

The signal peptide prediction by signalP

_Pm_ClipSP1



>*PmClipSP1* length = 70

# Measure	Position	Value	Cutoff	signal peptide?
max. C	26	0.542	0.32	YES
max. Y	26	0.652	0.33	YES
max. S	17	0.994	0.87	YES
mean S	1-25	0.884	0.48	YES
D	1-25	0.768	0.43	YES

Most likely cleavage site between pos. 25 and 26: VTS-QG

The N-Glycosylation sites prediction with NetNglyc sever

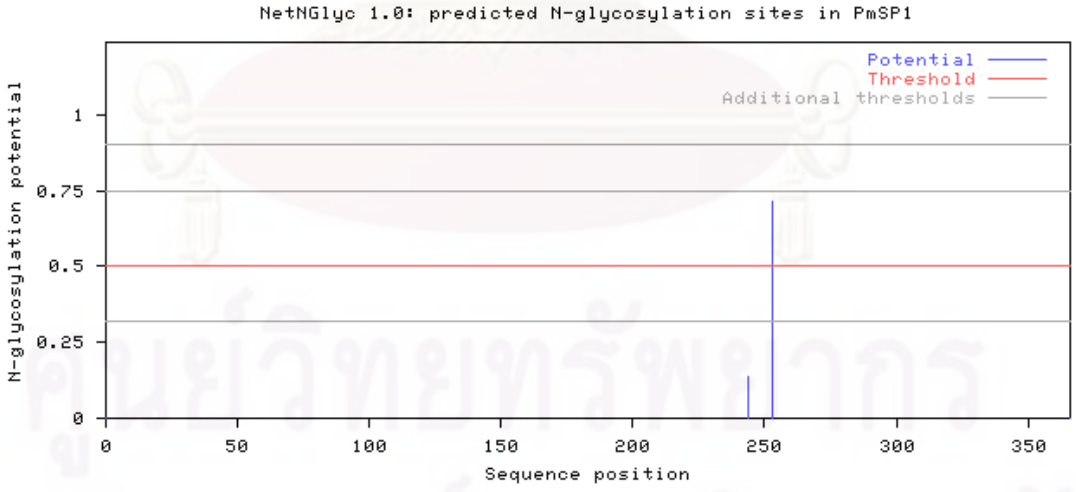
*Pm*ClipSP1

Output for 'PmClipSP1'

```
Name:  PmClipSP1   Length:  366
MNIKRGCVAWLVPAVLLVVAQQVTSQGADCVRSQCISIRECPALLKLLQDPTRINIRKLQDATCYVRNREPMVCCPSITT 80
TETPTIPTKSLLPENCGHSAHLNRIVGGEVAPLDAYPWKAVLGYKDKGLAAIEFLCGGSVINERYVLTAAHCVDPGTLGT 160
RRLEVVRLGEWDLTTTEDCESTNSGGVFCAPPVQDFEAEEIIGHPSYNTRVRFSDDIALIRLNRPINFQESAGFVLPVCL 240
PPSNFSPRTAAGNKSAIAAGWGFTETGSASNKIKHVKLPLVDSTECSQVYKGSTVSEQLCAGGNAGEDSCGGDSGGPLVL 320
AGTFGPPYQQIGIVSYGPVSCGQQGVPGIYTSVSSYRTWIEQNLKP
.............................................................................. 80
.............................................................................. 160
.............................................................................. 240
...........N.................................................................. 320
.......................................... 400
```

(Threshold=0.5)

SeqName	Position	Potential	Jury agreement	N-Glyc result
PmClipSP1	244	NFSP	0.1360 (9/9)	---
PmClipSP1	253	NKSA	0.7128 (8/9)	+

