

ลักษณะสมบัติเชิงหน้าที่ของคลิปปโดเมนซีรีนโปรตีนส *PmClipSP2*  
จากกุ้งกุลาดำ *Penaeus monodon*

นางสาวกัญญ์พัชญ์ พรหมรุ่งเรือง

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FUNCTIONAL CHARACTERIZATION OF A CLIP DOMAIN SERINE  
PROTEINASE *PmClipSP2* FROM THE BLACK TIGER SHRIMP

*Penaeus monodon*

Miss Kanyanat Promrungreang

A Thesis Submitted in Partial Fulfillment of the Requirements  
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Department of Biochemistry

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Thesis Title           FUNCTIONAL CHARACTERIZATION OF A CLIP  
DOMAIN SERINE PROTEINASE *PmClipSP2* FROM  
THE BLACK TIGER SHRIMP *Penaeus monodon*  
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กัญญ์ วรรณรุ่งเรือง: ลักษณะสมบัติเชิงหน้าที่ของคลิปโดเมนซีรีนโปรติเนส *PmClipSP2* จากกุ้งกุลาดำ *Penaeus monodon*. (FUNCTIONAL CHARACTERIZATION OF A CLIP DOMAIN SERINE PROTEINASE *PmClipSP2* FROM THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร.อัญชลี ทศนาขจร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ปิติ อ่ำพ่าย, 113 หน้า.

คลิปโดเมนซีรีนโปรติเนสเป็นองค์ประกอบสำคัญในกระบวนการส่งสัญญาณในระบบภูมิคุ้มกันของสัตว์ที่ไม่มีกระดูกสันหลัง จากฐานข้อมูล EST ของกุ้งกุลาดำ *Penaeus monodon* พบว่าลำดับนิวคลีโอไทด์ที่สมบูรณ์ของยีน *PmClipSP2* ประกอบไปด้วย open reading frame จำนวน 1,107 คู่เบส ซึ่งเป็นรหัสของโปรตีนที่มีกรดอะมิโน 369 ตัว โดยมี signal peptide จำนวน 25 กรดอะมิโน ซึ่งโปรตีนมีขนาด 40.18 กิโลดาลตัน โครงสร้างของโปรตีน *PmClipSP2* ประกอบด้วยคลิปโดเมนทางด้านปลายอะมิโนและซีรีนโปรติเนสโดเมนทางด้านปลายคาร์บอกซิล ในงานวิจัยนี้เราได้ทำการยับยั้งการแสดงออกของยีน *PmClipSP2* โดยการฉีดอาร์เอ็นเอสายคู่พบว่าประสบความสำเร็จในการลดระดับการเกิดทรานสคริปชัน และสามารถลดแอกทิวิตีของเอนไซม์ฟีนอลออกซิเดสในกุ้งที่ได้รับอาร์เอ็นเอสายคู่ และพบว่าผลการแสดงออกของยีน *PmClipSP2* มีผลทำให้กุ้งมีอัตราการตายเพิ่มขึ้น 100% และมีจำนวนเชื้อแบคทีเรียในเลือดเพิ่มมากขึ้นเมื่อฉีดด้วยเชื้อ *Vibrio harveyi* เป็นที่น่าสนใจมากเมื่อพบว่าการฉีดกุ้งที่ลดการแสดงออกของยีน *PmClipSP2* ด้วยผนังเซลล์ของจุลชีพมีผลทำให้กุ้งตายอย่างเฉียบพลัน โดยพบว่ากุ้งมีจำนวนเม็ดเลือดลดลงอย่างมีนัยสำคัญ จากการศึกษาหน้าที่ของรีคอมบิแนนท์ *PmClipSP2* พบว่าสามารถจับกับ  $\beta$ -1,3-glucan และ LPS นอกจากนี้โปรตีนรีคอมบิแนนท์ยังสามารถกระตุ้นฟีนอลออกซิเดส-แอกทิวิตีและมีคุณสมบัติของโปรติเนสแอกทิวิตี จากการวิเคราะห์การแสดงออกของโปรตีนโดยวิธีเวสเทิร์นบลอต (Western blot) ด้วยแอนติบอดีที่จำเพาะต่อโปรตีน *PmClipSP2* พบโปรตีน *PmClipSP2* ในเม็ดเลือดกุ้งแต่ไม่พบในพลาสมา จากข้อมูลทั้งหมดนี้บ่งชี้ว่า *PmClipSP2* เป็นโปรตีนที่จับกับผนังเซลล์ของแบคทีเรียโดยตรงและกระตุ้นระบบโพธิฟีนอลออกซิเดสผ่านโปรติเนส นอกจากนี้อาจมีหน้าที่รักษาสมดุลของเม็ดเลือด โดยช่วยลดพิษที่เกิดจาก LPS

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KANYANAT PROMRUNGREANG: FUNCTIONAL CHARACTERIZATION OF A CLIP DOMAIN SERINE PROTEINASE *PmClipSP2* FROM THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR: PITI AMPARYUP, Ph.D., 113 pp.

Clip domain serine proteinases (clip-SPs) are the essential components of signaling cascades in the innate immune system of invertebrates. From the shrimp *Penaeus monodon* EST database (<http://pmonodon.biotec.or.th>), a full-length cDNA of *PmClipSP2* was identified and characterized. It contains an open reading frame of 1,107 bp encoding a predicted protein of 369 amino acids including a 25 amino acid signal peptide. The predicted molecular mass of the mature protein is 40.18 kDa. *PmClipSP2* exhibits a characteristic sequence structure of clip-SPs consisting of an N terminal clip domain and a C terminal SP domain. Knockdown of the *PmClipSP2* gene by double-stranded RNA (dsRNA) of *PmClipSP2* gene, significantly reduced the *PmClipSP2* transcript level and significantly reduced the total phenoloxidase (PO) activity. Silencing of the *PmClipSP2* gene led to the 100% mortality rate and high number of bacteria in hemolymph of shrimp systemically infected with *Vibrio harveyi*. Interestingly, injection of microbial cell wall components had lethal effect on the *PmClipSP2* knockdown shrimp that showed significantly decreased in the number of hemocytes. Functional analysis revealed that the recombinant *PmClipSP2* could bind to  $\beta$ -1,3-glucan and LPS and activates phenoloxidase (PO) activity and has a proteolytic activity. Western blot analysis using the antibody raised against the r*PmClipSP2* revealed that *PmClipSP2* was present in hemocytes, but not in cell free plasma. Taken together, these results suggest that *PmClipSP2* directly binds to bacterial cell wall components and activates the proPO system through the proteinase cascade and it probably involves in the hemocyte homeostasis by neutralizing LPS toxicity.

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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
DEPC	Diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
dsRNA	double stranded ribonucleic acid
EF1 $\alpha$	elongation factor 1 alpha
EST	expressed sequence tag
EtBr	ethidium bromide
GFP	green fluorescence protein
HLS	hemocyte lysate supernatant
Hr	Hour
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
Laminarin	$\beta$ -1,3-glucan
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
°C	degree Celsius
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAP, PPAAE, ppA	prophenoloxidase activating enzyme
PCR	polymerase chain reaction
<i>PmClipSP2</i>	serine proteinase 2 of <i>Penaeus monodon</i>
PO	Phenoloxidase
PPAF	prophenoloxidase activating factor
proPO	Prophenoloxidase
<i>rPmClipSP2</i>	recombinant <i>Penaeus monodon</i> serine proteinase 2 mature protein
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	Ribonuclease
RT	reverse transcription
SDS	sodium dodecyl sulfate
S	Second
SP	serine proteinase
SPH	serine proteinase homolog
UTR	untranslated region
WSSV	white spot syndrome virus
YHV	yellow head virus
µg	Microgram
µl	Microliter
µM	Micromolar

# CHAPTER I

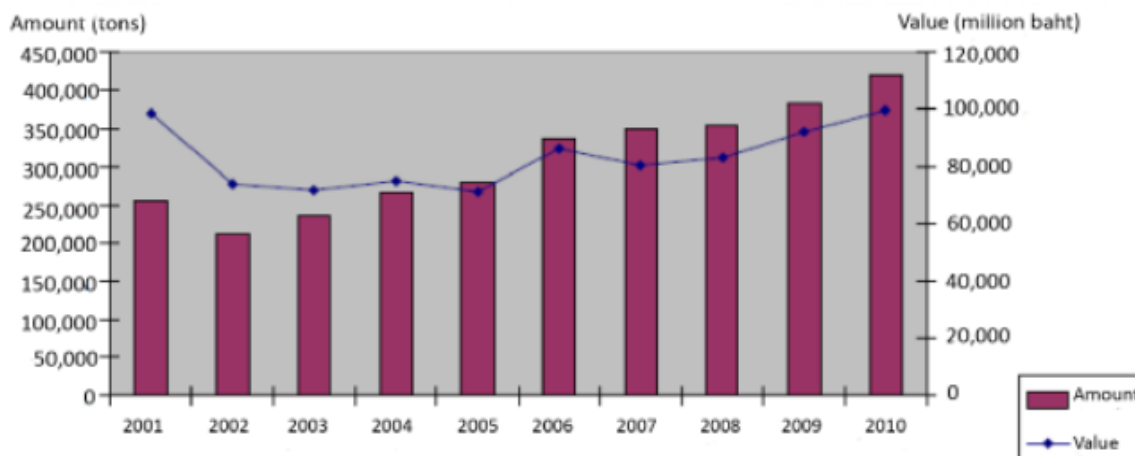
## INTRODUCTION

### 1.1 General introduction

Black tiger shrimp (*Penaeus monodon*) is a marine crustacean that is being cultured worldwide. Shrimp culture has spreaded in many countries especially in the tropical countries in South East Asia such, Central and south America. Asian countries are the major shrimp producer up to 75% of the world production. China and Thailand are the leading countries for shrimp production (Source: FAO databases, 2007). In Thailand, shrimp farm began in 1970s and to be the leader for exportation of the black tiger shrimp, *Penaeus monodon* (Wyban, 2007). The location of shrimp farms are in the provinces in the central regions (Samut Sakhon, Samut Songkhram), the East (Chanthaburi), and the coasts of Southern (Nakorn Sri Thammarat, Surat Thani).

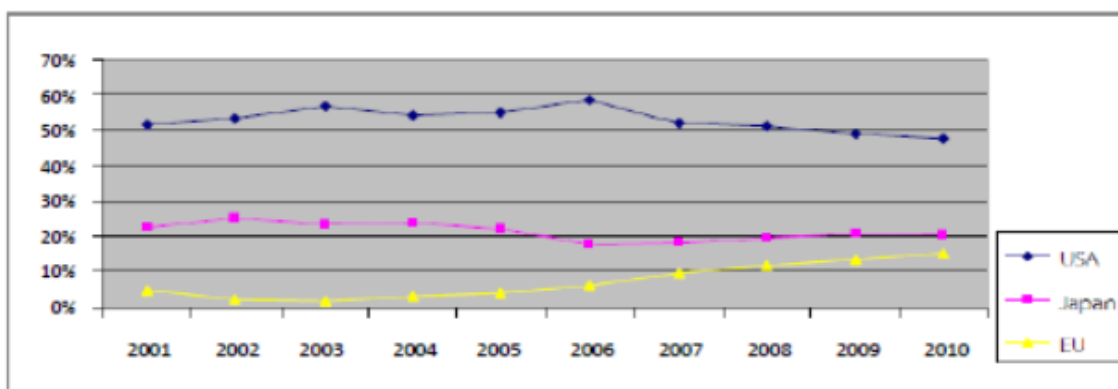
*Penaeus monodon* (The black tiger shrimp) is the major species farmed in Asia. The other species are *P. indicus*, *P. vannamei*, and *P. chinensis*. *P. monodon* is the fastest growing and largest of all shrimp species and it is tolerant to a wide range of salinities, but is highly susceptible to pathogen infection. During 2001-2010, shrimp aquaculture exportation from Thailand has reached an average exportation at 309,256 tons per year valued to 83,200 million baht per year. The shrimp aquaculture exportation increased every year (Figure. 1.1).





**Figure 1.1** Thailand's shrimp exportation during 2001-2010 (Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand).

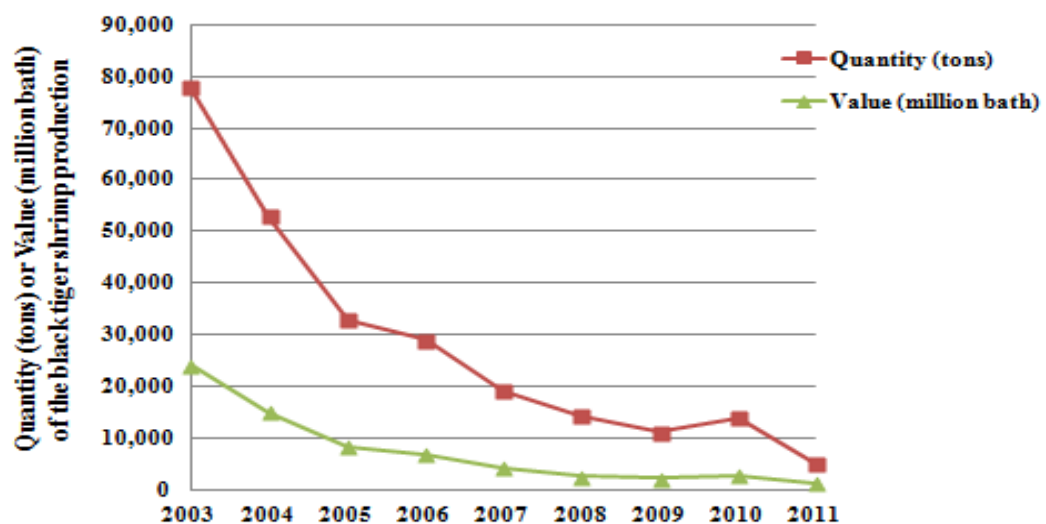
Shrimp production is exported to various countries all over the world. USA is the important trader (53% of the shrimp market share), followed by Japan and the European union (21% and 7%, of the shrimp market share) (Figure 1.2).



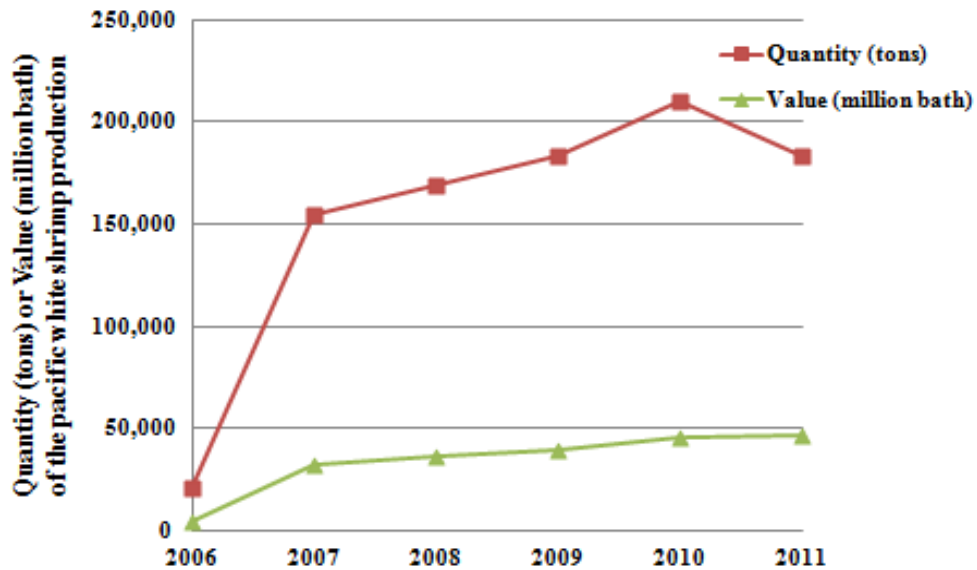
**Figure 1.2** Shrimp aquaculture exported market share in USA, Japan and European Union during 2001-2010 (Source: Fisheries Foreign Affairs Division, Department of Fisheries, Thailand).

In the last decades, shrimp production has seriously affected by infectious diseases. Generally, infectious diseases caused by virus and bacteria

(Bachère, 2000). This is the major problem for production losses. Since 2003, the production of black tiger shrimp had been decreased because smaller size and the high mortality of shrimp. Whilst the production of the black tiger shrimp was still reducing, the pacific white shrimp (*Litopenaeus vannamei*) was more popular (since 2006) because genetic selection of the pacific white shrimp was successfully performed that led to the effective growth rate, disease resistance and high survival rate in larval rearing. Moreover, the pacific white shrimp are easy to domesticate and being a tolerant species. While in the black tiger shrimp, the genetic information has been studied less than the pacific white shrimp (Wyban, 2007). Therefore, the pacific white shrimp has become the main cultured shrimp species instead of the black tiger shrimp (Figure 1.3 and 1.4).



**Figure 1.3** The black tiger shrimp (*Penaeus monodon*) production during 2003-2011. (Source: [www.oae.go.th/oe\\_report/export\\_import/export.php](http://www.oae.go.th/oe_report/export_import/export.php)).



**Figure 1.4** The pacific white shrimp (*Litopenaeus vannamei*) production during 2006-2011. (Source:www.oae.go.th/oae\_report/export\_import/export.php).

## 1.2 Taxonomy of *Penaeus monodon*

*Penaeus monodon*, the giant tiger shrimp, is a penaeid shrimp species that are classified into the largest phylum in the animal kingdom, the Arthropoda. This group of animal is characterized by the presence of pair appendages and a protective cuticle or exoskeleton that covers the whole animal. The taxonomic definition of the black tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

**Phylum** Arthropoda

**Subphylum** Crustacea Brünnich, 1772

**Class** Malacostraca Latreille, 1802

**Subclass** Eumalacostraca Grobben, 1892

**Order** Decapoda Latreille, 1802

**Suborder** Dendrobranchiata Bate, 1888

**Superfamily** Penaeoidea Rafinesque, 1815

**Family** Penaeidae Rafinesque, 1985

**Genus** *Penaeus* Fabricius, 1798

**Species** *Penaeus monodon* Fabricius, 1798

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

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

**F.A.O. Names:** Giant tiger prawn, Crevette gigante tigre, Camaron tigre gigante.

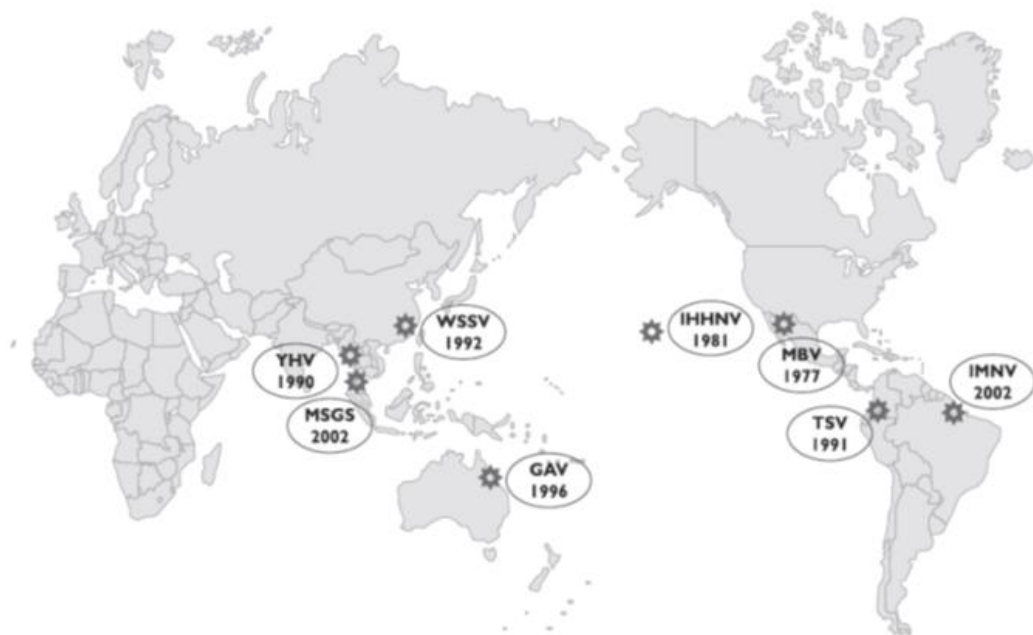
### **1.3 Shrimp Diseases**

Diseases are one of the major problems in shrimp aquaculture. Shrimp diseases can be classified into two groups, noninfectious and infectious (Lightner and Redman, 1998). The noninfectious diseases are nutritional imbalances, toxicants, environmental extremes, and genetic factors (Lightner, 1988; Johnson, 1995). Infectious diseases occur from various pathogen, e.g, fungi, bacteria, parasites, and virus. Virus and bacteria cause the most serious diseases in shrimp farming.

### 1.3.1 Viral diseases

Seven viral pathogens are discovered and studied in Thailand, e.g., white spot syndrome virus (WSSV), yellow-head virus (YHV), monodon baculovirus (MBV), Taura syndrome virus (TSV), hepatopancreatic parvovirus (HPV), infectious hypodermal and hematopoietic virus (IHHNV), Laem Singh virus (LSNV). White spot syndrome virus (WSSV) and yellow-head virus (YHV) are two of the major viral pathogens of the shrimp which cause white spot syndrome and yellow head disease, respectively. These are the serious problems in shrimp mortality.

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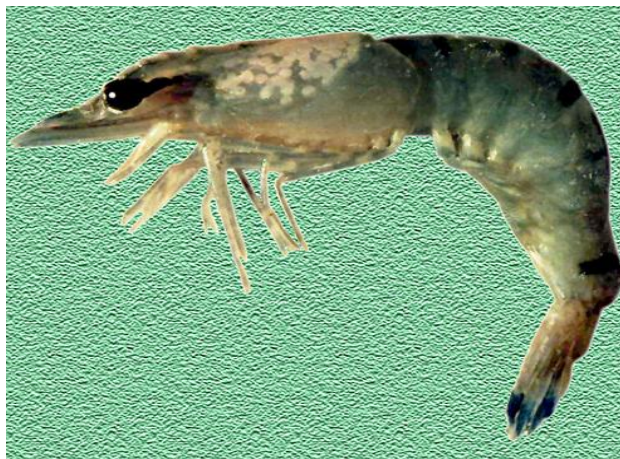
**Figure 1.5** History of the emergence of the major pathogens of farmed shrimp. GAV, gill-associated virus; IHHNV, infectious hypodermal and haematopoietic necrosis virus; IMNV, infectious myonecrosis virus; MBV, monodon baculovirus; MSGS, monodon slow growth syndrome; TSV, Taura syndrome virus; WSSV, white spot syndrome virus; YHV, yellow head virus (P.J. Walker and C.V. Mohan, 2009).

### **1.3.1.1 White spot syndrome virus (WSSV)**

White spot syndrome virus (WSSV) causes high mortality in shrimp. This virus has the large circular double stranded DNA of ~292 to 305 kb, and is an enveloped rod-shaped particle with a single filamentous appendage-like tail at one end of the nucleocapsid (Vlak et al. 2004, Yang et al., 2001). Infection of WSSV in shrimp can cause 100% of cumulative mortality within 3-10 days (Chou et al., 1995, Flegel, 2006). This problem leads to loss of large economic in the shrimp culture industry. The obvious sign of WSSV infection is the various sizes of white spot that observed on the cuticle of the shrimp (Figure 1.6). The causative carrier was a new bacilliform virus (Takahashi et al., 1994). However, the disease can occur without the presence of white spots. Other signs of the WSSV infection in shrimp also include the body surface and appendages turning to red or pink, loosing shell, lower food consumption and show lethargic behavior (Liu et al, 2009).

In 1992, WSSV was first reported in the Chinese province Fujian, from where it spread quickly (Flegel, 1997). In the same year WSSV spreaded quickly in Taiwan (Chen, 1995). In 1994, the outbreak of disease was extended in Japan, Korea, Thailand, India and Malaysia and in 1996 it had affected in East Asia and South Asia. Nowadays WSSV has spreaded to almost all shrimp farming of the world. The disease is dispersed by contamination of water, decomposing tissue or fecal matter, fluid from infected females and cannibalism. Direct transmission can happen among unrelated crustacean

species. It is difficult to protect and inhibit the infection due to this virus is alive for long time in the environment (2 years in shrimp habitat). The major target tissues for replication are gills, stomach and body cuticular epithelium, hematopoietic tissues, lymphoid organ and antennal glands (Tan et al., 2001; Durand and Lightner, 2002; Escobedo-Bonilla et al., 2007). The WSSV infection can be detected by several diagnostic methods such as PCR, *in situ* hybridization, mini-array, observation of tissues subjected to staining, and immunological methods using specific antibodies to WSSV proteins (Okumura et al., 2004).



**Figure 1.6** Gross signs of WSSV infection. White inclusions are found in the shrimp cuticle at the late stage of infection (Source: [www.flickr.com](http://www.flickr.com)).

### 1.3.1.2 Yellow Head Virus (YHV)

Yellow head disease (YHD) can occur in shrimp and prawn. Particularly, in *P. monodon* that is one of the two major species in shrimp culture. This disease is contagious and highly lethal in shrimp (Wongteerasupaya et al., 1995). In 1990, first report of YHV occurs in black

tiger shrimp (*P. monodon*) farms in Thailand. This disease occurs in the juvenile to sub-adult stages of shrimp and causes 100% of mortality within 3-5 days from first apparent signs (Lightner, 1996). YHV can also experimentally or naturally infect in the other penaeid shrimp (Munro and Owens, 2007). The major target tissues for replication by this virus is the lymphoid organ (Kanobdee et al., 2002; Soowannayan et al., 2002). The onsets of YHD-infected shrimp have been observed consuming feed at high rate for each day. Feeding suddenly ceases and within 1 day, the moribund shrimp swim slowly near the surface of pond edges. After that the dorsal cephalothorax of YHV-infected shrimp occur the light yellow and in few hours, shrimp will die (Figure 1.7).

YHV was first conceived to be a baculovirus. But, it was later reported during characterization that its morphology differed from that of baculoviruses (Boonyaratpalin et al., 1993). YHV is rod-shaped. The viral genomes of YHV compose of single-stranded RNA (ssRNA) of positive sense with a helical nucleocapsid. 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). The prognosis of YHV infection could be analyze by using immuno-histochemistry, the monoclonal antibody aggregated with a surface glycoprotein and the nucleocapsid protein of virus (Sánchez-Barajas et al., 2009). Moreover, pathology was determined



by RT-PCR (Wongteerasupaya et al., 1997) or *in situ* hybridization (Wongteerasupaya et al., 1996; Tang et al., 1999).



**Figure 1.7** Gross signs of yellow head infection in shrimp are shown on the left. Generally, infected shrimp show yellowish discoloration of the cephalothorax. Shrimp on right are normal (Chantanachookin et al., 1993).

### 1.3.2 Bacterial Disease

In Thailand, vibriosis is a major disease that affect in shrimp and prawn (Nash et al., 1992; Austin and Zhang, 2006). This bacterial causes high mortality in shrimp up to 100%, it could infect in many generations of shrimp, e.g., larvae, post-larvae, juveniles, sub-adults or adults (Lightner, 1983). High mortalities usually occur in post-larvae and young juvenile shrimp. In 1982, there is the first report about *Vibrio harveyi* that was discovered in a sandbar shark (*Carcharhinus plumbeus*) at the National Aquarium in Baltimore, USA (Grimes et al., 1984) and this bacteria is called *V. carchariae*. The research about pathogenicity of *Vibrio* infection in *P. monodon* were studied in Extracellular products (ECP) and live bacteria that caused virulence of *Vibrio*

(Liu et al., 1996). Characterization of *V. harveyi*, it is gram-negative bacteria. Consequently, cell wall consists of two membranes, e.g., outer membrane that composed of lipopolysaccharides and inner cytoplasmic membrane. Peptidoglycan layer is between outer and inner membrane. *V. harveyi* is a facultative anaerobe, therefore it has living by using either respiration oxygen or fermentation. *V. harveyi* is bioluminescent bacteria, the capacity of light synthesis depends on the amount of bacteria in substrate (i.e., sea water or special medium in lab). Oxygen needs for the synthesis of luminescence reaction (Showalter et al., 1990). Bioluminescent bacteria synthesize auto-inducer that is specific chemical. High concentrations of auto-inducer can induce luminescence reactions in bacteria but in lower concentrations, cells do not synthesize the light. *V. harveyi* can be found free-swimming in tropical marine waters, commensally in the gut microflora of marine animals, and as both a primary and opportunistic pathogen of marine animals (Austin and Zhang, 2006).

*V. harveyi* is one of the major disease in fish and invertebrates, including seabass, seahorses, sharks, lobster, and shrimp. Pathogenicity of *V. harveyi* depends on the number of bacteria cells. The symptoms of *V. harveyi*-infected shrimp are vasculitis, eye-lesions, gastro-enteritis, and luminous vibriosis. Luminous vibriosis causes high mortality in shrimp farm. *V. harveyi* gets into host by the mouth and then forms plaques. There are the reports, this bacteria is a pathogen in white gut disease and loose shell syndrome in *P. monodon* in

india (Jayasree et al., 2006). For contamination of this pathogen, it can spread through egg and larval tanks that is a big problem for shrimp farmer. In the past shrimp farming used antibiotic to control the bacterial disease and then occurred the problem of drug resistance (Karunasagar et al., 1994). Vibrio-selective (TCBS) or general marine agar plate was used for simple detection of vibrio infection in shrimp hemolymph. Moreover, the effect of copper concentration could decrease luminescence and toxin of *V. harveyi* (Nakayama et al., 2007) (Figure 1.8).



**Figure 1.8** Vibriosis in shrimp aquaculture. Vibriosis usually causes high mortality in post larvae and young juveniles. Adult shrimp with vibriosis show reddening of the body with red to brown gills (Source: [www.engormix.com](http://www.engormix.com)).

#### **1.4 The crustacean defense system**

Invertebrate animals lack adaptive immune systems but they have been developed other biological systems of host defense that called innate immunity, the innate immune system consists of cellular and humoral immune responses. Cellular immune defense are phagocytosis, encapsulation, and formation of

nudules. Humoral immune defense are agglutinins (e.g. lectins), coagulation system, antimicrobial peptides and prophenoloxidase activating system (melanin synthesis).

The circulatory system of crustacean involves in both cellular and humeral defenses. The hemocytes and plasma protein are weapons for first defense process by recognizing pathogens. Hemocytes have function in phagocytosis, cytotoxicity, cell-cell communication and melanization (proPO system) (Jiravanichpaisal et al., 2006). Moreover, there are many enzymes and proteins in hemolymph plasma, e.g., hemocyanin, antimicrobial peptide, proteinase inhibitors, phenoloxidase etc.

#### **1.4.1 Blood cells in crustacean**

In crustacean, hemocytes have been classified into three types: semigranular, granular cell and hyaline. Each type of hemocyte cell has different function in immune system, e.g., phagocytosis, cytotoxicity, encapsulation, cell adhesion, degranulation, and haemolysis, (Johansson et al., 2000). In total hemocytes, semigranular is the most amounts of total hemocyte cells (75 % of total). Semigranular consists of small granulars that expose in the process of phagocytosis. Moreover, semigranular has important function in encapsulation and degranulation. Granular cell is 10-20 % of total hemocyte cells; it contains many the secretory large granulars. The important function of semi-granular and granular cell are storage of the important factors for

activation of proPO system and the cytotoxic reaction (Smith et al., 1983). The hyaline plays important role in the phagocytosis and coagulation, smallest hemocytes do not have cytoplasmic granules (Söderhäll et al., 1986).

#### **1.4.2 Pattern recognition protein**

The innate immune system have been recognized the pathogen-associated molecular patterns (PAMPs) by using the pattern recognition receptors. PAMPs are on the surface of pathogens but not in the host (Medzhitov et al., 2002). For example of PAMPs are lipopolysaccharide (LPS) from gram-negative bacteria, peptidoglycan (PGN) from gram-positive bacteria, glucan from fungi, double-stranded RNA from viruses, and the manan from yeast (Hoffmann et al., 1999; Kurata et al., 2006). Plasmatic protein and hemocyte were used for the procedure of recognition of invading pathogen (Medzhitov et al., 1997).

Microbial cell wall composes of carbohydrates. For agglutination reaction, lectin or hemagglutinin could bind with specific carbohydrates that presented on different cell surfaces. There are many reports about the  $\beta$ -1,3-glucan binding protein (BGBP) in many crustaceans, e.g., freshwater crayfish *P. leniusculus* (Duvic et al., 1990), brown shrimp *P. californiensis* (Vargas-Albores et al., 1996), chinese white shrimp *P. chinensis* (Du et al., 2007), pacific white shrimp *P. vannamei* (Vargas-Albores et al., 1997; Jiménez-Vega et al., 2002), and black tiger shrimp *P. monodon*

(Sritunyalucksana et al., 2002). Even though BGBP has glucanase-like motif but it has not glucanase activity. BGBP could bind with glucan and then activates immune response (Muta, 1995; Seki et al., 1995). Lipopolysaccharide and  $\beta$ -1,3-glucan binding protein (LGBP) in *P. monodon* that is important factor for activation of the prophenoloxidase system (Amparyup et al., 2012).

### **1.4.3 Antimicrobial Peptides (AMPs)**

In the innate immune system, Antimicrobial Peptides (AMPs) are important components. These are the part of first process in immune defense for against pathogens (Hancock et al., 2006; Brown and Hancock, 2006). For determination, generally AMPs have *in vitro* antimicrobial activity and then kill microorganism. Moreover, sometime they are modurator in immunity for preventing the host (Hancock and Diamond, 2000). Penaeid shrimps, There are many AMP sequences that reported in Genbank database. The major AMPs in penaeid shrimps are anti-lipopolysaccharide factors (ALFs), crustins, penaeidins that consist of various isoforms. AMPs are expressed in hemocytes and they localize to respond in bacterial infection (Muñoz et al., 2002). Different isoforms of shrimp AMPs have been studied that involved in activation by different pathogens. The major isoforms of each AMP family have been characterized the biological activities by using their synthetic peptides and recombinant proteins (Bachère et al., 2004; Zhao and Wang, 2008; Smith et al., 2008).

#### 1.4.4 The prophenoloxidase (proPO) system

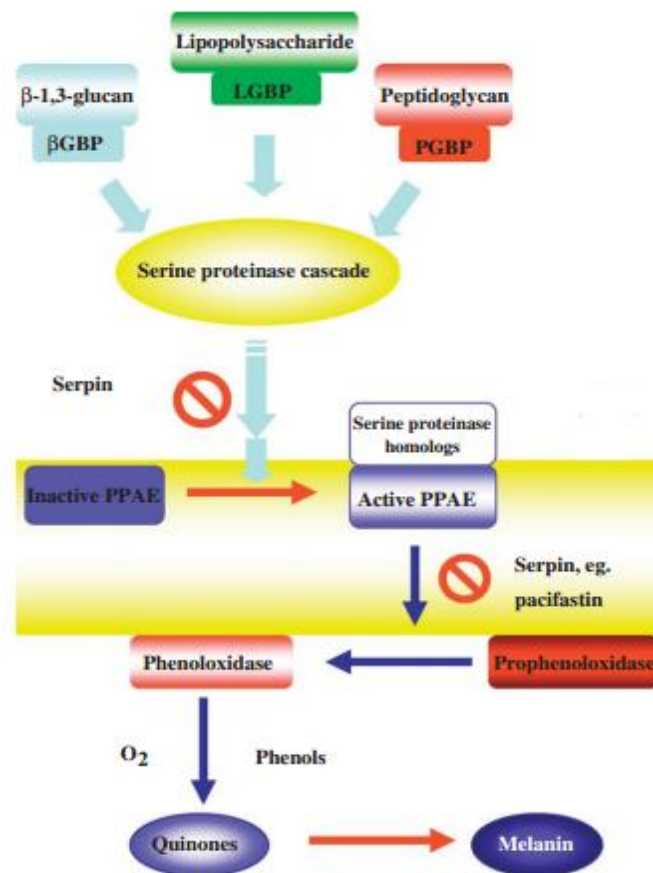
The major innate immune system in invertebrate is the prophenoloxidase activating or melanin synthesis (proPO system). First report in 1977, this system presents in insects and crustacean that responds to pathogens (Unestam and Soderhall, 1977). This defense system is turned into active form by activation of pathogen associated molecular patterns (PAMPS), e.g. lipopolysaccharides (LPS) or peptidoglycans from bacteria  $\beta$ -1,3-glucans from fungi that bind to specific recognition proteins from host and then activate serine proteinase cascade. The final result of this activation, prophenoloxidase (proPO) is converted to phenoloxidase (active form) (Johansson and Soderhall, 1985). Phenoloxidase (PO) is present as a proenzyme that called prophenoloxidase (proPO). PO performs catalization, they hydroxylate monophenols and oxidise odiphenols to quinones, then quinones are polymerized to melanin (Cerenius et al., 2004). In arthropods, activation of proPO required either one serine proteinase homologue or more and non-catalytic proteinase (Sritunyaluksana et al., 2000). This system affects to induce encapsulation and nodule formation which caused first against to pathogen entering host (Ratcliffe et al., 1985; Gotz, 1986). The proPO mRNA in *P. monodon* is expressed in hemocyte (Sritunyaluksana et al., 1999). In crustacean, proPO system is in semigranular and granular hemocytes that is localized in vesicles (Liang et al., 1992) while in insects their localization are in either hemocytes or plasma. Proteins that were isolated and purified from

the insect *Bombyx mori* and the freshwater crayfish *Pacifastacus Zeniusculus*, involve in proPO-system (Soderhall et al., 1990; Soderhall. 1992).

There is the first report of proPO sequence was from crayfish in 1995 (Aspan et al.,1995). Amino acid sequence of proPO from *P. monodon* has highly similarity to proPO from crayfish (74%). Two proPO in *P. monodon* were identified (Amparyup et al., 2009) that are more similarity to other proPO in penaeid shrimp than proPO in other crustacean. In the other crustaceans and shrimp, proPO system has been examined recently by using *in vivo* gene knockdown. Even though, there are the reports in some insects, the proPO system is not important for immune defense against pathogen infection (Leclerc et al., 2006; Schnitger et al., 2007). In crayfish, the proPO gene was suppressed by using RNA interference (RNAi), the result suggested that proPO gene plays important role in proPO system of crayfish immunity (Liu et al., 2007). In addition, the silenced proPO in crayfish increased *Aeromonas hydrophila* in hemolymph and then reduces survival bacteria in infected crayfish (Liu et al., 2007). Moreover, in penaeid shrimp the proPO system is essential for immune defense. In *F. Chinensis*, infection of *V. anguillarum* causes the up-regulation in proPO transcripts (Gao et al., 2009), while in *L. vannamei*, infection of WSSV causes the down-regulation in the both of proPO1 and proPO2 expression levels (Ai et al., 2008 ; Ai et al., 2009). In *P. monodon*, the roles of proPOs were first explained. Silencing of two proPOs (*PmproPO1* and *PmproPO2*) and other clip-SPs (*PmPPAE1*,



*PmPPAE2*, and *PmClipSP1*) increased the infection of *V. harveyi* and the mortality rate of shrimp after infection of bacteria *V. harveyi*. Moreover silencing of two proPOs and two PPAEs significantly reduced the total PO activity in hemolymph, while silencing of *PmClipSP1* was not reduce the total PO activity. (Amparyup et al., 2009; Amparyup et al., 2010; Charoensapsri et al., 2009; Charoensapsri et al., 2011) (Figure 1.9).



**Figure 1.9** The prophenoloxidase activating system in arthropods β-1,3-glucan from fungi, lipopolysaccharide from Gram-negative bacteria, and Peptidoglycan from Gram-positive bacteria are recognized by pattern-recognition proteins (PRPs) are β-1,3 glucan binding protein (BGBP), LPS and β-1,3 glucan binding protein (LGBP), and peptidoglycan binding protein (PGBP). After that serine proteinase cascade was

activated and then converted prophenoloxidase to phenoloxidase for melanin synthesis (Jiravanichpaisal., 2006).

#### **1.4.5 The prophenoloxidase activating enzymes (PPAEs)**

In arthropod, proPO cascade has serine proteinases (SPs) that called proPO activating enzyme (PPAE). In crayfish, PPAE that presents in granule hemocyte cells, is inactive form. When pathogens come into host, PPAE was secreted from hemocytes to plasma and changed to active form (Aspàn et al., 1991; Aspàn et al., 1995). In the silkworm *B. mori*, PPAE was in many tissues, e.g., salivary glands, hemocytes, integument but not in the mid gut or fat body (Satoh et al., 1999). In crayfish, PPAE is associated in proceeding both peroxinectin and proPO (Lin et al., 2007). In *P.monodon*, *PmPPAE1* and *PmPPAE2* were characterized for their role in proPO system. *PmPPAE1* is closely related to the PPAE in crustacean, whereas *PmPPAE2* is very similar to the PPAEs in insect (Wang et al., 2001; Charoensapsri et al., 2011).

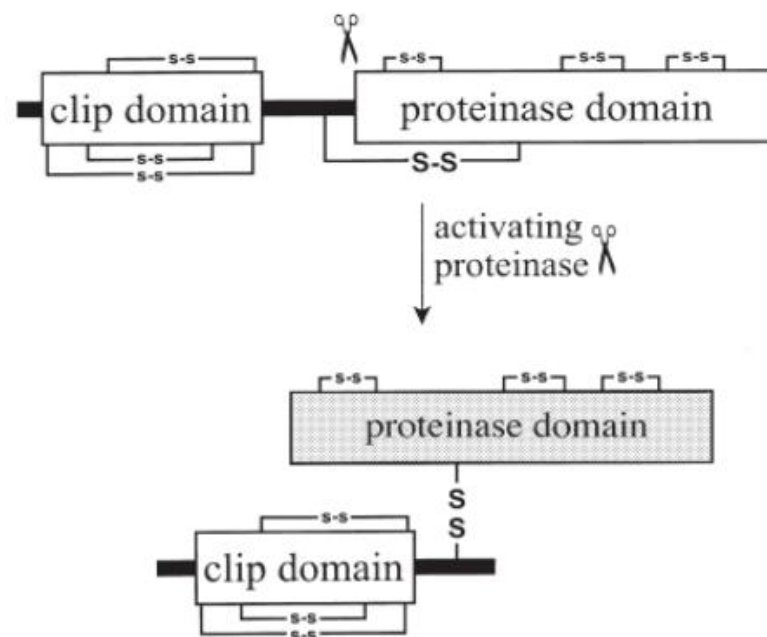
#### **1.4.6 Clip domain serine proteinases (Clip-SPs)**

Clip domain serine proteinases (clip-SPs) in invertebrates have the function that involved in immune responses and embryonic development (Jiang and Kanost, 2000; Krem and Cera, 2002). The other functions of clip-SPs were also characterized and involved in the melanin synthesis by proPO system (Jiang et al., 2000; Jang et al., 2008; Charoensapsri et al., 2009). Clip-SPs consist of two parts, the clip domain at N-terminus and the serine proteinase domain at C-terminus. Serine proteinases domain contains three conserved

amino acid residues, Histidine, Aspartate, and Serine. Conservation of six cysteines in clip domain for forming three disulfide bonds that is similar to  $\beta$ -defensin in invertebrate and vertebrate (Wang et al., 2001; Ganz, 2004) but when analysis of these structures by x-ray crystallography that are different (Piao et al., 2005). First call of clip domain is Iwanaga et al. (1998) because the disulfide bridges are formed that look like a paper clip. Clip domain and SP domain (catalytic domain) were cleaved for activation site but the two domains still linked by a disulfide bond after activation (Jiang et al., 2000) (Figure 1.10).

First identification of clip domain serine proteinase has been reported from horseshoe crab, *Tachypleus tridentatus* which has function in coagulation system (Muta et al., 1990). Proteolytic cascade of clotting system in horseshoe crab is activated by pathogen cell wall (Kawabata et al., 1996). In *D. melanogaster*, the clip-domain SPs was called snake and easter that controls the development of embryo. Activation system was named Toll signaling pathway. The *Drosophila* Toll pathway is important immune system in post-embryonic state but understanding in this system was not clear. Five SP were characterized that involved in Toll signaling pathway. Normally four of SPs are activated by fungi that associated in Toll signaling pathway while another one responded to gram positive bacteria (Kambris et al., 2006).

In arthropod, there are many studies about serine proteinase cascade in proPO activating system. The serine proteinase in *B. mori* (Sato et al., 1999), *H. diomphalia* (Kim et al., 2002), and *P. leueusculus* (Wang et al., 2001) are named prophenoloxidase activating enzyme (PPAE). Serine proteinase in *M. Sexta* (Gupta et al., 2005) and *D. Malanogaster* (Tang et al., 2006) are named prophenoloxidase activating proteinase (PAP) and melanization proteinase (MP) respectively. Hemolymph proteinase 14 (HP14) from *M. Sexta* is some SPs that auto-activated when microbial cell wall invaded into host (Gorman et al., 2007).



**Figure 1.10** Structure of clip-domain proteinases. The proteinases consist of a clip domain and a proteinase domain typical of the chymotrypsin family. Two domains were linked by a disulfide bond. This form is inactive form. Specific proteolysis at N-terminus of proteinase domain occurs for activation of proenzyme but after activation, two domains still attach by a disulfide bond (Jiang et al., 2000).