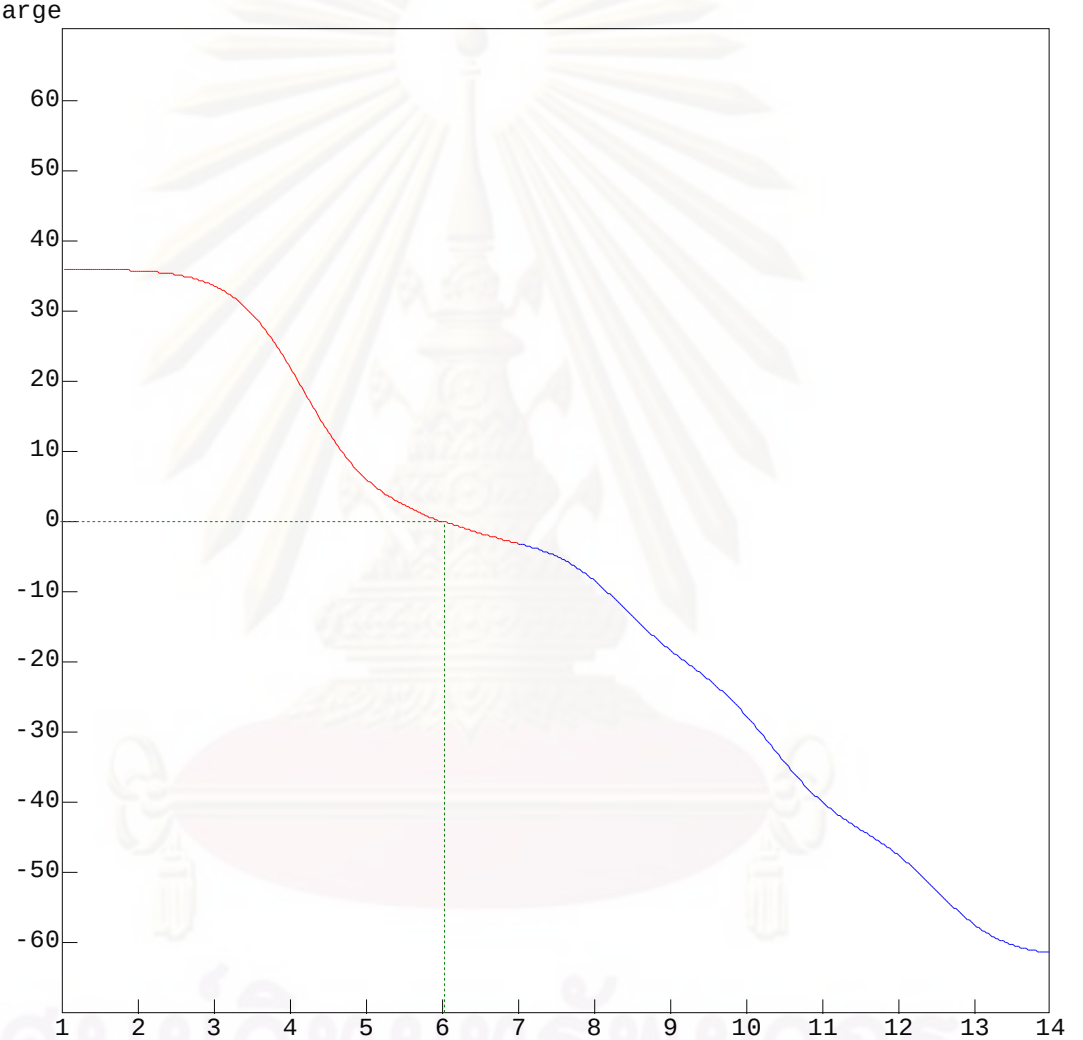


The prediction of putative pI by Ganetyx program

*Pm*ClipSP1

```
[GENETYX-WIN : Caluculate isoelectric point]
Date              : 2009.03.25
Filename          : Sequence1
Sequence size     : 376
Sequence position : 1 - 376
```

Charge



Amino Acid	Number	pKa
Arg (R)	18	12.5
His (H)	5	6.0
Lys (K)	13	10.5
Asp (D)	14	3.9
Cys (C)	17	8.3
Glu (E)	20	4.3
Tyr (Y)	10	10.1

```
N-terminal Met (M)  9.3
C-terminal  ( )  0.0
Isoelectric point: 5.98
```