#### **1.4.7 clip domain serine proteinase homologues (Clip-SPHs)**

Clip domain serine proteinase homologues (Clip-SPHs) lack proteolytic activity due to the replacement of the active site serine residue by glycine. Even though, SPH has not proteolytic activity but it acts in other biological function in immune system, e.g., as an immune molecule for cell adhesion (Huang et al., 2000; Lin et al., 2006). Generally in many organisms, SPH acts as SP cofactor in the proPO activation system, e.g., *H. Diomphalia*, *M. Sexta*, and *Tenebrio molitor* (Lin et al., 2006; Wang et al., 2008) but for proPO activation in *B. moli*, PPAE does not require SPH (Sato et al., 1999). The first report of SPH is in 1990 that involved in stabilization of muscle attachment in embryo from *D. Malanogaster*. This SPH was named masquerade (Mas) (Murugasu-Oei et al., 1995). SPHs need the proteolytic activation similar to SP. In crayfish, SPH in hemocyte was cleaved for activation reaction such as pattern recognition, granulocyte adhesion and opsonization but not involved in prophenoloxidase activation (Lee et al., 2001). In *P.monodon*, silencing of *PmMasSPH1* and *PmMasSPH2* significantly decreased the total PO activity in shrimp hemolymph. This result suggested that the two SPHs are involved in the proPO system (Amparyup et al., unpublished data).

#### **1.4.8 Proteinase inhibitor**

Proteinase inhibitors are important factors in the immune system for regulation of functional of proteinases. Proteinase inhibitors regulate some proteinase cascades, e.g.,  $\alpha$ -macroglobulin is antiprotease that acts as inhibitor

in clotting system (Vargas-Albores et al., 1996; Armstrong et al., 1999), serine proteinase inhibitors in the Serpin and Kazal families (Kanost et al., 2001; Kanost et al., 2001; De Gregorio et al., 2002; De Gregorio et al., 2002). Like clotting system, in prophenoloxidase system has serine proteinase inhibitors for regulation cascade (Kanost et al., 1996). For instance,  $\alpha$ -macroglobulin and pacifastin could inhibit proPO activation in crayfish (Aspàn et al., 1990). Moreover, in *M. sexta*, serpin-6 could inhibit proPO-activating proteinase-3 (PAP-3) (Wang et al., 2004). In proPO activating system, host cells are protected by proteinase inhibitors from damaging of toxical product in this system. Serpin is proteinase inhibitor that consists of reactive site loop that interacts the active site of a proteinase and then forms the complex of serpin-proteinase (Stone et al., 1997). In *F. chinensis* shrimp, serpin is highly expressed when challenged with *Staphylococcus aureus* or *Vibrio anguillarum*, and WSSV (Liu et al., 2009). The Kazal is one type of serine proteinase inhibitors that found in many shrimp, e.g., *P. monodon*, *P. chinensis*, *P. vannamei* etc. (Jarasrassamee et al., 2005; Jiménez-Vega et al., 2005; Kong et al., 2009). The Kazal-type SPIPm2 could inhibit subtilisin that shown it acts as defend element against pathogenic proteinases from bacteria (Somprasong et al., 2006). In crayfish,  $\alpha$ -Macroglobulin and pacifastin could inhibit PPO activation system (Aspàn et al., 1990).

## 1.5 RNA interference (RNAi)

RNA interference (RNAi) is the immune defense in eukaryotic organism. When eukaryotic cells were infected by virus (e.g., RSV, poliovirus, HPV, HIV-1 etc.), the double stranded RNA (dsRNA) could protect them. After that sequence-specific was induced for prevention of gene expression (Bagasra et al., 2004). First step, RNAaseIII-like enzyme, e.g., Dicer in *D. melanogaster* (Elbashir et al., 2001; Agrawal et al., 2003; Bernstein et al., 2003) attached to dsRNA. After dsRNA was cleaved into short interfering RNA (siRNA) by this enzyme (Hammond et al., 2000). The siRNA will be assembled with exo-, endo-nucleases, helicase, RecA and other protein for forming RNAi-induced silencing complex (RISC). The target mRNA is binded and cleaved by the RISC at the region complement to siRNA. Therefore, mRNA is degraded leading to suppressing of gene expression. The first data of RNAi was reported in plant (Matzke et al., 1989). The dsRNA silenced gene in *Caenorhabditis elegans* (Fire et al., 1998). Many organism have been successes in silencing of gene, e.g., *Planaria* (Sánchez Alvarado et al., 1999), Zebrafish (Wargelius et al., 1999), *Drosophila* (Misquitta et al., 1999), and plants (Jensen et al., 2004). In crustacean, RNAi technique was used as the tool for studying of functional immune in crayfish (Liu et al., 2007) and *P. monodon* (Charoensapsri et al. 2009).

## 1.6 Previous studies

A full-length cDNA of *PmClipSP2* was analyzed for its role in shrimp immune system. The *PmClipSP2* consists of a signal peptide, the clip domain at the N-terminus and the serine proteinase domain at the C-terminus. Moreover, the previous study showed that transcripts of *PmClipSP2* is up-regulated in hemocytes at 3 hr after a pathogenic bacterial *Vibrio harveyi* infection using real time PCR analysis (Amparyup et al., unpublished data). These results indicated that *PmClipSP2* is likely involved in shrimp immune responses.

## 1.7 Objectives

The objective of this thesis is to characterize the function of *PmClipSP2* in shrimp immunity. RNA interference (RNAi) technique was used as a tool for characterization the role of *PmClipSP2* in shrimp proPO and defense systems. The recombinant protein of *PmClipSP2* was expressed in the *E. coli* system and the function of *rPmClipSP2* were analyzed. This study investigates the role of a clip domain serine proteinase in the shrimp immune responses that will lead to understanding of shrimp immunity.



# CHAPTER II

## MATERIALS AND METHODS

### 2.1 Equipments and Chemicals

#### 2.1.1 Equipments

-20 °C Freezer (Whirlpool), -80 °C Freezer (ThermoForma)

Costar<sup>®</sup> 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 (Mettler)

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)

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

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

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

PD-10 column (GE Healthcare)

pH meter Model # SA720 (Orion)

Pipette tips 10, 20, 100 and 1000  $\mu$ l (Axygen Scientific, USA)

Power supply, Power PAC 3000 (Bio-RAD Laboratories, USA)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Touch mixer Model # 232 (Fisher Scientific)

TCBS (thiosulfate–citrate–bile–sucrose) (Becton, Dickinson)

Sterring hot plate (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Mettler)

### **2.1.2 Chemicals, Reagents and Biological substance**

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

2-Mercaptoethanol, C<sub>2</sub>H<sub>6</sub>OS (Fluka)

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

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

Absolute methanol, CH<sub>3</sub>OH (Scharlau)

Acrylamide (Plus one)

Agarose (Sekem)

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

Ammonium persulfate,  $(\text{NH}_4)_2\text{S}_2\text{O}_8$  (USB)

Ampicillin (BioBasic)

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid,  $\text{BH}_3\text{O}_3$  (MERCK)

Bovine serum albumin (Fluka)

Bromophenol blue (MERCK)

Calcium chloride ( $\text{CaCl}_2$ ) (MERCK)

Chloramphenicol (Sigma)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

Diethyl pyrocarbonate (DEPC),  $\text{C}_6\text{H}_{10}\text{O}_5$  (Sigma)

Dithiothreitol (Pharmacia)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid disodium salt dehydrate (EDTA)

Formaldehyde,  $\text{CH}_2\text{O}$  (BDH)

Formamide deionized (Sigma)

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

Glacial acetic acid ,  $\text{CH}_3\text{COOH}$  (J.T. Baker)

Glucose,  $\text{C}_6\text{H}_{12}\text{O}_6$  (Ajax chemicals)

Glycerol,  $\text{C}_3\text{H}_8\text{O}_3$  (Scharlau)

Glycine,  $\text{NH}_2\text{CH}_2\text{COOH}$  (Scharlau)

Hydrochloric acid (HCl) (MERCK)

Imidazole (Fluka)

Isopropanol,  $\text{C}_3\text{H}_7\text{OH}$  (MERCK)

Isopropyl- $\beta$ -D-thiogalactoside (IPTG),  $\text{C}_9\text{H}_{18}\text{O}_5\text{S}$  (USBiological)

Laminarin from Laminaria (Sigma)

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

Magnesium chloride ( $\text{MgCl}_2$ ) (MERCK)

Methanol,  $\text{CH}_3\text{OH}$  (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)

pET28b(+) vector (Novagen)

Phenol, saturated (MERCK)

Prestained protein molecular weight marker (Fermentas)

RNA markers (Promega)

Skim milk powder (Mission)

Sodium acetate,  $\text{CH}_3\text{COONa}$  (Carlo Erba)

Sodium cacodylate trihydrate (CAC),  $(\text{CH}_3)_2\text{AsO}_2\text{Na} \cdot 3\text{H}_2\text{O}$ , (Sigma)

Sodium chloride,  $\text{NaCl}$  (BDH)

Sodium dihydrogen orthophosphate,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (Carlo Erba)

Sodium dodecyl sulfate,  $\text{C}_{12}\text{H}_{25}\text{O}_4\text{SNa}$  (Sigma)

Sodium hydroxide,  $\text{NaOH}$  (Eka Nobel)

Triethanolamine (Unilab)

Tris-(hydroxy methyl)-aminomethane,  $\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$  (USB)

Tri reagent (Molecular Research Center)

Tryptic soy broth (Difco)

Urea (Fluka, Switzerland)

### **2.1.3 Enzymes and Kits**

ImProm-II<sup>TM</sup> Reverse Transcription system kit (Promega)

Mini Quick Spin RNA Columns (Roche Applied Science)

NucleoSpin<sup>®</sup> Extract II Kits (MACHEREY-NAGEL)

QIAprep<sup>®</sup> Miniprep kits (QIAGEN)

pGEM<sup>®</sup>-T Easy Vector Systems (Promega)

T & A Cloning vector Kit (RBC Bioscience)

T7 RiboMAX<sup>(TM)</sup> Express RNAi System (Promega)

*Taq* DNA polymerase (Fermentus)

Advantage<sup>®</sup> 2 Polymerase Mix (Clontech)

*Pci*I (Biolabs)

*Xho*I (Biolabs)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

T7 RNA polymerase (Roche)

T4 DNA ligase

Trypsin (Sigma)

#### **2.1.4 Microorganisms**

*Escherichia coli* strain Rosetta (DE3)

*Escherichia coli* strain JM109

*Vibrio harveyi* 639

#### **2.1.5 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](http://smart.embl-heidelberg.de/smart/set_mode.cgi?GENOMIC=1))

## **2.2 Sequence analysis**

Nucleotide and amino acid sequence data were analyzed by sequence analysis tools such as GENETYX program, BlastX program, SMART program, ClustalW program, PHYLIP program, and TREEVIEW program. The GENETYX software program (Software Development Inc.) was used to edit and translate the full length cDNA sequence. The BlastX program (Altschul et al., 1997) from the GenBank (<http://www.ncbi.nlm.nih.gov>) was used to analyze the sequence to compare with database. The SMART program was used to investigate domains and putative motifs. The ClustalW program was used to align the related sequences that were searched from GenBank. The PHYLIP program (Felsenstein, 1993) was used to analyze phylogenetics. The TREEVIEW program (<http://taxonomy.zoology.gla.ac.uk/rod.html>) was used to illustrate the phylogenetic trees.

**Table 2.1 Nucleotide sequences of the primers used**

Primer	Sequence (5'-3')	Purpose
<i>PmClip</i> SP2-F	5'GGACATGTCCGGATGTCACCGAGACGAATG3'	Recombinant Protein
<i>PmClip</i> SP2-R	5'GCCTCGAGCTAATGATGATGATGATGATGGGGACAGCACACCCATGGCT3'	Recombinant Protein
SP2T7-F	5'GGATCCTAATACGACTCACTATAGGGGCGTTGGTCTTCACTGCTCTC3'	<u>RNAi</u>
SP2T7-R	5'GGATCCTAATACGACTCACTATAGGCAGAAGTGCCTTCCAAGGATAG3'	<u>RNAi</u>
<i>Clip</i> SP2-F	5'GGCGTTGGTCTTCACTGCTCTC3'	<u>RNAi</u>
<i>Clip</i> SP2-R	5'CAGAACTGCCTTCCAAGGATAG3'	<u>RNAi</u>
GFPT7-F	5'TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA3'	<u>RNAi</u>
GFPT7-R	5'TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA3'	<u>RNAi</u>
GFP-F	5'ATGGTGAGCAAGGGCGAGGA3'	<u>RNAi</u>
GFP-R	5'TTACTTGTACAGCTCGTCCA3'	<u>RNAi</u>
EF1 $\alpha$ -F	5'GGTGCTGGACAAGCTGAAGGC3'	RT-PCR
EF1 $\alpha$ -R	5'CGTTCCGGTGATCATGTTCTTGATG3'	RT-PCR



### 2.3 Preparation of dsRNA

For preparation of dsRNA, gene specific primers (named ClipSP2-F and ClipSP2-R) (Table2.1) were used to amplify *PmClipSP2* (342 bp) gene and then PCR products were purified by using Nucleospin extraction kit. After that the sense strand template and the anti-sense strand template were synthesized by SP2T7-F/ClipSP2-R primers and SP2T7-R/ClipSP2-F primers, respectively (Table2.1). T7 RiboMAX Express (Promega) was used to generate single stranded RNAs. Double stranded RNAs were synthesized by mixture and annealing of single strand RNAs. The exogenous gene (the GFP gene) for a negative control was amplified by GFP specific-primer pairs using the pEGFP-1 vector as template. For the sense strand template and the anti-sense strand template of GFP gene were synthesized by using GFPT7-F/GFP-R and GFP-F/GFPT7-R, respectively (Table2.1). PCR products were analyzed by 1.2% agarose gel electrophoresis and stored at -80 °C for further *in vivo* experiments.

### 2.4 Shrimp preparation

Selection of shrimp (*P. monodon*) for experiment was performed by selection from materially local farm. When shrimp were shipped onto the laboratory, shrimp were kept in seawater tanks least 7 days before proceeding in experiment.

## **2.5 Injection of dsRNA into shrimp**

For gene knockdown experiments, shrimp (~3 g) were injected twice with *PmClipSP2* dsRNA (2.5 µg dsRNA per 1 g shrimp). The dsRNA were dissolved in 25 ml of injection buffer (150 mM NaCl). Shrimp was injected at the third segment of ventral by 0.5 ml of syringes. For the control groups, GFP dsRNA and 150 mM NaCl were injected into the shrimp. At 24 h after the second injection, hemolymph were collected from shrimp.

## **2.6 Extraction of total RNA and synthesis of cDNA**

The total RNA was extracted from hemolymph by using TRI REAGENT<sup>®</sup> (Molecular Research Center, USA). The process was described in the protocol. The purity of RNA was analyzed by 1.2% agarose gel electrophoresis and then measured the concentration at 260 nm by spectrophotometer. The first strand cDNA was synthesized from total RNA by using ImProm-II<sup>™</sup> Reverse Transcriptase System kit (Promega, USA) and then stored at -80 °C.

## **2.7 Analysis of gene expression in silenced shrimp by semi-quantitative RT-PCR**

Semi-quantitative RT-PCR technique was used to determine the efficiency of *PmClipSP2* dsRNA for knockdown of *PmClipSP2* transcript levels by using gene specific primers of ClipSP2-F and ClipSP2-R (Table 2.1). Shrimp (~3 g) were injected twice with 7.5 µg of *PmClipSP2* dsRNA. For

the control groups, GFP dsRNA and 150 mM NaCl were injected into the shrimp. At 24 h after the second injection, the hemolymph were collected from each group of shrimp (2 shrimps/group), and then extracted the total RNA from hemolymph for synthesis of the first strand cDNA. The first strand cDNA was analyzed by RT-PCR technique. Elongation factor-1 $\alpha$  gene (EF-1 $\alpha$ ) was used as internal control by using EF1 $\alpha$ -F and EF1 $\alpha$ -R primers (Table 2.1). The PCR products were analyzed by 1.2% agarose gel electrophoresis.

## **2.8 The specificity of gene knockdown by semi-quantitative RT-PCR**

RNAi silencing specificity by *PmClipSP2* dsRNA was analyzed by semi-quantitative RT-PCR using gene-specific primers for the other clip-domain serine proteinases of *P. monodon* including genes of clip-SPs (*PmPPAE1*; FJ595215, *PmPPAE2*; FJ620685, and *PmClipSP2*; FJ620687) and clip-SPHs (*PmMasSPH1*; DQ455050, *PmMasSPH2*; FJ620686 and *PmMasSPH3*; FJ620689). GFP dsRNA and NaCl were used as control. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

## **2.9 PO activity in hemolymph of silenced shrimp**

Determination of PO activity in hemolymph of silenced shrimp, shrimp were injected with *PmClipSP2* dsRNA (2.5  $\mu$ g dsRNA per 1 g shrimp). One day later, shrimp were again injected. GFP dsRNA and 150 mM NaCl were used as control. At 24 h post injection, hemolymph was collected from silenced

shrimp. After that 65  $\mu$ l of L-dopa (3 mg/ml in water) was added into hemolymph (2 mg total protein of hemolymph) and adjusted final volume (500  $\mu$ l) by 10 mM Tris-HCl pH 8.0 and then incubated for 30 min at room temperature. After finished time of incubation, each mixture was added with 500  $\mu$ l of 10% (v/v) acetic acid for stop reaction. PO activity was measured at 490 nm by spectrophotometer. For the following of enzyme reaction, dopachrome or o-quinone (substrate oxidation) was measured the absorbance. The experiment was repeated three times. PO activity was reported as  $\Delta A_{490}/\text{mg total protein}/\text{min}$ . Distilled water was used as control for measurement.

## **2.10 Cumulative mortality assay of the *PmClipSP2* silenced shrimp after injection with *Vibrio harveyi***

To study cumulative mortality assay of *PmClipSP2* silenced shrimp survival post the pathogenic bacteria *V. harveyi* infection, shrimps (~ 4 g ) were injected with *PmClipSP2* dsRNA (2.5  $\mu$ g dsRNA per 1 g shrimp). GFP dsRNA and 150 mM NaCl were used as control groups. At 24 h after the first injection, shrimp were again injected with either *PmClipSP2* dsRNA or GFP dsRNA solution that was contained with  $2 \times 10^5$  CFUs of *V. harveyi* in 25 ml of final volume. The record of cumulative mortality was showed for 5 day post injection. The experiment was repeated three times.

## **2.11 Bacterial cell counts in *PmClipSP2*-silenced shrimp**

For bacterial cell counts in the hemolymph experiment, shrimps (~ 4 g ) were injected twice with either *PmClipSP2* dsRNA (2.5 µg dsRNA per 1 g shrimp) or GFP dsRNA (control group). After second injection, shrimp were again injected with  $2 \times 10^5$  CFUs of *V. harveyi* in 25 ml of final volume. At 6 h after injection, hemolymph were collected. The total plate count was used to determine the amount of bacterial cells in hemolymph. The process of this technique was dilution of each hemolymph sample serially. After that the serial dilution of each sample was dropped (10 µl) and spotted onto LB-agar plate. Finally LB-agar plates were incubated at 30 °C for 24 h. The measurement of viable bacterial numbers were detected by following colony-forming unit (CFUs).

## **2.12 Lethal effect of LPS and $\beta$ -1,3-glucan on the *PmClipSP2* silenced shrimp**

To study the effect of LPS and  $\beta$ -1,3-glucan in the *PmClipSP2* silenced shrimp, shrimp (~4 g) were injected with either *PmClipSP2* dsRNA or microbial cell wall (LPS and  $\beta$ -1,3-glucan). This experiment, shrimps were divided into 6 groups. Group 1, shrimp were injected with *PmClipSP2* dsRNA. Group 2, shrimp were injected with LPS. Group 3, shrimp were injected with  $\beta$ -1,3-glucan. Group 4, shrimp were injected with both *PmClipSP2* dsRNA and LPS. Group 5, shrimp were injected with both *PmClipSP2* dsRNA and

$\beta$ -1,3-glucan. Group 6, shrimp were injected with *PmClipSP2* dsRNA,  $\beta$ -1,3-glucan and LPS. The mortality was recorded for 5 days post injection. The experiment was repeated three times.

## **2.13 Hemocytes counts in *PmClipSP2*-silenced shrimp**

For total hemocyte counts in *PmClipSP2*-silenced shrimp, shrimp were injected twice with either *PmClipSP2* dsRNA or GFP dsRNA (control). At the same time, the other groups of shrimp were injected twice with dsRNA, and at 24 h after second injection, shrimp were again injected with LPS (10  $\mu$ g LPS/1 g of shrimp) and incubated 1 h. Hemolymph were collected and analyzed, then the number of hemocytes were determined by light microscope.

## **2.14 Construction of plasmid for expression of recombinant *PmClipSP2* Protein**

### **2.14.1 Amplification of the mature *PmClipSP2* gene**

DNA fragments of the mature *PmClipSP2* gene from *P. monodon* were amplified by using gene specific primers (named *PmClipSP2*-F and *PmClipSP2*-R) (Table 2.1). The amplification reactions were 25  $\mu$ l/reaction that were consisted of 1x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 1  $\mu$ M each primer, 1  $\mu$ l cDNA sample and 1 units Advantage 2 *Taq* DNA polymerase (Clontech). After that following thermal cycle conditions were: pre-denaturing at 94 °C for 10 min, 30 cycles of denaturing step 94 °C for

30 seconds, annealing step 55 °C 30 seconds, extension step 72 °C for 30 seconds, and the final extension was carried out 72 °C for 10 min.

### **2.14.2 Agarose gel electrophoresis**

Agarose gel electrophoresis was used to separate and analyze DNA. PCR products were determined by using 1.2% (w/v) agarose gel electrophoresis. Solid agarose was dissolved in 1x TBE (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0), and then boiled the solution by microwave oven. After solution cool down at 55-60 °C, then the gel was poured into the tray. Plastic comb was placed into fluid to form sample wells in the agarose. PCR products were mixed with 6X loading dye and then loaded into each well. The standard DNA marker was GeneRuler™ 100 bp or 1 kb DNA Ladder plus (Fermentas). Electrophoresis was performed at 150 mV 30 min. The 2.5 µg/ml of ethidium bromide (EtBr) solution was used to stain gel for 1.5 min and then destained in water for 15 min. DNA fragments were detected by the UV transilluminator and photographed.

### **2.14.3 Purification of PCR product from agarose gel**

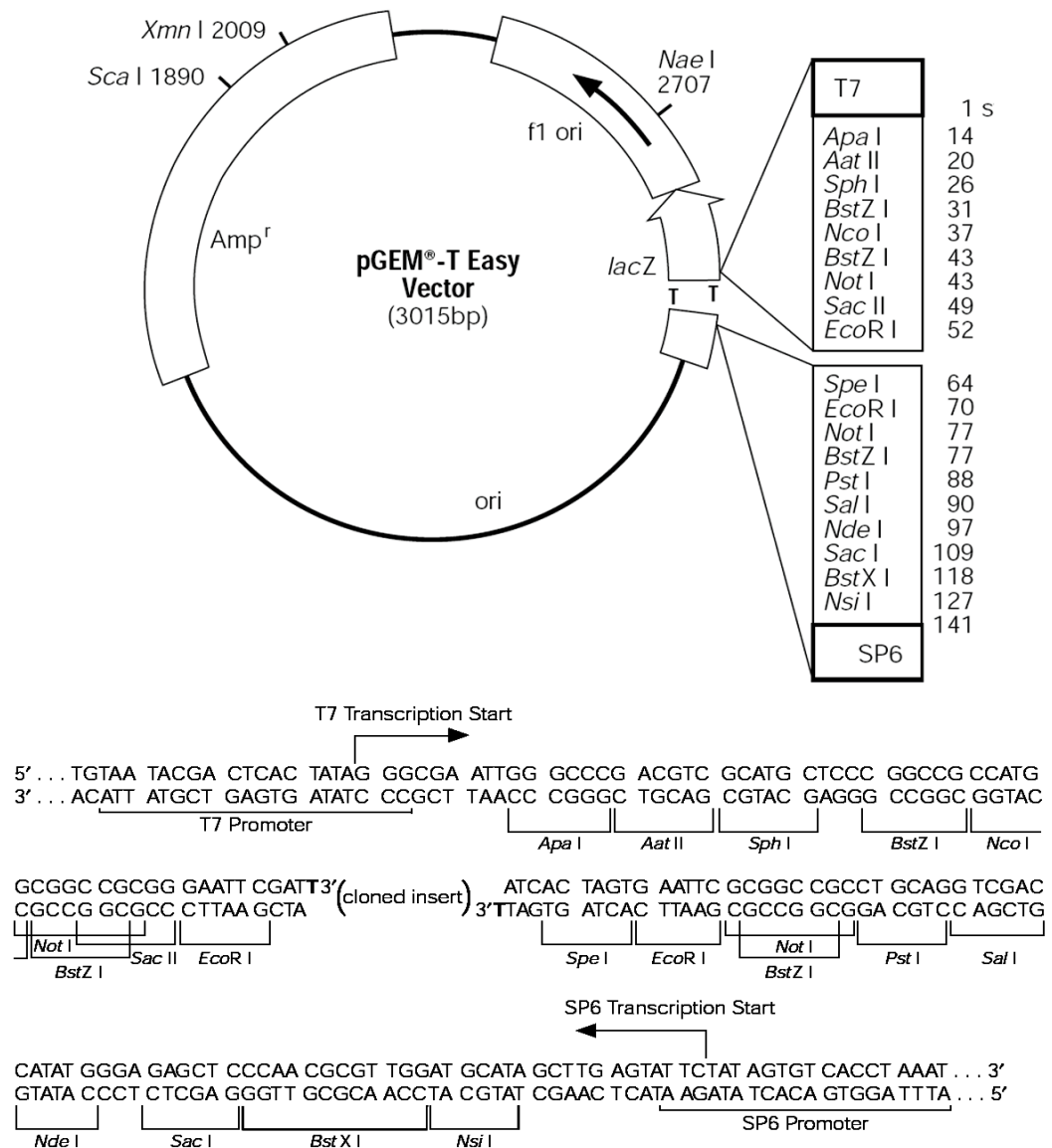
The expected PCR bands were cutted from the gel. PCR products were purified by NucleoSpin® Extract II Kits (MACHEREY-NAGEL). The process was described in the protocol. First, gel fragment was cutted and added with 700 µl of NT buffer and then incubated at 60 °C until gel completely dissolved. The solution was filled into NucleoSpin® column and then centrifuged

at 13,000x g for 1 min. After that the supernatant was removed, then column was washed by using 600 µl of NT3 buffer and centrifuged at 13,000 x g for 1 min. After that the column was centrifuged again to remove ethanol from NT3 buffer. Finally, DNA was eluted by using the ultrapure water. Eluted product was stored at -20 °C

#### **2.14.4 Construction of *PmClipSP2*-T&A vector**

After PCR products were amplified with specific primers of *PmClipSP2* gene (Table 2.1) and then used the NucleoSpin<sup>®</sup> Extract II Kits (MACHEREY-NAGEL) to purify PCR product. After that the purified PCR was ligated into T&A cloning vector (RBC Bioscience). The ligation products were transformed into *E. coli* strain JM109. The positive colonies were confirmed by colony PCR and then digested by *PciI/XhoI*. The plasmid was extracted by using QIAprep<sup>®</sup> Miniprep kits (QIAGEN) and then sequenced at Macrogen Inc (Korea).





**Figure 2.1** pGEM<sup>®</sup>-T easy vector map (A) and multiple cloning site sequences (B) (Promega)

## 2.15 Recombinant protein expression, purification and antibody production

After amplification of *PmClipSP2* gene, this gene was contained 5' flanking *PciI/XhoI* restriction enzyme sites and 3' flanking hexa-histidine

tag fused in-frame. *PciI* and *XhoI* were used to digest purified PCR product and ligated into (*PciI* /*XhoI* sites) the pET28b(+) expression vector and then transformed into *E.coli* JM109. The positive clones were confirmed by nucleotide sequencing. The selected recombinant plasmid (pET28b-*PmClipSP2*) was transformed into *E.coli* Rosetta (DE3) pLysS cells. The transformed *E. coli* was grown in LB medium that was contained with kanamycin. 1 mM IPTG was used for induction of expression, and then harvested the cultured cells at 0, 2, 4, and 6 hr. Induction of *E.coli* cells were detected by 12.5% SDS-PAGE gel and the coomassie brilliant blue for staining of the gels. After that *E.coli* cells were sonicated by sonicator and then harvested recombinant *PmClipSP2* protein (*rPmClipSP2*). The *rPmClipSP2* protein was purified by using Ni-NTA affinity chromatography column. The purified *rPmClipSP2* was used to synthesize antibodies at Biomedical Technology Research Unit, Chiang Mai University, Thailand.

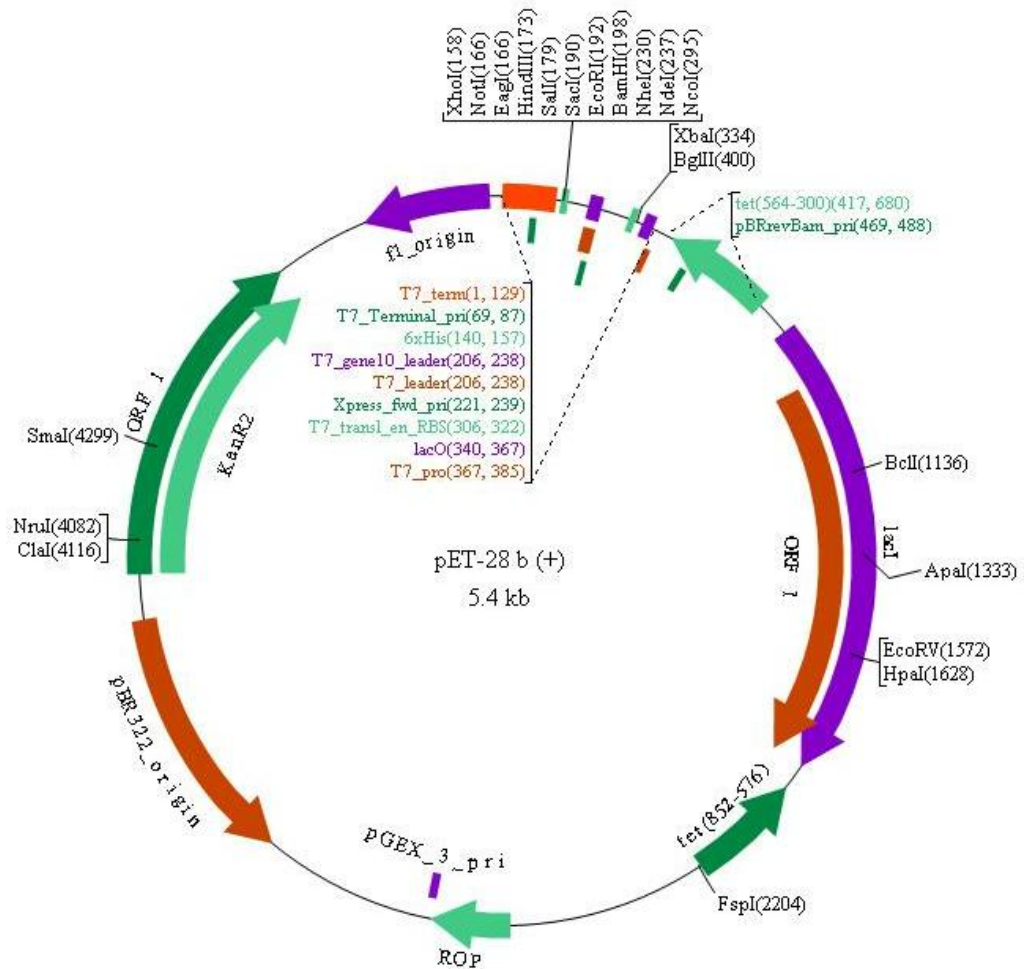


Figure 2.2 pET28b(+) vector map

## 2.16 Analysis of *PmClipSP2* protein in hemolymph of shrimp by using Western blot

Shrimp was collected hemolymph from the segment of ventral by 0.5 ml of syringes and then kept in an anticoagulant solution. After that hemolymph was centrifuged at 800 x g at 4 °C for 10 min and then separated the supernatant (plasma solution) from the pellets (hemocyte cells). Hemocyte cells were homogenized with CAC buffer (0.1 M CaCl<sub>2</sub>, 10 mM sodium

cacodylate, pH 6.5), and then broke cells by centrifugation at 25,000 x g at 4 °C for 20 min. Finally, the hemocyte lysate supernatant (HLS) was harvested in the supernatant. For analysis by using Western blot, 12.5% SDS-PAGE gel was used to analyze by loading of 20 µg of HLS and 100 µg of plasma protein and then electro-blotted onto nitrocellulose membrane. After that 5% of non-fat dry milk in Tris-buffered saline (TBS) and 0.05% Tween 20 was used to block membranes overnight. After washing, *PmClipSP2* protein was determined by using 15 ml of a 1:500 dilution of the rabbit polyclonal anti-m*PmClipSP2* antisera (primary antibody) in TBS, then membrane was incubated and washed. After that 15 ml of a 1:10,000 dilution of the anti-rabbit alkaline phosphatase conjugated antibodies (secondary antibody) in TBS was added and incubated. After washing, protein-alkaline phosphatase antibody complex was determined by using 5-Bromo-4-chloro-3-indolyl Phosphate/Nitroblue Tetrazolium (BCIP/NBT) as substrate.

## **2.17 Binding assay of r*PmClipSP2* protein**

Enzyme-linked immunosorbent assay (ELISA) was performed to determine functional r*PmClipSP2* protein. First, either lipopolysaccharide (LPS) or  $\beta$ -1,3 glucan (2 µg/well) was coated onto each well of 96-well microtiter plates (Costar) and incubated at 37°C for 24 h. After that plate was fixed by incubation at 60°C for 2 h and then added 5% bovine serum albumin for blocking non-specific binding sites at 4°C for 3 h and then washed by Tris-buffered saline (TBS) three times. After washing, r*PmClipSP2* protein was

varied the concentration (12.5 - 200  $\mu\text{g/ml/well}$ ), and then incubated onto each well at 4°C for 3 h. After washing, the bound protein was immunochemically detected. 100  $\mu\text{l}$  of a 1:10000 dilution of the rabbit polyclonal anti-*mPmClipSP2* antisera (primary antibody) in TBS was added and incubated at 37°C for 3 h, washed and then incubated for 3 h with 100  $\mu\text{l}$  of a 1:5,000 dilution of the anti-rabbit alkaline phosphatase conjugated antibodies (secondary antibody) in TBS and washed. Finally, 50  $\mu\text{l}$  of *p*-nitrophenyl phosphate in the diethanolamine buffer (1.0 mg/ml) was added and incubated at 37°C for 30 min and then stopped the reaction by using 100  $\mu\text{l}$  of 0.4 M NaOH. The alkaline phosphatase antibody-protein complex was detected by measurement at 405 nm with micro spectrophotometer reader plate.

## **2.18 PO Activation Assay of the *rPmClipSP2* Protein**

Hemolymph were collected from normal shrimp. The *rPmClipSP2* protein (20  $\mu\text{M}$ ) and activator (LPS or  $\beta$ -1,3-glucan) were added into hemolymph (250  $\mu\text{g}$  total protein). The mixtures were incubated for 15 min at room temperature and then added L-DOPA (3 mg/ml) in each reaction. After that the each mixture was incubated for 30 min at room temperature and the reactions were stopped by using 10% (v/v) acetic acid. PO activity was determined at 490 nm. The experiment was repeated three times. PO activity was reported as  $\Delta A_{490}/\text{mg}$  total protein/min. Bradford assay kit (Bio-Rad) was used in Protein concentration measurement.

## 2.19 Measurement of Proteinase Activity

To determine proteinase activity of recombinant *PmClipSP2* protein (*rPmClipSP2*), hemolymph from normal shrimp were collected. After that hemolymph were incubated with *rPmClipSP2* protein and LPS. N-Benzoyl-Phe-Val-Arg-p-nitroanilide-hydrochloride (B-7632) was used as substrate and the enzymatic activity was detected at 405 nm. The experiment was repeated three times.

## 2.20 Activation of PO activity in hemolymph of knockdown shrimp

To examine the involvement of  $\beta$ -1,3-glucan and LPS in the activation of *PmClipSP2* in the shrimp proPO system. Shrimp were injected twice with *PmClipSP2* dsRNA (2.5  $\mu$ g dsRNA per 1 g shrimp) and then collected hemolymph from the knockdown shrimp at 24 h after the second injection. Then, the total protein in hemolymph was incubated with trypsin (exogenous proteinase that directly activates the proPO to PO),  $\beta$ -1,3-glucan or LPS. GFP dsRNA or 150 mM NaCl was used as control. PO activity levels were detected by using L-DOPA as the substrate and measured at 490 nm. The experiment was repeated three times. PO activity was reported as  $\Delta A_{490}/\text{mg total protein}/\text{min}$ .

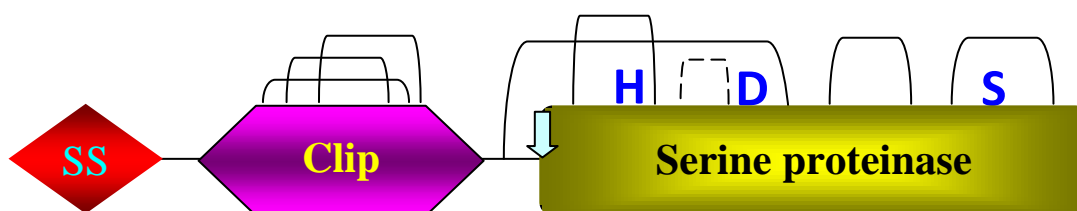
## CHAPTER III

### RESULTS

#### 3.1 Sequence analysis of the *PmClipSP2* from *Penaeus monodon*

Four clip domain serine proteinases (Clip-SPs) were identified from *Penaeus monodon* EST database (<http://pmonodon.biotec.or.th>) (Tassanakajon et al., 2006). They are prophenoloxidase activating enzyme 1 (*PmPPAE1*), prophenoloxidase activating enzyme 2 (*PmPPAE2*), clip domain serine proteinase 1 (*PmClipSP1*) and clip domain serine proteinase 2 (*PmClipSP2*). In this research, we selected *PmClipSP2* for further characterization. The complete cDNA sequence of *PmClipSP2* (FJ620687) was obtained from the previous research (Amparyup et al., 2009b). The full-length sequence of *PmClipSP2* cDNA is composed of 1,317 bp, containing an ORF encoding a polypeptide of 369 amino acid residues. The molecular mass of the mature protein is 40.18 kDa with the isoelectric point (pI) of 5.14.

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 *PmClipSP2* (Figure 3.1). The domain organization of *PmClipSP2* is shown in Figure 3.1.



**Figure 3.1** Predicted organization of *PmClipSP2* 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 *PmClipSP2*

Sequence analysis by BlastX revealed that the deduced amino acid sequence of *PmClipSP2* was similar to a Clip domain serine proteinase 1 (*PmClipSP1*) (FJ620688) (74% sequence similarity), the prophenoloxidase activating enzyme I (*PmPPAE1*) (HQ008365) (62%), the prophenoloxidase activating enzyme II (*PmPPAE2*) (HQ008366) (60%), the prophenoloxidase activating factor I (*HdPPAF1*) (BAA34642) from *Holotrichia diomphalia* (54%), the melanization protease I (*DmMP1*) (NP\_649450) from *Drosophila melanogaster* (52%) and the prophenoloxidase activating proteinase I (*MsPAP1*) (AY789465) from *Manduca sexta* (51%) (Table 3.1).



**Table 3.1** The BLASTX results and percentages of similarity of *PmClipSP2* sequence to other clip-SPs in GenBank database.

<b>Clip-SPs</b>	<b>Closest species</b>	<b>% similarity</b>	<b>Accession No.</b>
<i>PmClipSP1</i>	<i>Penaeus monodon</i>	74%	FJ620688
<i>PmPPAE1</i>	<i>Penaeus monodon</i>	62%	HQ008365
<i>PmPPAE2</i>	<i>Penaeus monodon</i>	60%	HQ008366
<i>HdPPAF1</i>	<i>Holotrichia diomphalia</i>	54%	BAA34642
<i>DmMP1</i>	<i>Drosophila melanogaster</i>	52%	NP_649450
<i>MsPAP1</i>	<i>Manduca sexta</i>	51%	AY789465

Multiple sequence alignment of the deduced amino acid sequence of *PmClipSP2* with those of other clip-SPs in arthropods revealed the six conserved clip domain cysteines at the N-terminus. Moreover, the eight conserved cysteine residues in the SP domain that participate in the formation of four disulfide bonds were found in *PmClipSP1*, *PmPPAE1*, *PmPPAE2*, *MsPAP1*, *HdPPAF-I*, *DmMP1* and *P1PPAE* (Figure 3.2).

PmClipSP2 MNKQRPSTSPVALVFTALLLFHGAAS-----  
PmClipSP1 MNIKRG--CVRNLFVFLVLLVAVQVTS-----  
PmPPAE2 MHYRVFTISCRAAVILLVLLVTSGGAT-----  
HdPPAFI MGVHFF--IIVFFVLLNLYSIKQAG-----  
DmMP1 MEPHFFITVWMLMGTSSTYRQ-----  
MsPAP1 MRNHIVTIFVAVVWTCVFSQ-----  
PmPPAE1 MGVTVVLLWVCVVAWAAAVGD---EQLSGKKREPOQPNFNFPGNGSFRPSNN-----RPGFTPAGQSDCTGNRRNSRR--CQNRP  
PIPPAE MKVVVELLWLSLVTAAVGVGVGVNVTQEEAHSRVKRFQFNRGRGGNPHRNNA PGHGHG--HGGFIPPGHQ--GGNLHNIGRPGAGR

## Clip domain

PmClipSP2 -----QGTGCH-----RDECTLITDCPKLL  
PmClipSP1 -----QGADCV-----RSCCSIRECPALL  
PmPPAE2 -----RDRROARCS-----AGAPCVLVDSCPVK  
HdPPAFI -----CRTNGENARCVINNCKIIV  
DmMP1 -----EITGYCRTPDENSITCINIRECGYIF  
MsPAP1 -----SCTIPQGADSNCSISIECPQLL  
PmPPAE1 SGGQALGQGGVSSAFQOIPWLSQLSRDQON---LLIPNLPKTPSGGAQNRFFLLGTGKPVVFPQCCVTRPFRFGHCRYLQHCIOPE  
PIPPAE PS-AQTLGQGHNLN---NQLSWFNDVVS GTNNEITLKLGLPRLPLRQAQNTFFQLGVGQPVVFPQCCRTIPKSERGCCRFPQYCILPE

Clip domain 5-5 5-5 5-5  
PmClipSP2 DLIKNE--TLDSTGELQATCFINKRQFVCCP---APVTEPK--VIKES-----LLPNCGLVGDV---R  
PmClipSP1 KLIQDP--TRINIRKLDATCYVRNREEMVCCPS---ITTEITPT---IFTKS-----LLPENCGHSAHLN--R  
PmPPAE2 AIFLSP--NAGDKHRADQLICGREGHRLKVVCCGS---SNVITTRERIDVTPTSNFGNG--NGQLLPSNCGQTSNLN--K  
HdPPAFI DSVLTSDF--EVIRFTRASOCGY--NGQLVCCG--SSASYQPPHTSASIRNRRPELLP-----NDCGYQVEAD--K  
DmMP1 ELIQSEEVTEQDRRFLQASQCGYRNGQVLICANSRMRNQQBQWGNHCPTQITKPKTKRSGTKLLPMAP-----NCG-ENFGD--R  
MsPAP1 SAFEQRPLPSPVVNYLRKSCQCFDGYTERVCCGGLPQOASRCPTEAPVPIRAPFVNPGGVDPYDDESSPAPRNCQGVDMNGD--R  
PmPPAE1 FANNFNV-----FLRYVCFIEGVYVGVCCEDTFNNINATFPFPPTIKPTIRPVTP-----PSQSRGCGLVAKRPPTR  
PIPPAE FAQNFOA-----FLOYVCFIQTYVGACCTITVNNVGVTAFPFPPTPTPTPTPT-----KSEANGCGLVAKRPPTR

## Serine proteases domain

PmClipSP2 VVGGEDAPIDAYFWKAVLGYRIGGLPEIHTECGGSVINERYIMTAACHVNANIINERELELAVIRLGEWDLSTEMDCTINTSNGSRFC  
PmClipSP1 IVGGEVAPLDAYFWKAVLGYKDKGLAAIEELCGGSVINERYVLTAAHCVDPGTLGTR--REEVVRLGEWDLTTEDCESTNSGVVFC  
PmPPAE2 IFGGEATGVGEFWMAVLGVNSG---SLDWECCGALINDRYVLTAAHCGDPDFLFGS--ILTAIRLGEYDFSKSKDCNSAAD---FC  
HdPPAFI ILNGDDIVVEEFPWTAMIGYKNS--NFEQACGGSLINRNYVITAACHVAGRVLRVVG--ALNKVRLGEWNTADPDCYGAVR--VCV  
DmMP1 VVGGENETKREFWMALIENIKPE--NVKGHRCGSLINRNYVLTAAHCVS--AIPSDW-ELTGVRLGEWDASTINPDCYVGNRRDC  
MsPAP1 TYGGQITDLDLDEFWMALDGLYLRTR--GSTTYCCGGVLINRNYVLTAAHCTIGAVEREVG-KLITVRLGEYDQNSVDGVDDV---C  
PmPPAE1 IVGGKQADAPQEFWMAALMRDQAS---SYCGGVLTIDSHILTAACHVDR---FDRNTITVRLGEYTDLADQ-----  
PIPPAE IVGGKPADDEREFWVAALLROGST---QYCGGVLTINQHVLTAAHCVRG---FDQTTITVRLGEYDFEKQTS-----