APPENDIX B

```
ATGCATCATCATCATCATCATCAGGGTGCAGATTGTGTACGCAGTCAGTGTATCTCAATT  60
 M  H  H  H  H  H  H  Q  G  A  D  C  V  R  S  Q  C  I  S  I
CGAGAATGTCCAGCTCTGCTAAAACTTTTACAGGATCCTACACGAATCAATATCAGGAAG  120
 R  E  C  P  A  L  L  K  L  L  Q  D  P  T  R  I  N  I  R  K
CTACAAGATGCCACCTGCTATGTCAGGAACCGGGAACCTATGGTATGCTGTCCATCTATA  180
 L  Q  D  A  T  C  Y  V  R  N  R  E  P  M  V  C  C  P  S  I
ACTACAACTGAAACACCGACGATTCCCACAAAGTCTCTCCTCCCAGAAAATTGTGGGCAC  240
 T  T  T  E  T  P  T  I  P  T  K  S  L  L  P  E  N  C  G  H
AGTGCTCACTTGAACAGAATTGTGGGTGGAGAAGTAGCCCCACTTGATGCATACCCATGG  300
 S  A  H  L  N  R  I  V  G  G  E  V  A  P  L  D  A  Y  P  W
AAAGCTGTTCTAGGATATAAAGATAAAGGATTAGCTGCCATTGAATTTCTCTGCGGGGGT  360
 K  A  V  L  G  Y  K  D  K  G  L  A  A  I  E  F  L  C  G  G
TCAGTCATTAACGAGAGATATGTTCTTACTGCTGCTCATTGTGTAGACCCTGGTACACTT  420
 S  V  I  N  E  R  Y  V  L  T  A  A  H  C  V  D  P  G  T  L
GGCACACGAAGATTGGAAGTAGTTCGACTGGGTGAATGGGACCTCACCACCACTGAAGAC  480
 G  T  R  R  L  E  V  V  R  L  G  E  W  D  L  T  T  T  E  D
TGTGAGAGCACAAATAGTGGAGGGGTATTCTGTGCTCCTCCAGTTCAAGATTTCGAGGCT  540
 C  E  S  T  N  S  G  G  V  F  C  A  P  P  V  Q  D  F  E  A
GAGGAAATTATCGGTCATCCCTCATACAACACTCGTGTGAGATTCTCCGATGACATTGCA  600
 E  E  I  I  G  H  P  S  Y  N  T  R  V  R  F  S  D  D  I  A
CTCATCAGACTCAACAGGCCCATTAACTTCCAGGAATCAGCAGGATTTGTGTTGCCTGTG  660
 L  I  R  L  N  R  P  I  N  F  Q  E  S  A  G  F  V  L  P  V
TGCCTGCCTCCATCTAACTTCTCCCCTCGTACAGCAGCTGGTAACAAATCAGCAATTGCA  720
 C  L  P  P  S  N  F  S  P  R  T  A  A  G  N  K  S  A  I  A
GCTGGATGGGGCTTCACTGAAACTGGCTCTGCAAGTAACAAAATTAAGCATGTAAAGCTG  780
 A  G  W  G  F  T  E  T  G  S  A  S  N  K  I  K  H  V  K  L
CCATTGGTTGACAGTACTGAGTGTAGTCAGGTGTACAAAGGCAGTACAGTCAGTGAACAA  840
 P  L  V  D  S  T  E  C  S  Q  V  Y  K  G  S  T  V  S  E  Q
CTCTGTGCCGGTGGCAATGCTGGTGAAGACTCGTGCGGTGGAGACTCTGGTGGTCCCTTG  900
 L  C  A  G  G  N  A  G  E  D  S  C  G  G  D  S  G  G  P  L
GTACTTGCCGGTACTTTTGGTCCTCCCTACCAGCAGATTGGCATTGTTTCCTACGGTCCT  960
 V  L  A  G  T  F  G  P  P  Y  Q  Q  I  G  I  V  S  Y  G  P
GTCAGCTGTGGCCAGCAGGGGGTACCTGGTATCTACACATCTGTAAGCAGCTACAGGACA  1020
 V  S  C  G  Q  Q  G  V  P  G  I  Y  T  S  V  S  S  Y  R  T
TGGATTGAGCAGAACTTAAAGCCATAA                                   1050
 W  I  E  Q  N  L  K  P  *
```