PmClipSP2 APFVQDFDFEEVTEHPSYDNRILFS--DDIALIRLSKPINELTSAGFVQVCLFPADLSLSAEARSQGAIVAGVGVTEKG--IQSDRLQ  
PmClipSP1 APFVQDFEAEEIIGHPSYNTRVRF--DDIALIRLNRPINQESAGFVLFVCLFSPSFPSPRTANGKSAIAGWGFTEIG--SASNKIK  
PmPPAE2 LPFVQDITPEQVVLHPSFNKRAPES--DDIALIRLNRRVQLNAG---VHPICLBAAGLVNVSFLNGDAIVIGWGHTEIG--TINTOVLQ  
HdPPAFI PPKPIDLGIETITQHDYVDGSKDRYHDIALIRLNROVEETNY---IRFVCLFQFNE---EVQVQRLLTVGWGRTEIG--QYSTIKQ  
DmMP1 NEFYVDYFVEERFPHQYPGNSRDQINDIALIRLRDEVOYSDF---ILFVCLFHLASQHNNIFLGKVVVAGWGRTEIN--FTSNIKL  
MsPAP1 RDEFPQNIPIEAAYPHSGYSDNNKNRKDDIALVRLTRAYTY---VKPICLAHNE---RLATENDVFAVAGWGRKLSG--KSSPIKL  
PmPPAE1 -TGHVDFKVADEMRHRSYDITTYVN--DIALIRKQGSTINFNVD---IRFVCLFEGDE---SYEGHTGTVIGWGTIYYGPFVSTLQ  
PIPPAE -TGAQITGVKIKKEHEAYDITTYVN--DIALITLTKSTEFNAD---IRFVCLFEGDE---TYVDRQGTIVGWGTIYYGPFVSVLM

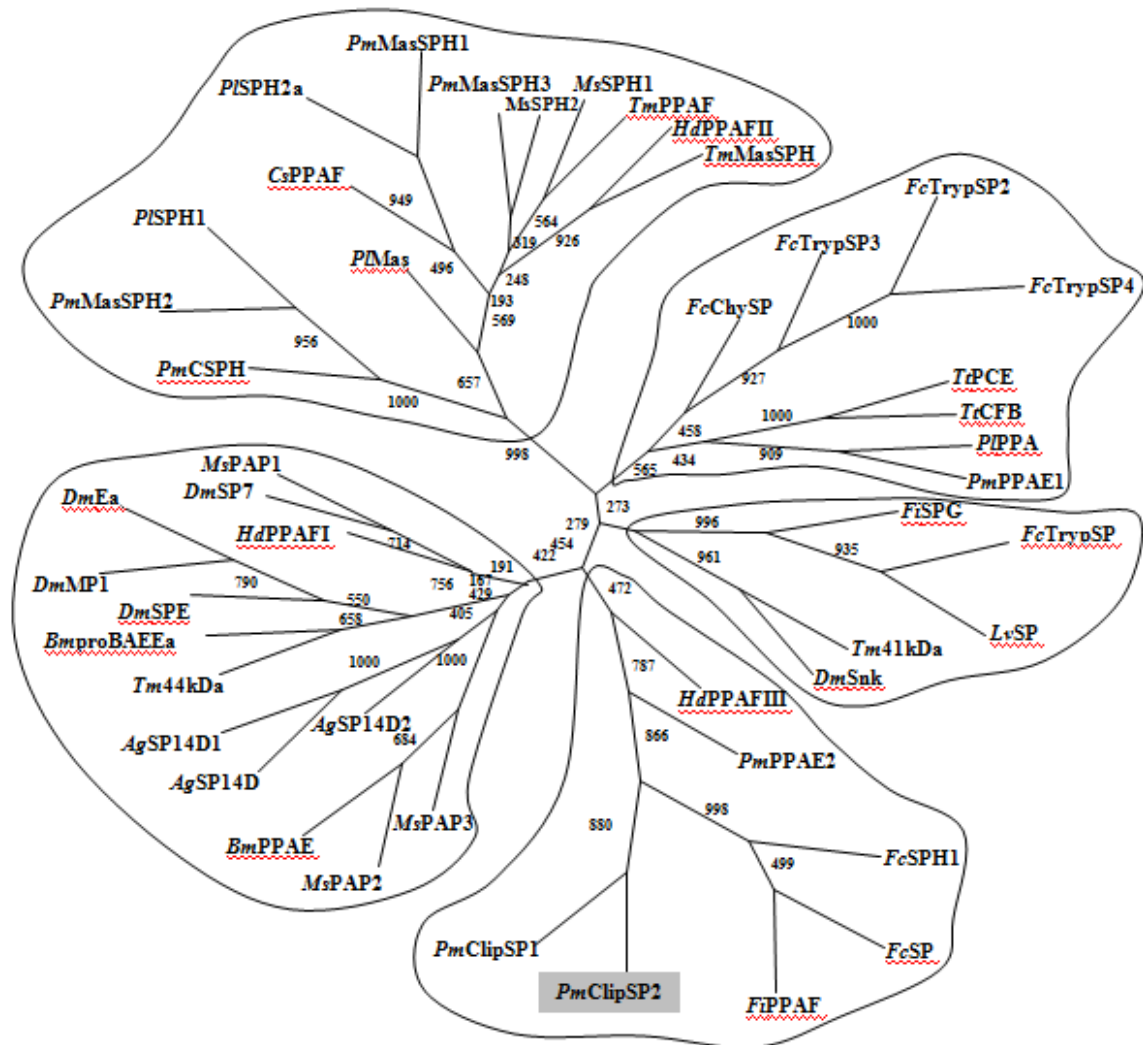
PmClipSP2 HLLIPEFVENKECNERV--RGN-LVAEQICMGGEAG-KDSRREGDGGPLIMKGSEREVSMQIGTVSYGFTSCGQKGFPGVYTSVSHY  
PmClipSP1 HWKLPPLVDSTECQVMV--KBS-IVSEQLCAGGNAG-EDSCGGDGGGPLVIL-AGTFGPPYQQIGTVSYGFVSCGQOQVPGGIYTSVSSY  
PmPPAE2 KVSLEPFVDLGTCKRIH--AGETLVNEQVCFGGFRAG-QDSCNGDGGGPLFNLAVPG---TILGTVSKG-GACGSPGVPPIYTDVASY  
HdPPAFI KLAVFVVAEQCAKTFGAAGVVRVRSQQLCAGGEKA-KDSCGGDGGGPLLAERANQ--QFFLEGLVSGF-ATCGTEGWPGIYTKVGY  
DmMP1 KAEIDTIFPTSECNQRMATORRIVTITQMCAGGVEG-VDSRGGDGGGPLLLEDYSNGNSNYIAGVSYGFTFCGLKGWPGVYTRVEAY  
MsPAP1 KLGMPITFDKSDCASKVRNLGAELTDKQICAGGVFA-KDTCRGGDGGGPLMORREP--INEVVGIVSFG-NRCGLDGPVGVYSVAVY  
PmPPAE1 EMTVPIWTKACDDAN---EQNIIDKQLCAGATDGGKDCQGGDGGGPLLLQGSSEN-RRAVVGVSWSG-IRCAEPGNPVGIVYTRVSKY  
PIPPAE EWSIPITWADDAAN---GQDIIDKQLCAGDKAGGKDCQGGDGGGPLMLQGSAN-RRAVVGVSWSG-IRCAEASPGVYTRISKY

PmClipSP2 RSWVEEILRE--- 369  
PmClipSP1 RWIECNLKE--- 366  
PmPPAE2 RGWIVQNLKE--- 371  
HdPPAFI RWIEGNIRE--- 365  
DmMP1 LNWIENNVR--- 390  
MsPAP1 SDWILSLRSTNV 383  
PmPPAE1 VDWIKNAV---- 463  
PIPPAE TDWIRANQ---- 468

**Figure 3.2** Multiple alignment of amino acid sequence of *Penaeus monodon* PmClipSP2 with other arthropods clip serine proteinases. The amino acid sequence of *P. monodon* Clip SP2 (PmClipSP2, FJ620687) was aligned with those of *Penaeus monodon* Clip SP1 (PmClipSP1, FJ620688), PPAE1 (PmPPAE1, FJ595215) and PPAE2 (PmPPAE2, FJ620685); *Holotrichia diomphalia* PPAF-I (HdPPAF-I, BAA34642); *Drosophila melanogaster* MP1 (DmMP1, NP\_649450); *Manduca sexta* PAPI (MsPAP1, CAL25132) and *Pacifascatus leniusculus* PPAE (PIPPAE, CAB63112). Red line show clip domain part. Purple line show serine proteinase domain part. Blue colour show conserve cysteine. There are six conserved cysteines in the clip domain forming three disulfide bonds.

### 3.3 Phylogenetic analysis

To examine the relationship of the PmClipSP2 protein to clip-SP proteins in other arthropods, the NJ distance based method was used for the construction of phylogenetic tree by comparison of the amino acid sequences of the SP domain (Figure 3.3). NJ analysis categorized clip-SPs and clip-SPHs in arthropods into two groups: clip-SPs and clip-SPHs. The clip-SPs group can be categorized into four subgroups: The first subgroup is the insect PPAEs (*BmPPAE*, *HdPPAFI*, *MsPAP1*, *MsPAP2*, *MsPAP3*) and the insect clip-SPs (*AgSP14D*, *AgSP14D2*, *DmMP1*, *DmSP7*, *DmSPE*, *DmEa*, *BmproBAEEase*, and *Tm44kDa*). The second subgroup consists of horseshoe crab clip-SPs (*TtCB* and *TtPCE*) and crustacean PPAEs (*PmPPAE1* and *PIPPA*). The third subgroup contains *DmSnk* (*Drosophila melanogaster* snake) and *Tm41* (*Tenebrio molitor* 41 kDa zymogen) and the final subgroup is the shrimp clip-SP or clip-SPH (*PmClipSP1*, *PmClipSP2*, *PmPPAE2*, *FcSPH*) and insect PPAF (*HdPPAFIII*).



100

**Figure 3.3** Bootstrapped unrooted neighbour-joining tree of the serine proteinase domain of clip-SPs and clip-SPHs from arthropods: *Penaeus monodon* clip-SP2 (*PmClipSP2*; ACP19561), clip-SP1 (*PmClipSP1*; ACP19562), PPAE1 (*PmPPAE1*; ACP19558), PPAE2 (*PmPPAE2*; ACP19559), Mas-like SPH1 (*PmMasSPH1*; ABE03741), Mas-like SPH2 (*PmMasSPH2*; ACP19560), Mas-like SPH3 (*PmMasSPH3*; ACP19563), Mas-like protein (*PmCSPH*; AY600627); *Penaeus chinensis* SPH (*FcSPH1*; DQ318859); *Pacifastacus leniusculus* PPA (*PIPPA*; CAB63112), Mas-like protein (*PIMas*; Y11145), SPH1 (*PISPH1*; AY861652), SPH2a (*PISPH2a*; EU552456); *Callinectes sapidus* PPAF (*CsPPAF*; AY555734); *Anopheles gambiae* serine protease 14D (*AgSP14D*; FJ653845), serine protease 14D2 (*AgSp14D2*; AF117749); *Drosophila melanogaster* melanization protease 1

(*DmMP1*; NM\_141193), Spätzle-Processing enzyme (*DmSPE*; NM\_142911), snake (*DmSnk*; NM\_079614), easter (*DmEa*; NM\_079638), serine protease 7 (*DmSP7*; NM\_141477); *Bombyx mori* PPAE (*BmPPAE*; NM\_001043367), SP zymogen (*BmproBAEEase*; NM\_001043379); *Holotrichia diomphalia* PPAFI (*HdPPAFI*; AB013088), PPAFII (*HdPPAFII*; AJ400903), PPAFIII (*HdPPAFIII*; AB079666); *Manduca sexta* PAPI (*MsPAP1*; AY789465), PAP2 (*MsPAP2*; AY077643), (*MsPAP3*; AY188445), SPH1 (*MsSPH1*; AF518767), SPH2 (*MsSPH2*; AF518768); *Tenebrio molitor* PPAF (*TmPPAF*; AJ400904), Mas-like SPH (*TmMasSPH*; AB084067), 41 kDa zymogen (*Tm41kDa*; AB363979), 44 kDa zymogen (*Tm44kDa*; AB363980); *Tachypleus tridentatus* proclotting enzyme (*TiPCE*; M58366) and coagulation factor B (*TiCFB*; D14701). Bootstrap values indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

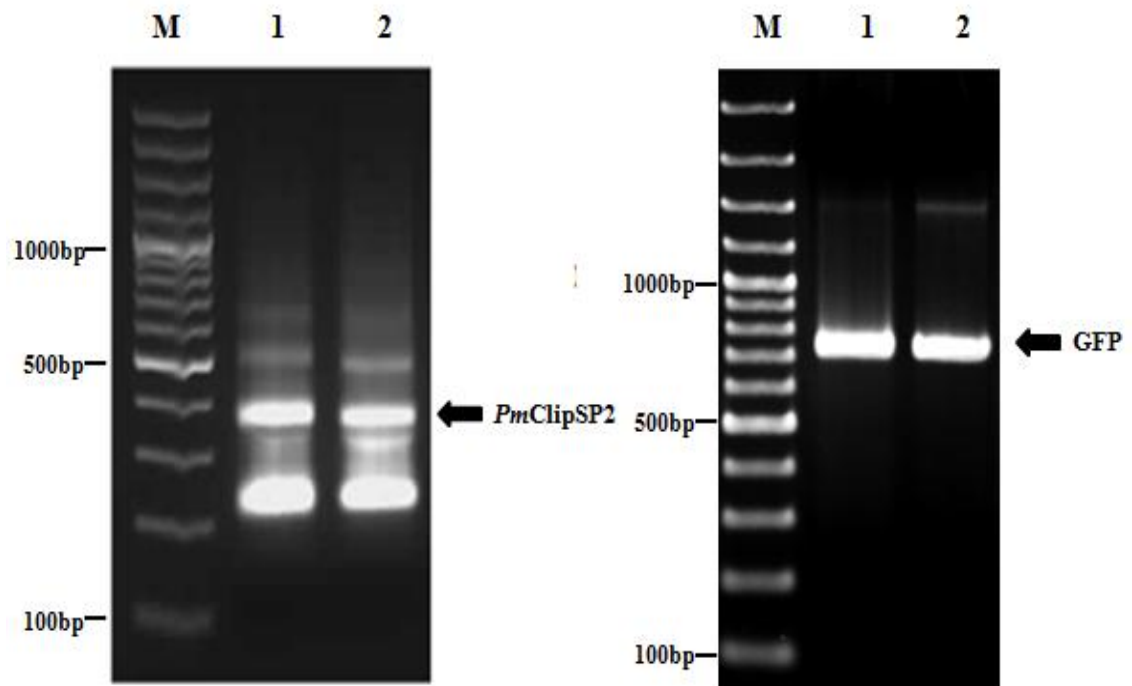
### **3.4 Silencing of the *PmClipSP2* gene transcript by RNAi (RNA interference)**

To study the potential role of *PmClipSP2* in shrimp immunity, double stranded RNA (dsRNA) interposed RNA interference (RNAi) was used to suppress the *PmClipSP2* mRNA in *P.monodon*. Knockdown efficiency of *PmClipSP2* transcripts was determined at the mRNA level using semi-quantitative RT-PCR technique. The *PmClipSP2* knockdown shrimp was assayed for the enzymatic activity of phenoloxidase, the bacterial count, the total hemocyte number and the cumulative mortality.

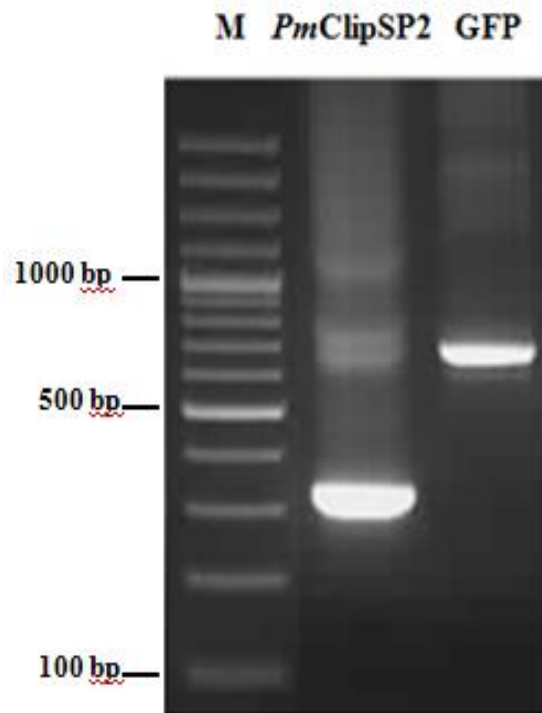
#### **3.4.1 Preparation of dsRNA**

The DNA fragment of *PmClipSP2* (342 bp) was amplified from the recombinant plasmid that contained the full length *PmClipSP2* cDNA with

a pair of the gene-specific amplification primers (named ClipSP2-F and ClipSP2-R). For dsRNA synthesis, primers contain the T7 promoter recognition sites that flanked the 5' end. The sense strand template and the anti-sense strand template were synthesized by SP2T7-F/ClipSP2-R primers and SP2T7-R/ClipSP2-F primers, respectively. The exogenous gene for a negative control, the GFP gene was amplified by GFP specific-primer pairs using the pEGFP-1 vector as template. For the sense strand template and the anti-sense strand template of GFP gene were synthesized by using GFPT7-F/GFP-R and GFP-F/GFPT7-R, respectively. The expected 342 bp PCR product was gel-purified and was used to synthesize the dsRNA with a T7 RNA polymerase using a kit for T7 RiboMAX™ Express Large Scale RNA Production Systems. The ssRNAs of sense and antisense strands of *PmClipSP2* and GFP were synthesized and their concentrations were observed before annealing step (Figure 3.4). After the purification of DNase-treated dsRNA, an agarose gel of *PmClipSP2* and GFP dsRNAs revealed a major band of *PmClipSP2* and GFP dsRNAs (Figure 3.5).



**Figure 3.4** PCR products of *PmClipSP2* (342 bp): Picture on the left, lane 1 is *PmClipSP2* sense strand and lane 2 is *PmClipSP2* antisense strand templates. PCR products of GFP (740 bp): picture on the right, lane 1 is GFP sense strand and lane 2 is GFP antisense strand templates. Products were analyzed by 1.2 % agarose gelelectrophoresis. Lane M is GeneRuler™ 100 bp DNA ladder.



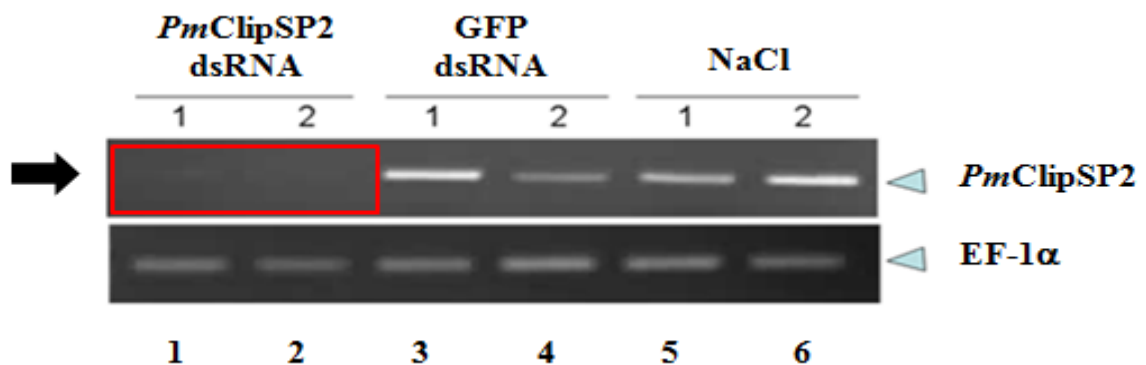
**Figure 3.5** Analysis of double strand RNA of *PmClipSP2* and GFP genes: lane 1 is *PmClipSP2* dsRNA and lane 2 is GFP dsRNA. Products were analyzed by 1.2 % agarose gelelectrophoresis. Lane M is GeneRuler™ 100 bp DNA ladder (Fermentas).

### 3.4.2 Gene silencing of *PmClipSP2*

To evaluate the knockdown efficiency of *PmClipSP2* dsRNA, shrimp (~3 g) were injected twice with 7.5 µg of *PmClipSP2* dsRNA. For the control groups, GFP dsRNA and 150 mM NaCl were injected into the shrimp. At 24 h after the second injection, the hemolymph were collected from each group of shrimp (2 shrimps/group), and analyzed by RT-PCR technique. For internal control, Elongation factor-1α gene (EF-1α) was used to detect the number of cDNA template and amplification capability between samples. The results

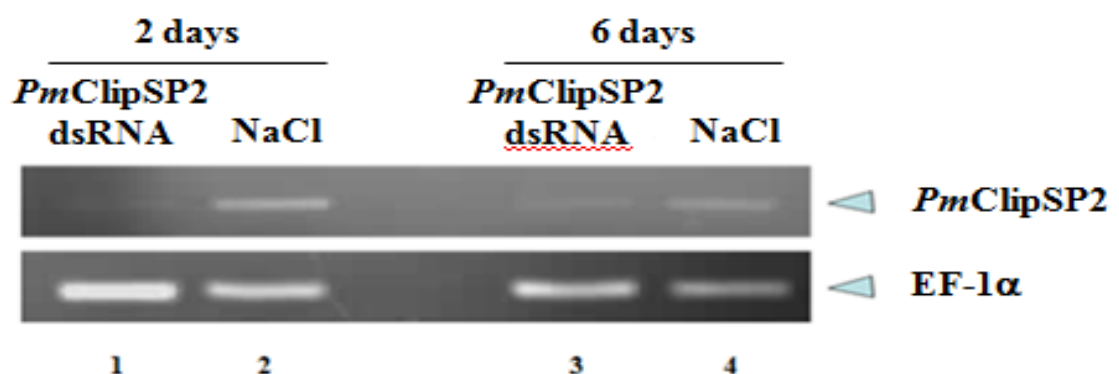


showed that the expression of *PmClipSP2* gene was suppressed by *PmClipSP2* dsRNA but not in control groups (GFP dsRNA and NaCl) (Figure 3.6).



**Figure 3.6** Suppression of *PmClipSP2* gene. Lanes 1 and 2 were shrimp that injected with *PmClipSP2* dsRNA, lanes 3 and 4 were shrimp that injected with GFP dsRNA and lanes 5 and 6 were shrimp that injected with NaCl. Elongation factor-1 $\alpha$  gene (EF-1 $\alpha$ ) was used as internal control. Two individuals were used per group.

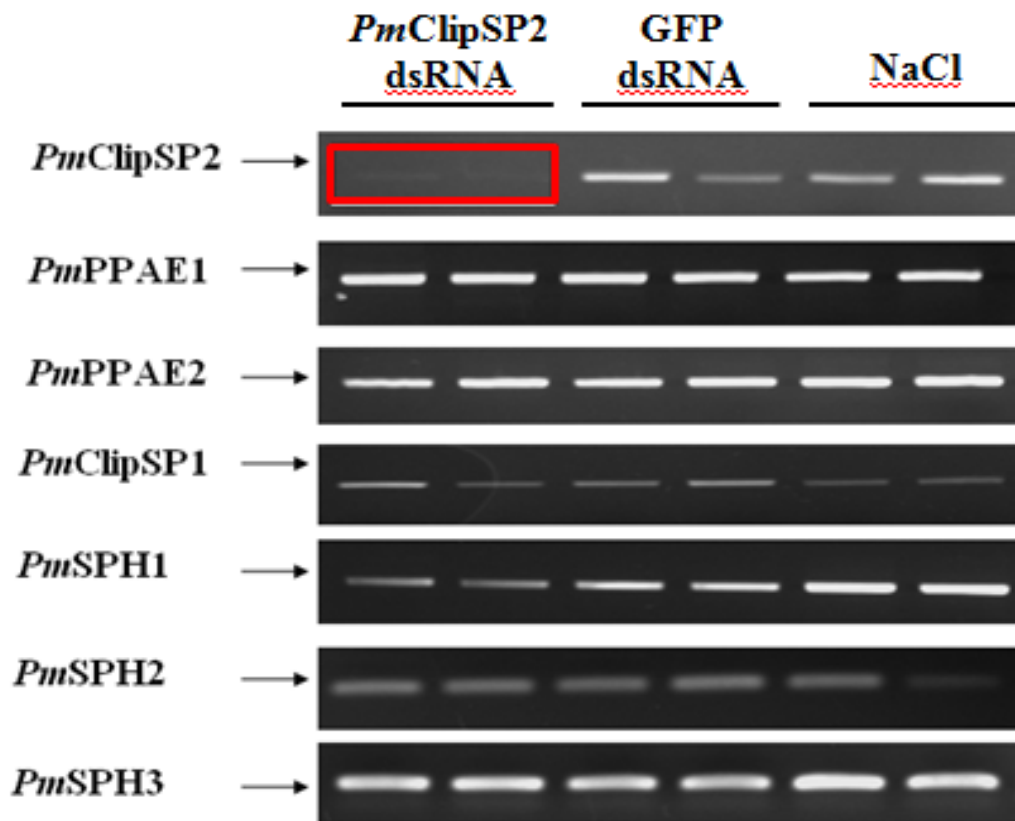
To determine silencing efficiency of *PmClipSP2* dsRNA, suppression of the *PmClipSP2* mRNA was followed for a period of 6 days. The result showed that *PmClipSP2* dsRNA could silence *PmClipSP2* gene up to 6 days after second injection (Figure 3.7).



**Figure 3.7** Silencing efficiency of *PmClipSP2* dsRNA determined at 2 and 6 days post injection: lane 1 and 2 are the groups that were injected with *PmClipSP2* dsRNA

and NaCl at day two. Lane 3 and 4 are the groups that were injected with *PmClipSP2* dsRNA and NaCl at day six.

RNAi silencing specificity by *PmClipSP2* dsRNA was further corroborated by RT-PCR using gene-specific primers for the other clip-domain serine proteinases of *P. monodon* including genes of clip-SPs (*PmPPAE1*; FJ595215, *PmPPAE2*; FJ620685, and *PmClipSP2*; FJ620687) and clip-SPHs (*PmMasSPH1*; DQ455050, *PmMasSPH2*; FJ620686 and *PmMasSPH3*; FJ620689). The results indicated that injection of *PmClipSP2* dsRNA did not suppress the transcriptional level of the other clip-SPs and clip-SPHs in *P. monodon* suggesting the specificity of *PmClipSP2* RNAi knockdown (Figure 3.8).

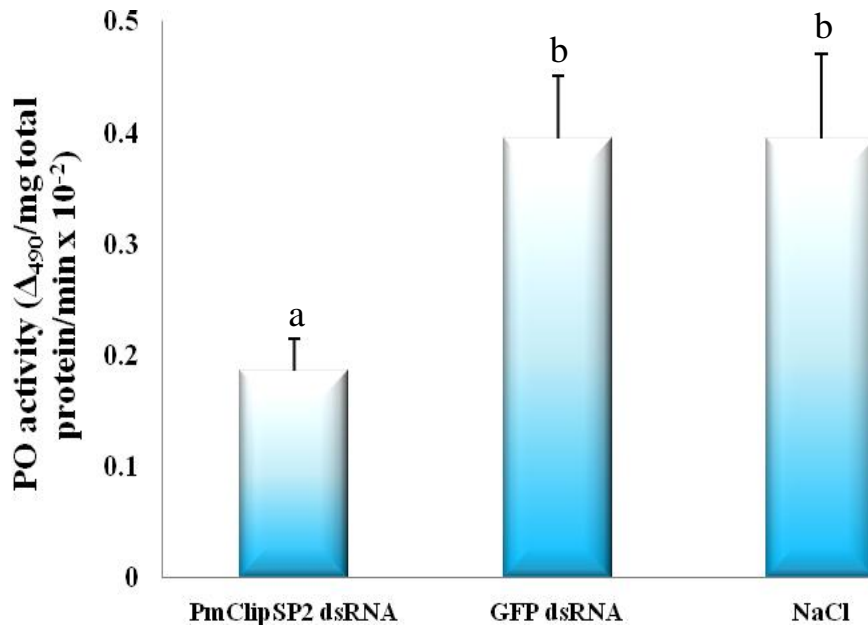


**Figure 3.8** Gene-specific silencing of *PmClipSP2* transcript levels in *P. monodon* hemocytes. The effect of *PmClipSP2* dsRNA on the transcription levels of *PmClipSP2*, and other shrimp clip-SPs (*PmPPAE1*, *PmPPAE2* and *PmClipSP1*) and clip-SPHs (*PmMasSPH1*, *PmMasSPH2* and *PmMasSPH3*) in *PmClipSP2* dsRNA, GFP dsRNA or NaCl injected shrimp was determined by RT-PCR. Specific primers were used to amplify and each lane represents cDNA from an individual shrimp.

### 3.4.3 Hemolymph PO activity of *PmClipSP2* silencing shrimp

The PO activity of the *PmClipSP2* silencing shrimp was detected. Shrimp hemolymph were collected at 24 hr after the second dsRNA injection and the PO activity was determined using L-DOPA as substrate. The PO activity was measured at 490 nm. PO activity was reported as  $\Delta A_{490}/\text{mg}$  total protein/min against control samples. The experiments were repeated three times. The results indicated that the PO activity in the *PmClipSP2* silencing

shrimp was decreased 39% when compared with both GFP dsRNA and NaCl (control groups). This suggested that *PmClipSP2* was involved in the regulation of the proPO system in shrimp (Figure 3.9).

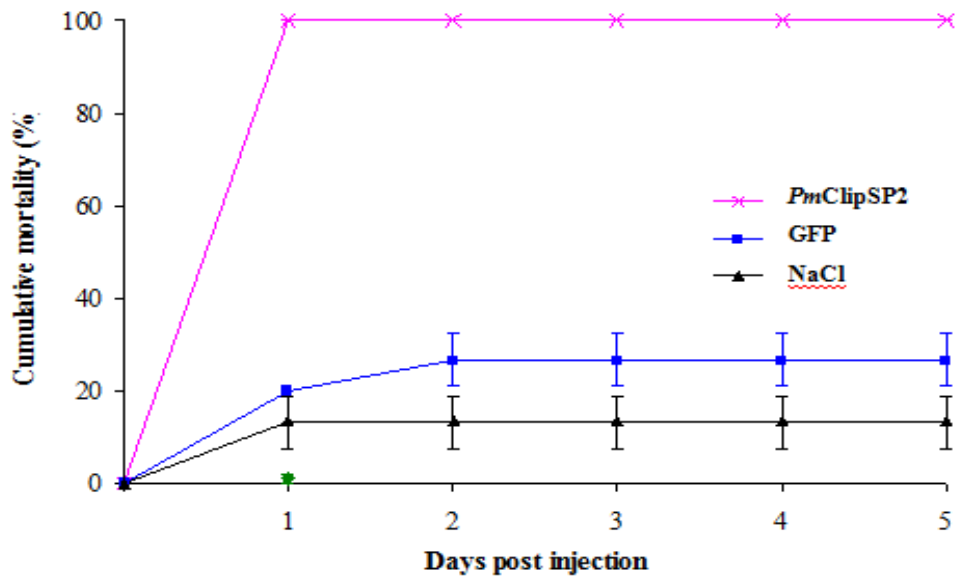


**Figure 3.9** Total PO activity in shrimp hemolymph injected with *PmClipSP2* dsRNA, GFP dsRNA and NaCl. PO activity was determined at 24 hr after the second dsRNA injection and recorded as  $\Delta A_{490}$ /mg total protein/min. Experiments were repeated three times and the data is shown as the mean  $\pm$  standard deviation. Means with the same lower case letters (above each bar) are not significantly different at the  $p < 0.05$  level.

#### 3.4.4 Cumulative mortality assay of the *PmClipSP2* knockdown shrimp after *V.harveyi* infection

To study the essential role of *PmClipSP2* gene in shrimp survival post injection with the pathogenic bacteria *V. harveyi*, shrimp (~4 g) were injected with *PmClipSP2* dsRNA and  $2 \times 10^5$  CFUs of *V. harveyi*. After the bacterial challenge, the mortality was recorded for 5 days post infection. The

*PmClipSP2* knockdown shrimp had 100% cumulative mortality within 24 h. For the control groups, shrimp that injected with GFP dsRNA and NaCl solution had 20% and 10% cumulative mortality, respectively (Figure 3.10).

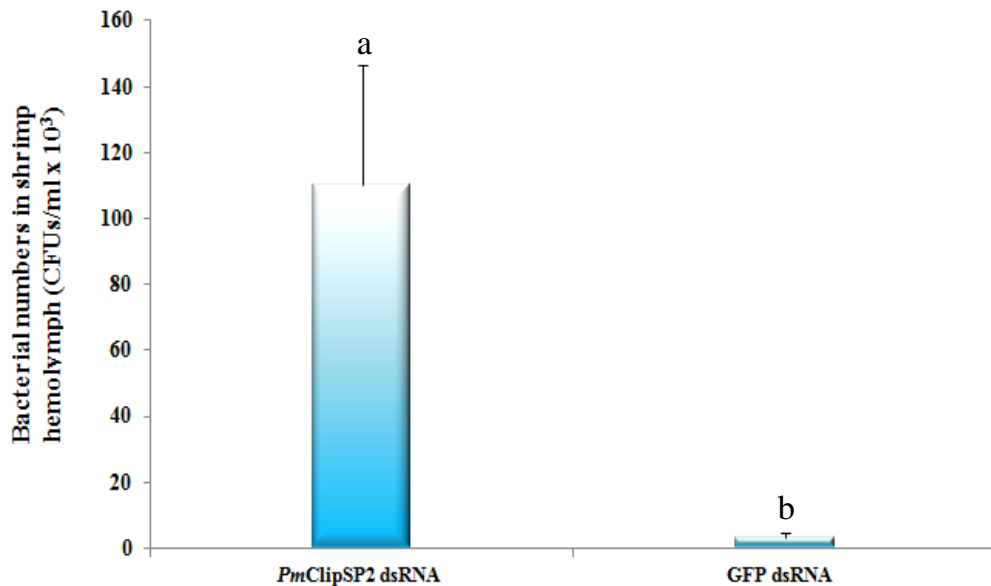


**Figure 3.10** Cumulative mortality of *PmClipSP2* knockdown shrimp challenged with *Vibrio harveyi* ( $2 \times 10^5$  CFUs). Control groups were injected with either NaCl or GFP dsRNA. Shrimp mortality was recorded for 5 days. The cumulative mortality (%) in each experimental group (8–10 shrimp/group) is presented as the mean  $\pm$  standard deviation, and is derived from triplicate independent experiments.

### 3.4.5 Bacterial count analysis in the hemolymph of *PmClipSP2*-silenced shrimp

Function of *PmClipSP2* in antibacterial defense was validated using RNAi and bacterial challenge approaches. Shrimp was silenced by *PmClipSP2* dsRNA and then was injected with *V. harveyi*. The total plate count was used to determine the number of bacterial cells in shrimp hemolymph. The results showed that the number of bacteria was markedly increased in *PmClipSP2*

knockdown shrimp but not in control groups with GFP dsRNA injection (Figure 3.11). After total plate count analysis, bacterial colonies (5 clones) were randomly selected for PCR amplification using 16S rDNA primers and subjected to DNA sequencing analysis. After Blast analysis, the results indicate that 16S rDNA sequences of the five bacterial colonies were highly similar to the DNA sequence of *V. harveyi*.



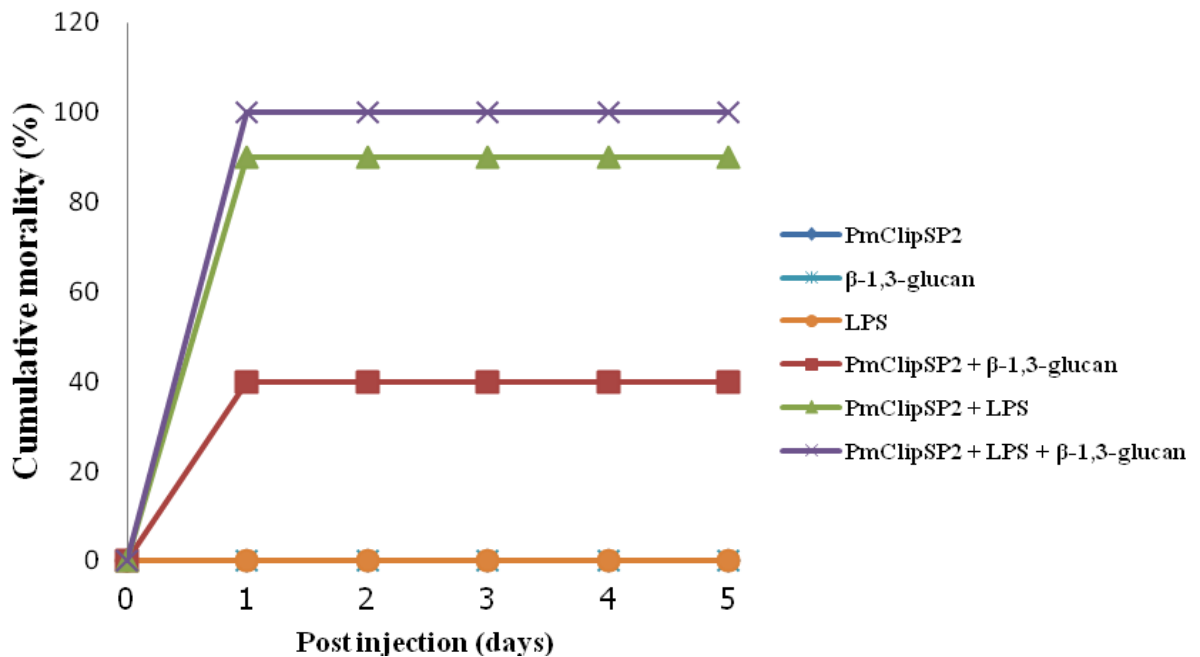
**Figure 3.11** The number bacterial count in the hemolymph of *PmClipSP2*-silenced shrimp. Shrimp were injected with either *PmClipSP2* dsRNA or GFP dsRNA (control) following *V. harveyi* injection. Shrimp hemolymph were collected to perform the total plate count. The data is shown as the mean  $\pm$  standard deviation. Means with the same lower case letters (above each bar) are not significantly different at the  $p < 0.05$  level.

Interestingly, we found that knockdown of the *PmClipSP2* gene followed by injection with LPS, resulted in shrimp death within 3-24 h. On the other hand, when the clip-SPs (*PmPPAE1*, *PmPPAE2*, and *PmClipSP1*)

families or other genes in proPO activating system from *P. monodon* were suppressed and then injected with LPS, shrimp did not die. These results suggest that *PmClipSP2* might be important in neutralization of the effect of LPS on shrimp survival.

#### **3.4.6 Lethal effect of LPS and $\beta$ -1,3-glucan on the *PmClipSP2* silenced shrimp**

To further investigate the effect of LPS and  $\beta$ -1,3-glucan in the *PmClipSP2* silenced shrimp, shrimp (~4 g) were injected with either *PmClipSP2* dsRNA or microbial cell wall (LPS and  $\beta$ -1,3-glucan). The experiments were divided into 6 groups. Group 1, shrimp were injected with *PmClipSP2* dsRNA. Group 2, shrimp were injected with LPS. Group 3, shrimp were injected with  $\beta$ -1,3-glucan. Group 4, shrimp were injected with *PmClipSP2* dsRNA and LPS. Group 5, shrimp were injected with *PmClipSP2* dsRNA and  $\beta$ -1,3-glucan. Group 6, shrimp were injected with *PmClipSP2* dsRNA,  $\beta$ -1,3-glucan and LPS. The mortality was recorded for 5 days post injection. The results showed that injection of only *PmClipSP2* dsRNA or microbial cell wall had no effect on shrimp mortality. While mortality rate of *PmClipSP2* silenced shrimp that were injected with  $\beta$ -1,3-glucan or LPS or both  $\beta$ -1,3-glucan and LPS were 40%, 90% and 100%, respectively (Figure 3.12).



**Figure 3.12** Cumulative mortality of shrimp after injection with *PmClipSP2* dsRNA or microbial cell wall (LPS or  $\beta$ -1,3-glucan) or both. Shrimp mortality was recorded for 5 days. The cumulative mortality (%) in each experimental group (8–10 shrimp/group) is presented as the mean  $\pm$  standard deviation, and is derived from triplicate independent experiments.

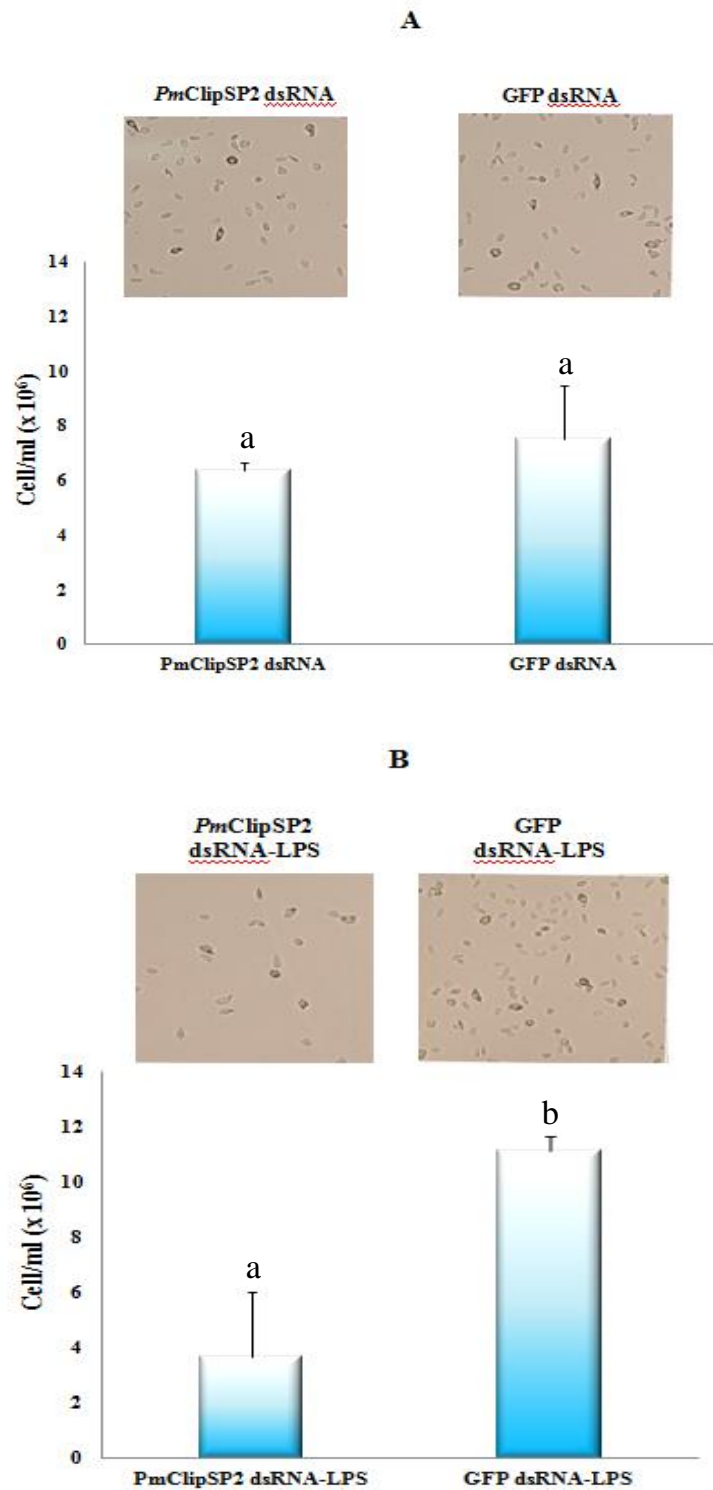
### 3.4.7 Total hemocytes count analysis in *PmClipSP2*-silenced shrimp

From previous result, *PmClipSP2*-silenced shrimp died from injection with LPS. Therefore, to further investigate the cause of death, we determined the total hemocytes in *PmClipSP2*-silenced shrimp.

For total hemocyte counts in *PmClipSP2*-silenced shrimp, shrimp were twice injected with either *PmClipSP2* dsRNA or GFP dsRNA (control). At 24 h after second injection, shrimp hemolymph were collected and analyzed. At the same time, the other groups of shrimp that twice injected with dsRNA were again injected with LPS (10  $\mu$ g LPS/1 g of shrimp). The result



showed that the number of hemocytes in *PmClipSP2* silenced shrimp with no LPS injection were not different from the control (Figure 3.13A) but in the *PmClipSP2* silenced group that injected with LPS, the number of hemocytes in *PmClipSP2*-silenced shrimp were significantly decreased when compare with the control (Figure 3.13B).



**Figure 3.13** Total hemocytes counts in *PmClipSP2*-silenced shrimp. Shrimp were divided into two groups: silenced shrimp with no LPS injection (A) and silenced shrimp injected with LPS (B). The numbers of hemocytes were determined by light

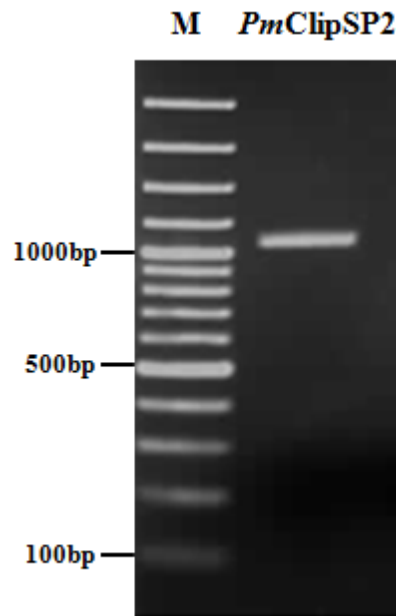
microscope. The data is shown as the mean  $\pm$  standard deviation. Means with the same lower case letters (above each bar) are not significantly different at the  $p < 0.05$  level.

### **3.5 Recombinant expression of a Clip domain serine proteinase 2 of (*PmClipSP2*) *Penaeus monodon* in the *E. coli* system**

To characterize the function of *PmClipSP2*, a mature protein of *PmClipSP2* gene was cloned and expressed in *E. coli* expression system using pET-28b(+) as an expression vector.