1. Nucleotide sequence and deduced amino acid sequence of recombinant mature *Pm*ClipSP1. Bold and underline showed His tag sequence for using purification.

```
  1 atgcatcatcatcatcatagaattgtgggtggagaagtagcc
     M  H  H  H  H  H  H  R  I  V  G  G  E  V  A
 46 ccacttgatgcatacccatggaaagctgttctaggatataaagat
     P  L  D  A  Y  P  W  K  A  V  L  G  Y  K  D
 91 aaaggattagctgccattgaatttctctgcgggggttcagtcatt
     K  G  L  A  A  I  E  F  L  C  G  G  S  V  I
136 aacgagagatatgttcttactgctgcccattgtgtagaccctggt
     N  E  R  Y  V  L  T  A  A  H  C  V  D  P  G
181 acacttggcacacggagattggaagtagttcgactgggtgaatgg
     T  L  G  T  R  R  L  E  V  V  R  L  G  E  W
226 gacctcaccaccactgaagactgtgagagcacaaatagtggaggg
     D  L  T  T  T  E  D  C  E  S  T  N  S  G  G
271 gtattctgtgctcctccagctcaagatttcgaggctgaggaaatt
     V  F  C  A  P  P  A  Q  D  F  E  A  E  E  I
316 atcggtcatccctcatacaacactcgtgtgagattctccgatgac
     I  G  H  P  S  Y  N  T  R  V  R  F  S  D  D
361 attgcactcatcagactcaacaggcccattaacttccaggaatca
     I  A  L  I  R  L  N  R  P  I  N  F  Q  E  S
406 gcaggatttgtgttgcctgtgtgcctgcctccatctaacttctcc
     A  G  F  V  L  P  V  C  L  P  P  S  N  F  S
451 cctcgtacagcagctggtaacaaatcagcaattgcagctggatgg
     P  R  T  A  A  G  N  K  S  A  I  A  A  G  W
496 ggcttcactgaaactggctctgcaagtaataaaattaagcatgta
     G  F  T  E  T  G  S  A  S  N  K  I  K  H  V
541 aagctgccattggttgacagtactgagtgtagtcaggtgtacaaa
     K  L  P  L  V  D  S  T  E  C  S  Q  V  Y  K
586 ggcagtacagtcagtgaacagctctgtgccggtggcaatgctggt
     G  S  T  V  S  E  Q  L  C  A  G  G  N  A  G
631 gaagactcgtgcggtggagactctggtggtcccttggtacttgcc
     E  D  S  C  G  G  D  S  G  G  P  L  V  L  A
676 ggtacttttggtcctccctaccagcagattggcattgtttcctac
     G  T  F  G  P  P  Y  Q  Q  I  G  I  V  S  Y
721 ggtcctgtcagctgtggccagcaggggtacctggtatctacaca
     G  P  V  S  C  G  Q  Q  G  V  P  G  I  Y  T
766 tctgtaagcagctacaggacatggattgagcagaacttaaagcca
     S  V  S  S  Y  R  T  W  I  E  Q  N  L  K  P
811 taa 813
     *
```

2. Nucleotide sequence and deduced amino acid sequence of recombinant *Pm*ClipSP1-SP domain. Bold and underline showed His tag sequence for using purification.

APPENDIX C

1. The proteinase activity assay of recombinant SP domain. Trypsin and Chymotrypsin were adopted as positive control.

proteinase	A_{410}		
	BPVApNA[1]	SAAPFpNA[2]	D,L-BApNA[3]
Trypsin (0.02/0.05 μM)*	0.3772	-	0.0542
Chymotrypsin (0.05 μM)	-	0.2094	-
SP1SPdomain (0.02/0.05 μM)*	0	0	0
SP1SPdomain (0.2/0.5 μM)*	0	0	0
SP1SPdomain (2.0/5.0 μM)*	0	0	0
SP1SPdomain (20.0/15.0 μM)*	0	0	0

1. N-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Trypsin substrate)
2. N-succinyl-Ala-Ala-Pho-Phe-*p*-nitroanilide (Chymotrypsin substrate)
3. N-benzoyl-D,L-aginine 4- nitroanilide hydrochlorid (Trypsin substrate)

* 0.02 , 0.2 , 2.0 , 20.0 μM proteine for substrate Benz
  0.05 , 0.5 , 5.0 , 15.0 μM proteine for substrate R-pNA , AApho

2. The proteinase activity assay of recombinant SP domain by varies $CaCl_2$ concentration. Trypsin was adopted as positive control.

Proteinase	Conc. $CaCl_2$	A_{410}	
	1. blank	0	0
Trypsin	2. 5 mM $CaCl_2$	0.5125	0.5135
	3. 25 mM $CaCl_2$	0.4423	0.4440
	4. 50 mM $CaCl_2$	0.5081	0.5076
	5. 100 mM $CaCl_2$	0.5033	0.5042
SP1 SPdomain	6. 5 mM $CaCl_2$	0.0190	0.0188
	7. 25 mM $CaCl_2$	0.0223	0.0225
	8. 50 mM $CaCl_2$	0.0104	0.0101
	9. 100 mM $CaCl_2$	0.0154	0.0160

*N-benzoyl-Phe-Val-Arg-p-nitroanilide (Trypsin substrate)

3. The proteinase activity assays of recombinant mature *Pm*ClipSP1. Trypsin was adopted as positive control and activator of recombinant *Pm*ClipSP1.

Proteinase	A_{410}	
1. blank	0	0
2. Trypsin (0.02 μM)	0.4800	0.4837
3. SP1 (0.02 μM)	0	0
4. SP1 (0.2 μM)	0	0
5. SP1 (2.0 μM)	0	0

*N-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Trypsin substrate)

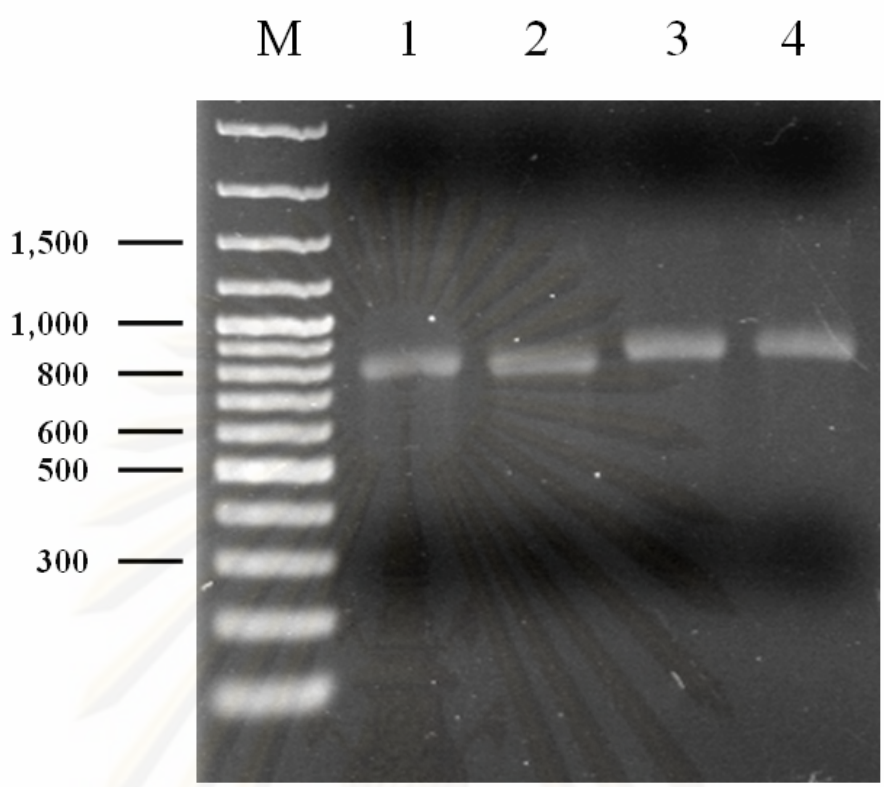
4. The PO activity assay of recombinant *Pm*ClipSP1

PO activity	HLS+ LPS+Laminarin	HLS+ LPS+Laminarin +SP1	HLS+ LPS+Laminarin +Trypsin
1	3.3	4.14	3.5
2	4.7	3.9	6.5
3	3.8	3.99	5.7
Average	4	4.02	5
SD	0.989949	0.169706	2.12132034

PO activity = OD490/min/mg HLS protein
Incubation time 15 min, 2 mg of HLS protein

5. The hemolymph PO activity from silencing gene shrimp measuring by L-dopa assay.

group	OD_{490}	PO activity	Average	SD
NaCl	0.3114	0.519	0.5549	0.06833
	0.3072	0.512		
	0.3802	0.6337		
GFP dsRNA	0.2761	0.4602	0.55143	0.10911
	0.4034	0.6723		
	0.3131	0.5218		
SP1 dsRNA	0.3341	0.55683	0.55791	0.01039
	0.3371	0.5481		
	0.3498	0.5688		



6. Synthetic dsRNA were analyzed by 1.2% agarose gel electrophoresis staining by ethidium bromide.

Lane M : GeneRuler™ 100 bp DNA ladder marker (Fermentas)

Lane 1 : *Pm*ClipSP1 ssRNA sense strand

Lane 2 : *Pm*ClipSP1 ssRNA antisense strand

Lane 3 : GFP dsRNA sense strand

Lane 4 : GFP dsRNA antisense strand

BIOGRAPHY

Mr. Kriangpol Wiriyaukaradecha was born on January 27, 1984 in Bangkok. He graduated with the degree of Bachelor of Science from the Department of Biology, Faculty of Science, SrinakharinWirot University in 2006. He has studied for the degree of Master of Science at program in Biotechnology, Chulalongkorn University since 2006.