#### **3.5.1 Amplification of a mature protein of the *PmClipSP2***

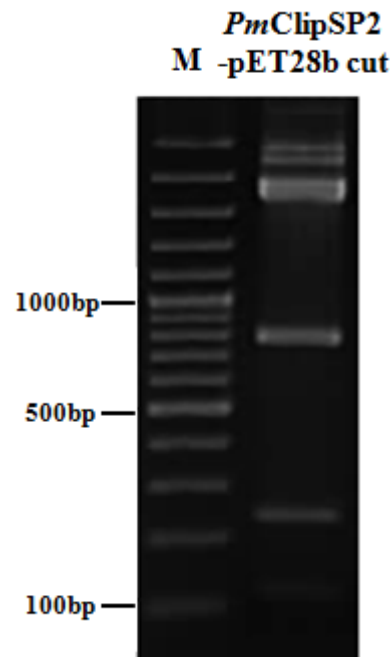
DNA fragments encoding a mature protein (m*PmClipSP2*) with hexa His-tag sequences at the C-terminus were amplified by using primers *PciISP2-F* and *XhoISP2-R*. After amplification, a single band of the protein was observed on agarose gel electrophoresis. The size of m*PmClipSP2* was approximately 1,032 bp (Figure 3.14). The amplified product was cloned and sequenced.



**Figure 3.14** Amplification of a mature protein of the *PmClipSP2* gene and PCR product was analyzed by 1.2% agarose gel electrophoresis. Lane M is GeneRuler™ 100 bp DNA ladder.

### 3.5.2 Construction of the recombinant plasmid pET-28b(+)-SP2

After DNA sequence was analyzed, the mature *PmClipSP2* gene was sub-cloned into an expression plasmid pET-28b(+) at the *PciI* and *XhoI* sites fused with six His encoded nucleotides at the C terminus. After that the recombinant plasmid pET-28b(+)-SP2 was transformed into *E. coli* JM109. The recombinant plasmid was extracted and confirmed by restriction enzyme (*PciI* and *XhoI*) digestion. The recombinant plasmid screening was detected by agarose gel electrophoresis (Figure 3.15).

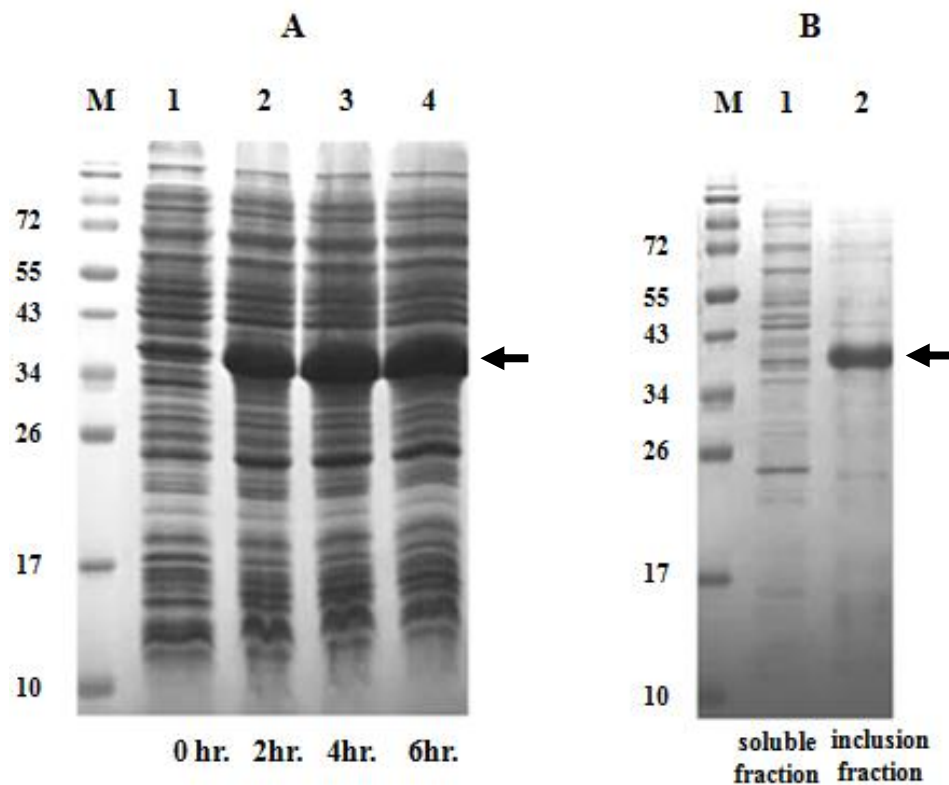


**Figure 3.15** Screening and detection of the recombinant plasmid. The mature *PmClipSP2* (m*PmClipSP2*) in pET28b(+) was digested with *Pci*I and *Xho*I ( Lane 1) and analyzed by 1.2% agarose gel electrophoresis. Lane M is a GeneRuler™ 100 bp DNA ladder marker.

### 3.5.3 Expression of the recombinant *PmClipSP2* protein

After the corrected clone was selected, *E. coli* Rosetta (DE3) pLysS cells were used to transform the plasmid for protein expression. After that at 37 °C, the single colony of transformed cells were grown in LB medium that were contained with kanamycin until they grew approximately an OD<sub>600</sub> of 0.6. For induction of expression, 1 mM IPTG was used for this condition and then harvested the cultured cells at 0, 2, 4, and 6 hr after IPTG addition. The induction was detected by using 12.5% SDS-PAGE gel and the Coomassie brilliant blue for staining of the gels. The protein was determined after 2 h of

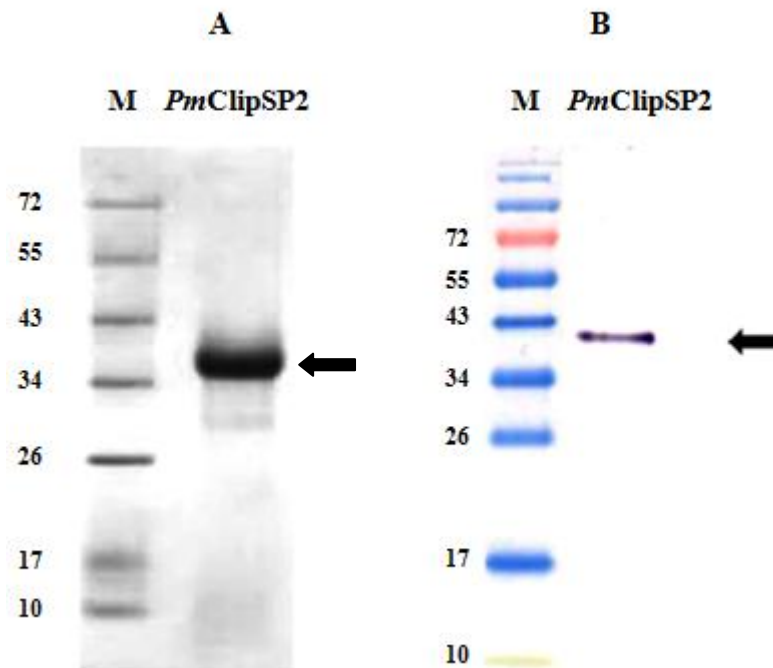
IPTG induction and evenly increased following induction period (Figure 3.16A). The recombinant protein was expressed the highest at 6 hr after induction. The 12.5 % SDS-PAGE was used to analyze both the supernatant and pellet fractions. The results showed that the protein with an expected molecular mass of 38.4 kDa was only expressed in the insoluble fraction (Figure 3.16B). Therefore, this condition was applied for a large-scale expression of the recombinant *mPmClipSP2*.



**Figure 3.16** Recombinant protein expression of *PmClipSP2* (*rPmClipSP2*). The *rPmClipSP2* was expressed after IPTG induction at 0, 2, 4 and 6 hr, respectively (A). Fractional analysis of the expressed protein showed that the protein was mainly expressed in inclusion body (B). Lane M is PageRuler™ prestrained protein ladder (Fermentus).

### **3.6 Purification of the recombinant proteins**

The recombinant *PmClipSP2* protein (*rPmClipSP2*) was purified from inclusion bodies by using Ni-NTA affinity chromatography. Dialysis step was used to refold the purified protein by removing urea. The purity of the recombinant protein was determined by using 12.5% SDS-PAGE gel (Figure 3.17A). Determination of *rPmClipSP2* protein was analyzed by western blot using anti-His-tag antibody. The result showed a major protein band, which corresponded to *PmClipSP2* with the predicted molecular mass of 38.4 kDa (Figure 3.17B).

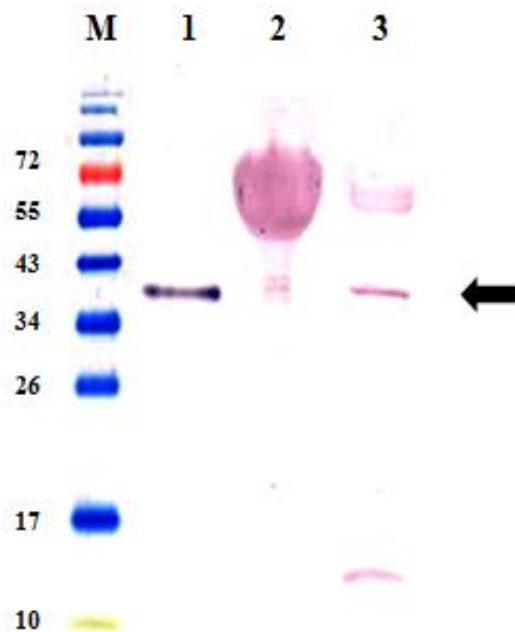


**Figure 3.17** Purification of *rPmClipSP2* protein by using Ni-NTA affinity chromatography analyzed onto 12.5 % SDS-PAGE gel (A) and western blot analysis using anti-His-tag antibody (B). The arrowhead indicates the expected *rPmClipSP2* that was ~38.4 kDa. Lane M is PageRuler™ prestrained protein ladder (Fermentus).

To determine endogenous *PmClipSP2* expression in hemocytes of shrimp, hemolymph were collected from normal shrimp and then kept in anticoagulant solution. Twenty  $\mu\text{g}$  of hemocyte lysate (HLS) and 100  $\mu\text{g}$  of cell-free plasma proteins were loaded into 12.5% SDS-PAGE gel and then transferred into nitrocellulose membrane. The rabbit polyclonal anti-*mPmClipSP2* antisera was used as primary antibody. The *rPmClipSP2* protein was used as control. The result showed one band of protein, which correlated to the predicted molecular mass of the *rPmClipSP2* protein (38.4 kDa).



The protein was determined in hemocytes but not in the cell-free plasma (Figure 3.18).



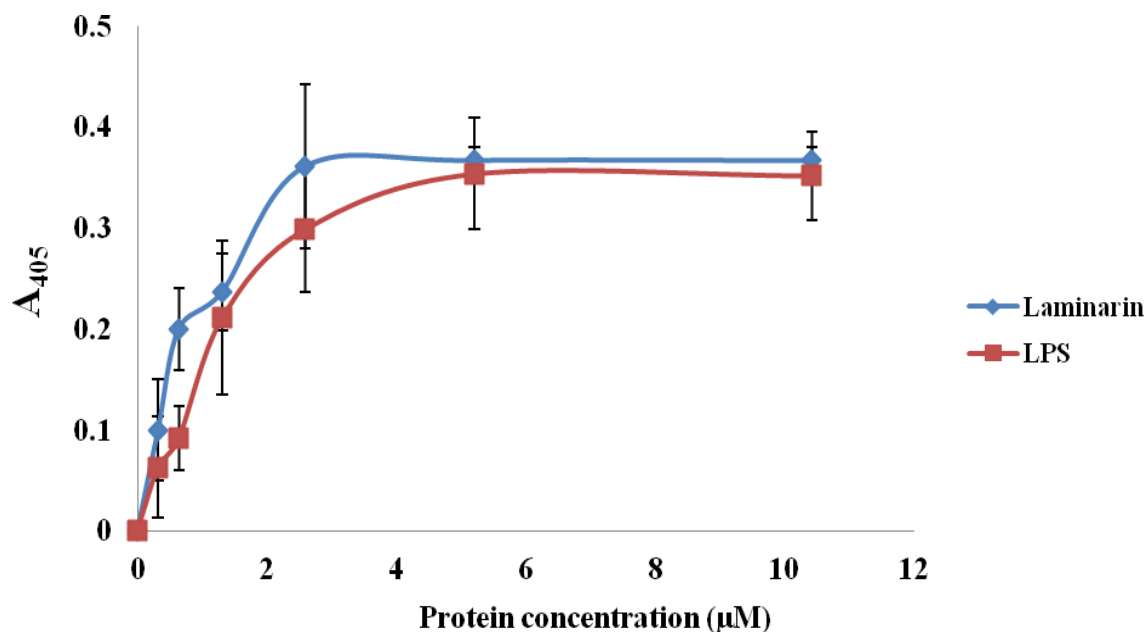
**Figure 3.18** Western blot analysis of hemocyte proteins or hemocyte lysate (HLS), cell-free plasma proteins and *rPmClipSP2* protein (control): Lane 1 is *rPmClipSP2* (1  $\mu$ g), lane 2 is cell-free plasma proteins (100  $\mu$ g), and lane 3 is HLS (20  $\mu$ g). The arrowhead indicates a protein corresponded to a predicted *rPmClipSP2* protein (38.4 kDa). The protein were analyzed by 12.5% SDS-PAGE and transferred to nitrocellulose membrane and detected by using anti-His-tag antibody. Lane M is PageRuler™ prestrained protein ladder (Fermentus).

### 3.7 Functional characterization of the recombinant *PmClipSP2*

The purified *rPmClipSP2* protein was assayed for the biological activity such as the binding activity, activation of PO activity, and proteinase activity.

### 3.7.1 Binding activity assay of *rPmClipSP2* protein

Enzyme-linked immunosorbent assay (ELISA) was performed to examine the binding of *rPmClipSP2* to  $\beta$ -1,3glucan or LPS. We coated  $\beta$ -1,3-glucan or LPS and then added various concentration of *rPmClipSP2* protein into each well of a microtiter plate. The bounded *rPmClipSP2* was detected by using the anti-*mPmClipSP2* antibody. The result showed that the *rPmClipSP2* could bind to  $\beta$ -1,3-glucan and LPS. The apparent dissociation constant ( $K_d$ ) of the *rPmClipSP2* to  $\beta$ -1,3-glucan and LPS were  $1.5 \times 10^{-6}$  and  $3.06 \times 10^{-6}$ , respectively (Figure 3.19). The dissociation constant ( $K_d$ ) was calculated by using saturation curve fitting according to the one-site binding model. The result supported that *rPmClipSP2* was a pathogen receptor proteins (PRPs) that could bind specifically to both  $\beta$ -1,3glucan and LPS.

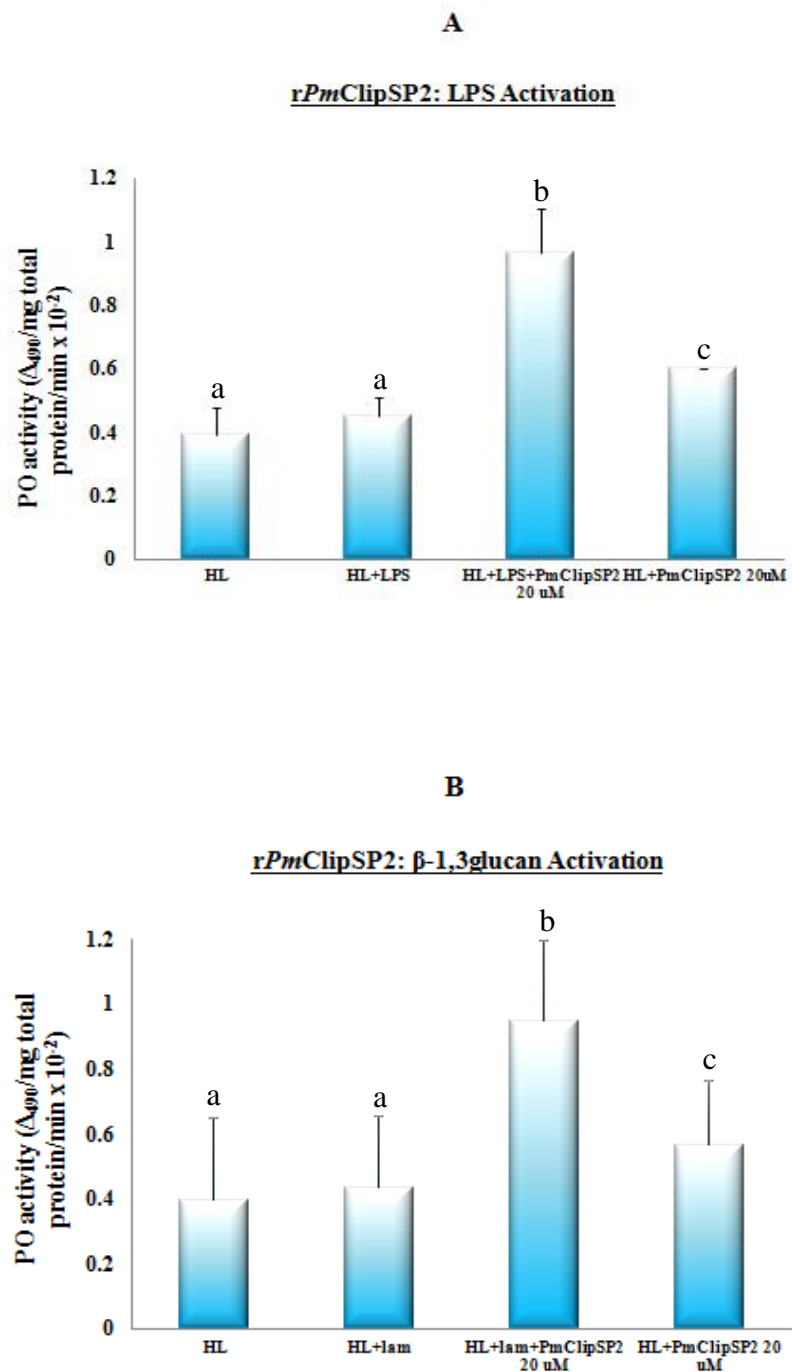


**Figure 3.19** Binding assay of the *rPmClipSP2* protein to the two microbial cell wall components, laminarin ( $\beta$ -1,3-glucan) and LPS. The result show the quantitative binding of *rPmClipSP2* (0 to 10  $\mu$ M) to immobilized LPS or beta-1,3-glucan, as detected by ELISA. Data are shown as the mean + 1 SD of three individual experiments. The data were curve fitted using a single-site binding model with  $R^2 = 0.92$  for laminarin ( $K_d = 1.5 \pm 0.17 \times 10^{-6}$ ) and  $R^2 = 0.96$  for LPS ( $K_d = 3.06 \pm 0.87 \times 10^{-6}$ ).

### 3.7.2 Activation of PO by *rPmClipSP2* protein

To study PO activation by the *rPmClipSP2* protein, hemolymph from normal shrimp were incubated with *rPmClipSP2* protein and activator (LPS or  $\beta$ -1,3-glucan). L-dopa (L-3,4-dihydroxyphenylalanine) was used as substrate. After that PO activity was detected at  $A_{490}$  nm. The results showed that adding of *rPmClipSP2* protein and LPS or  $\beta$ -1,3-glucan could significantly increased the total PO activity. The result supported that *rPmClipSP2* could activate

proPO system in the presence of LPS or  $\beta$ -1,3-glucan (Figure 3.20A and 3.20B).

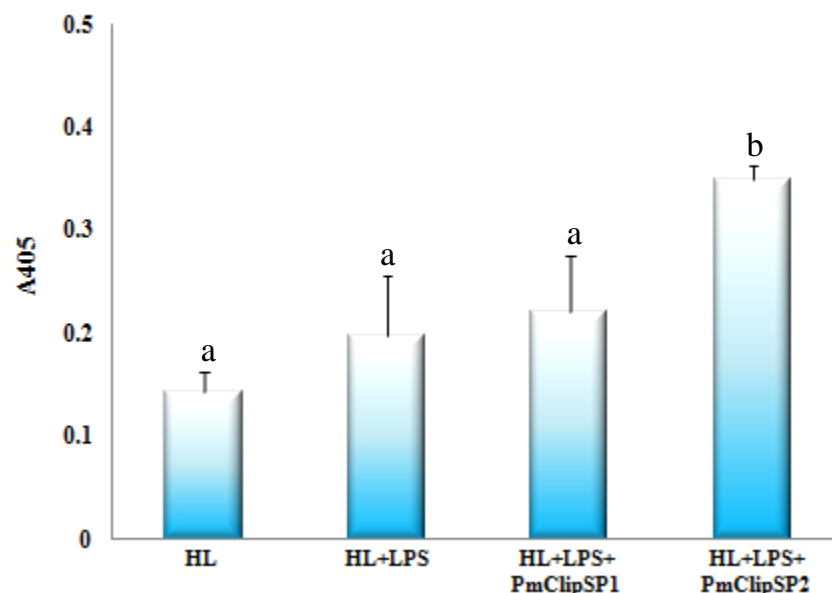


**Figure 3.20** Activation of PO by *rPmClipSP2* protein. The *rPmClipSP2* protein and activator (LPS or  $\beta$ -1,3-glucan) were added into normal shrimp hemolymph. (A) and

(B) showed activation of PO by adding LPS and  $\beta$ -1,3-glucan, respectively. L-dopa was used as substrate. PO activity was reported as  $\Delta_{A490}/\text{mg}$  total protein/min. The data are shown as the mean  $\pm$  1 SD (error bars) and are derived from three independently replicated experiments. Means with a different lower case letter (above each bar) are significantly different at the  $p < 0.05$  level.

### 3.7.3 Proteinase activity assay

Proteinase activity of the recombinant *PmClipSP2* protein (*rPmClipSP2*) was determined. Hemolymph from normal shrimp were collected and then incubated with *rPmClipSP2* and LPS. Finally N-Benzoyl-Phe-Val-Arg-p-nitroanilide-hydrochloride (B-7632) was used as substrate. The enzymatic activity was detected at  $A_{405}$  nm. The results showed that adding *rPmClipSP2* to the reaction mixture could increase proteinase activity while adding *rPmClipSP1* (control) did not affect the proteinase activity (Figure 3.21).



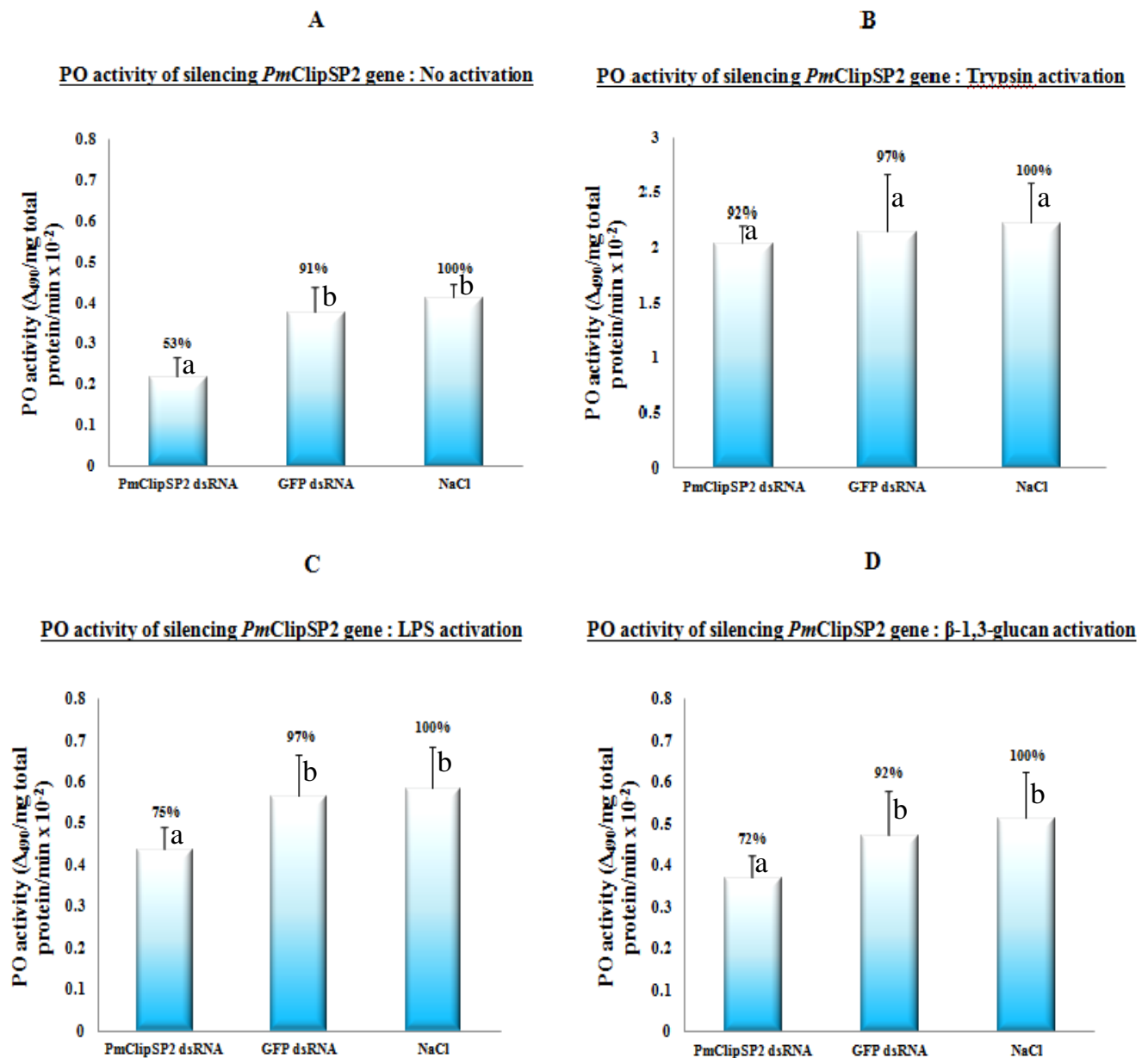
**Figure 3.21** Proteinase activity assay of the *rPmClipSP2* protein. Hemolymph were incubated with *rPmClipSP2* protein and LPS. The *rPmClipSP1* protein was used as control. N-Benzoyl-Phe-Val-Arg-p-nitroanilide-hydrochloride (B-7632) was used as

substrate. The enzymatic activity was detected at  $A_{405}$  nm. The data are shown as the mean  $\pm$  1 SD (error bars) and are derived from three independently replicated experiments. Means with a different lower case letter (above each bar) are significantly different at the  $p < 0.05$  level.

### **3.8 *In vitro* activation of PO system in hemolymph of knockdown shrimp**

To examine the involvement of  $\beta$ -1,3-glucan and LPS in the activation of *PmClipSP2* in the shrimp proPO system, dsRNA-mediated gene knockdown of *PmClipSP2* transcripts was performed. Hemolymph was collected from the knockdown shrimps at 24 h after the second dsRNA injection. Then, the total protein in hemolymph was incubated with trypsin (exogenous proteinase that directly activates the proPO to PO),  $\beta$ -1,3-glucan or LPS. PO activity was detected by using L-DOPA as the substrate and measured at 490 nm. The result showed that in the unstimulated-knockdown experiment (Figure 3.22A), PO activity in *PmClipSP2*-silenced shrimp was significantly decreased by 47% when compared with NaCl-injected shrimp (control groups). In the stimulated-knockdown experiment with trypsin (Figure 3.22B), PO activity in *PmClipSP2*-silenced shrimp was returned back to same level of PO activity in control groups (GFP dsRNA and NaCl). However, after activation with LPS or  $\beta$ -1,3-glucan, PO activities in *PmClipSP2*-silenced shrimp were still significantly decreased by 25% and 28%, respectively (Figure 3.18C and D), when compared with control groups. The results indicated that LPS and  $\beta$ -1,3-glucan could activate the proPO system. These results imply that

*PmClipSP2* may regulate the PO activity by LPS- or  $\beta$ -1,3-glucan-dependent activation.



**Figure 3.22** Activation of PO in hemolymph of knockdown shrimp. The hemolymph of knockdown shrimp were collected and divided into four reactions: (A) not added activator, (B) added trypsin, (C) added LPS, and (D) added  $\beta$ -1,3-glucan. PO activity was reported as  $\Delta A_{490}/\text{mg total protein}/\text{min}$ . Experiments were repeated three times and the data is shown as the mean  $\pm$  standard deviation. Means with the same lower case letters (above each bar) are not significantly different at the  $p < 0.05$  level.

# CHAPTER IV

## DISCUSSIONS

### **4.1 Sequence Characterization of a clip domain serine proteinase 2 (*PmClipSP2*) from black tiger shrimp *Penaeus monodon***

Clip domain serine proteinases (clip-SPs) in invertebrates have been reported to be involved in immune responses and embryonic development (Jiang and Kanost, 2000; Krem and Cera, 2002). The first description of clip-SP is identified as a pro-clotting enzyme that functions in blood clotting system of horseshoe crab, *Tachypleus tridentatus* (Muta et al., 1990). There were reports on the functions of Clip-SPs in the melanin synthesis by proPO system (Jiang et al., 2000; Jang et al., 2008; Charoensapsri et al., 2009) and in the synthesis of antimicrobial peptides (Iwanaga, 1993; Kawabata et al., 1996; Jiang and Kanost, 2000; Jang et al., 2008). Clip-SPs consist of two major domains, the clip domain at N-terminus and the serine proteinase domain at C-terminus. The clip domain consists of three disulfide bonds and it is always found at N-terminus of the SP domain (SP) or SP homologues (SPH).

Previously, several clip-SPs and clip-SPH were found in *P. monodon* EST database (<http://pmonodon.biotec.or.th>). *P. monodon* clip-SPs are divided into four types (*PmPPAE1*; FJ595215:(Charoensapsri et al., 2009), *PmPPAE2*; FJ62068: (Charoensapsri et al., 2011); *PmClipSP1*; FJ620688: (Amparyup



et al., 2010) and *PmClipSP2*; FJ620687) and clip-SPHs are divided into three types (*PmMasSPH1*; DQ455050: (Amparyup et al., 2007), *PmMasSPH2*; FJ620686 and *PmMasSPH3*; FJ620689).

In this research, sequence analysis of uncharacterized clip-SP of *P. monodon* named *PmClipSP2* showed that *PmClipSP2* exhibited the similarity to a melanization protease-1 (52%) of *Drosophila melanogaster*. Moreover, the previous study showed that transcripts of *PmClipSP2* is up-regulated in hemocytes at 3 hr after a pathogenic bacterial *Vibrio harveyi* infection using real time PCR analysis (Amparyup et al., unpublished data). These results indicated that *PmClipSP2* is likely to be involved in shrimp immune responses. A full-length cDNA of *PmClipSP2* was analyzed. The *PmClipSP2* consists of signal peptide and the clip domain at N-terminus and the serine proteinase domain at C-terminus. This domain organization of *PmClipSP2* is similar to clip-SP family in arthropods (Jiang et al., 2000; Jang et al., 2008).

From multiple amino acid sequence alignments of *PmClipSP2* with other arthropod clip-SPs, *PmClipSP2* is similar to *P. monodon* ClipSP1 (74%), *P. monodon* PPAE1 (62%), *P. monodon* PPAE2 (60%), *H. diomphalia* PPAF-I (54%), *D. melanogaster* MP1 (52%), and *M. sexta* PAP1 (51%) by conservation of six cysteines for forming three disulfide bonds at the clip domain (N-terminus) and conservation of three amino acid residues (His151,

Asp216, and Ser314) within its active site of the SP domain that is important for proteolytic activity (Phillips et al., 1992). This data suggested that *PmClipSP2* may be an active clip-SP.

However, from blast result, *PmClipSP2* is one of trypsin-like serine proteinase super families and is highly similar (74%) to clip-SP1 (*PmClipSP1*) of *P.monodon*. The phylogenetic tree indicated that the *PmClipSP2* was closely related to *PmClipSP1* but the percent identity (56%) of amino acid sequence between *PmClipSP1* and *PmClipSP2* was very low. This implies that both proteins might play different biological role in shrimp immunity. Moreover, from the phylogenic tree, *PmClipSP2* is located within the cluster of arthropod clip-SPs, supporting that *PmClipSP2* is an enzymatic clip-SP.

## **4.2 Effect of the *PmClipSP2* gene silencing by RNA interference**

RNA interference (RNAi) technique has been used to analyze the function of genes in immune system of several arthropod species (Reynolds et al., 2008). Double-stranded RNA (dsRNA) was used as a tool of gene silencing for gene functional study. In *D. melanogaster*, using RNAi to suppress two clip-SPs, showed that MP1 and MP2/SP7 were important in melanization cascade (Castillejo-Lopez and Hacker, 2005; Tang et al., 2006). In addition, the functions of three clip-SP genes from *P. monodon* (*PmPPAE1*, *PmPPAE2* and *PmClipSP1*) were already reported. Gene silencing of *PmPPAE1*, and

*PmPPAE2* but not *PmClipSP1* could decrease the total PO activity, indicating that, *PmPPAE1* and *PmPPAE2* are proteinases that required for PO activation in shrimp (Charoensapsri et al., 2009; Charoensapsri et al., 2011), while *PmClipSP1* was not involved in the PO activation. However, Gene silencing of *PmClipSP1* resulted in high mortality post *V.harveyi* injection suggested that *PmClipSP1* is involved in defense against *V.harveyi* infection (Amparyup et al., 2010). In this study, suppression of *PmClipSP2* gene resulted in a decrease in the total PO activity by 39%. Therefore, *PmClipSP2* plays important role in proPO system of shrimp.

In the previous reports, decreased PO activity in crayfish *P. leniusculus* using gene silencing experiment caused an increase in the number of a bacterium *A. hydrophila* (Liu et al., 2007). In addition, the gene silencing of two clip-SPs (*PmPPAE1* and *PmPPAE2*) and two proPOs (*PmproPO1* and *PmproPO2*) of shrimp *P. monodon* resulted in high mortality after *V.harveyi* challenge (Amparyup et al., 2009; Charoensapsri et al., 2009; Charoensapsri et al., 2011). This result suggested that the PO system is important in the shrimp immune defense against bacterial infection.

Moreover, induction of *in vitro* PO from *M.sexta* could reduce the growth in bacteria and fungi (Zhao et al., 2007) and activation of *in vitro* PO from crayfish *P. leniusculus* has affected in bacterial growth (Cerenius et al., 2010). In addition, *in vitro* PO activity could aggregate bacteria (Zhao et al.,

2007; Kan et al., 2008). In this study, it was shown that *PmClipSP2* is an essential factor in immune defense against *V. harveyi* infection. Silencing of the *PmClipSP2* gene strongly increased the shrimp mortality rate (100%) post *V. harveyi* injection within 24 hr. For bacterial counts in hemolymph post *V. harveyi* infection, the number of bacteria was highly increased in *PmClipSP2*-silenced shrimp. This is similar to the result of suppression of the two proPOs (*PmproPO1* and *PmproPO2*) that strongly increased the shrimp mortality rate post *V. harveyi* infection. Surprisingly, injection of LPS /Glucan into *PmClipSP2*-silenced shrimp caused rapid death whereas no effect was observed in *PmproPO1*-, *PmproPO2*- and *PmPPAE1*-silenced shrimp.

In crayfish, each LPS or  $\beta$ -1,3-glucan enters the proPO activating system through different pathway. When LPS activating pathway was inhibited, the proPO system could be activated by adding  $\beta$ -1,3-glucan (Soderhall., 1984). As the same result in *P.monodon*, the proPO system could be activated by adding LPS or  $\beta$ -1,3-glucan.

Previously, it has been shown that LPS causes a decrease in the number of circulating hemocytes in crustaceans (Lorenzon et al., 1999). In crayfish *P. leniusculus*, a cytokine-like factor in invertebrates called astakine that contains prokineticin (PK) domain is involved in blood cell differentiation and hematopoiesis response. Silencing of astakine gene did not significantly decrease the amount of hemocytes, while hemocyte numbers of

astakine-silenced crayfish that injected with LPS were dropped when compared with control (Soderhall et al., 2005). In this study, the numbers of hemocytes were not significantly decreased in *PmClipSP2*-silenced shrimp. However, injection of LPS into *PmClipSP2*-silenced shrimp substantial decrease in the number of hemocytes that resulted in shrimp death. The results imply that *PmClipSP2* might be responsible for LPS binding and could reduce LPS toxicity in shrimp.

### **4.3 Recombinant expression of *PmClipSP2* in the *E. coli* system, binding to bacterial cell wall components and PO activation**

To study the biological function of *PmClipSP2* protein, recombinant protein was produced by *E.coli* expression system (Baneyx, 1999). This system has many advantages, e.g., easy for protein synthesis and production, high capacity, low expense and high achievement (Cabrita et al., 2004). Nevertheless, there is restrictive weakness of this prokaryotic expression system for the expression of protein from eukaryote, e.g., the incapability to manage many post-translation modifications and protein expression to perform as inclusion body in the cells (Swartz, 2001). However, the efficiency to produce the recombinant protein by *E.coli* system has many factors such as promoter strength, gene dosage, mRNA stability, the efficiency of translation initiation, copy number vectors, codon usage, and temperature (Baneyx, 1999; Swartz, 2001; Jonasson et al., 2002). In this study, *E. coli* strain Rosetta (DE3)

and pET28b (+) were chosen as expression host and expression vector, respectively to produce the recombinant protein of *PmClipSP2*.

In this study, the mature recombinant *PmClipSP2* protein was successfully expressed, purified and refolded in *E. coli* expression system. Western blot analysis using antibody against *PmClipSP2*, showed that *PmClipSP2* was localized in hemocytes but not in the plasma of shrimp. Similar result was observed in a shrimp prophenoloxidase activating enzymes (*PmPPAE1*) that localizes in hemocyte (Charoensapsri et al., 2009). Previously, in *Manduca sexta*, the hemolymph proteinase-14 (proHP14) that is important in serine proteinase cascade of proPO system, could bind with LPS, LTA, DAP-PG, Lys-PG, laminarin, and  $\beta$ GRP1 (Wang and Jian, 2010).

In *P.monodon*, the masquerade-like serine proteinase homologue (*PmMasSPH*) interacts with lipopolysaccharide (LPS) and the dissociation constant (Kd) is  $2.51 \times 10^{-5}$  (Jitvaropas., 2009). Moreover, the recombinant *PmLGBP* protein (*rPmLGBP*) from *P.monodon* could interact with  $\beta$ -1,3-glucan or LPS (Amparyup et al., 2012). These reports are consistent with the result in *rPmClipSP2* protein that could bind with  $\beta$ -1,3-glucan or LPS. The dissociation constant (Kd) for the binding of *rPmClipSP2* protein to  $\beta$ -1,3-glucan and LPS are  $1.50 \times 10^{-6}$  and  $3.06 \times 10^{-6}$ , respectively. In human, thrombin, the important enzyme in coagulation cascade, binds to LPS and prevents lipopolysaccharide-induced shock from *P. aeruginosa* sepsis. This is

the additional role of thrombin in host defense (Papareddy et al., 2010). In our study, *rPmClipSP2* protein may interact with  $\beta$ -1,3-glucan and LPS and neutralize their toxicity. In addition, in the presence of either  $\beta$ -1,3-glucan or LPS, *rPmClipSP2* significantly enhances the total PO activity in hemolymph by 40% and 46%, respectively. This is similar to the result in *rPmLGBP* protein that increased the PO activity by activation of either  $\beta$ -1,3-glucan (72%) or LPS (88%) (Amparyup et al., 2012).

Our *in vivo* and *in vitro* experiments supported that *PmClipSP2* is a clip-SP participated in proPO activating system in *P.monodon* shrimp. It could interact with  $\beta$ -1,3-glucan or LPS to activate the serine proteinase cascade. Moreover *PmClipSP2* is important for the shrimp immune defense against *V. harveyi* infection, and it probably involves in the hemocyte homeostasis by neutralizing LPS toxicity. However, proPO system is a complicated biochemical process and the molecular mechanisms that regulate remain largely unknown. Therefore, continued effort to study the function of proteins in the proPO system is important for an understanding of the immunity in shrimp.

# CHAPTER V

## CONCLUSIONS

1. A clip-domain serine proteinase from *Penaeus monodon*, named *PmClipSP2*, was characterized. A full-length cDNA of *PmClipSP2* consisted of 1,317 bp encoding a 369 amino acid protein. The mature protein has a predicted molecular mass of 40.18 kDa with the isoelectric point (pI) of 5.14.
2. Sequence analysis revealed that *PmClipSP2* is composed of clip domain (N-terminal) with six cysteine residues forming three disulfide bonds and serine proteinase domain (C-terminal) containing three amino acid residues (His151, Asp216, and Ser314).
3. Sequence comparison using BLASTX program revealed that the deduced amino acid of *PmClipSP2* had a similarity of 74%, 62%, 60%, 54%, 52% and 51% to those of *P. monodon* ClipSP1, *P. monodon* PPAE1, *P. monodon* PPAE2, *H. diomphalia* PPAF-I, *D. melanogaster* MP1 and *M. sexta* PAP1, respectively.
4. Suppression of *PmClipSP2* by injection of double-stranded RNA (dsRNA) corresponded to the *PmClipSP2* gene can specifically silence the *PmClipSP2* gene but not other clip-SPs or clip-SPHs in *P. monodon*.



5. Silencing of *PmClipSP2* gene resulted in a significant decrease in the total PO activity. In addition, a high mortality rate and increase in bacterial number in the hemolymph were observed in the *PmClipSP2*-silenced shrimp followed *Vibrio harveyi* infection. These results suggest that *PmClipSP2* is directly involved in the proPO system in shrimp and is important in defense against bacterial infection.
6. Injection of bacterial cell wall components (LPS and  $\beta$ -1,3-glucan) into the *PmClipSP2*-silenced shrimp also led to a high mortality and a significantly decreased in the number of hemocytes. These results imply that *PmClipSP2* might be involved in neutralizing the LPS toxicity, thus, controlling hemocyte homeostasis in the LPS-injected shrimp.
7. The recombinant protein of *PmClipSP2* protein was successfully expressed in *E. coli*. The recombinant protein could bind to LPS and  $\beta$ -1,3-glucan and activate phenoloxidase (PO) activity and has a proteolytic activity.
8. Taken together, our result demonstrates that *PmClipSP2* is a clip-SP that directly binds to bacterial cell wall components and activates in the proPO-activating system and it is important in the shrimp immune defense against bacterial infection.

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## **APPENDICES**

**APPENDIX A**

## 1. Nucleotide sequence and deduce amino acid sequence of Clip domain serine proteinase 2 (*PmClipSP2*)

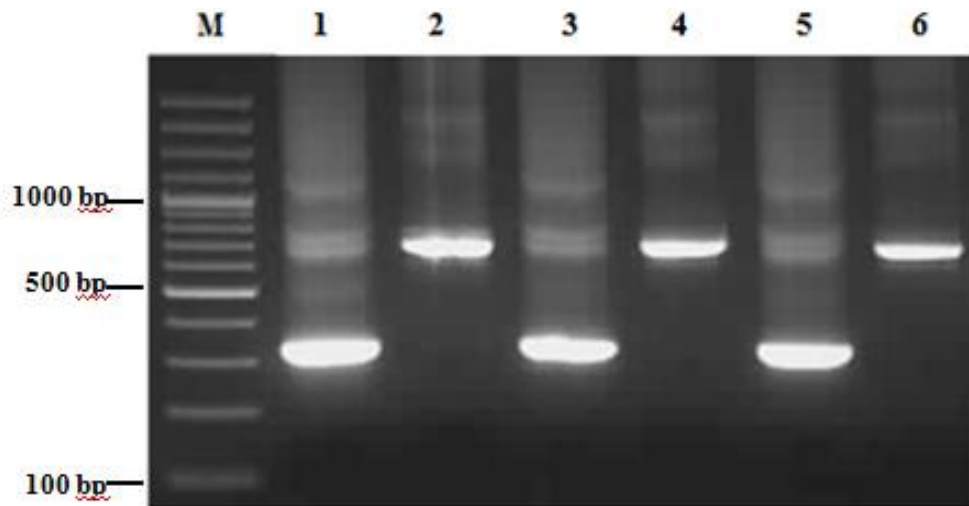
GAATTAACCGAGAAACACGTGTAAGGATACATCTCTGAAGTGCATCACTGGTTTCGAAAA  
 TGAACAAACAGAGACCAAGCACAAAGCCCGGTGGCGTTGGTCTTCACTGCTCTCCTGTTGT  
 M N K Q R P S T S P V A L V F T A L L L  
 TTGCTCACGGGGCGGCCAGCCAAGGAACAGGATGTCACCGAGACGAATGCACCTTGCTCA  
 F A H G A A S Q G T G C H R D E C T L L  
 CTGACTGCCCAAGCTCCTCGATCTCCTTAAGAACCCAACGCTGGACAGCATCGGGGAAC  
 T D C P K L L D L L K N P T L D S I G E  
 TACAGGCAGCTACGTGTTTCATTAACAAGCGACAGCCATGGGTGTGCTGTCCCGCCCTG  
 L Q A A A T C F I N K R Q P W V C C P A P  
 TCACCGAGCTCCTAAAGTCATCAAGGAATCCCTCCTGCCTCCGAACTGCGGCCTTGTGG  
 V T E P P K V I K E S L L P P N C G L V  
 GGGACGTCAGAGTGGTAGGAGGCGAAGACGCCCAATCGACGCCTATCCTTGAAGGCAG  
 G D V R V V G G E D A P I D A Y P W K A  
 TTCTGGGATATCGAATTGGAGGTTTACCCGAAATACACTTTGAATGCGGAGGTTTCAGTCA  
 V L G Y R I G G L P E I H F E C G G S V  
 TCAACGAGAGATATATCATGACGGCTGCTCACTGCGTCAATGCCAATATACTGAATGAGC  
 I N E R Y I M T A A H C V N A N I L N E  
 GAGAACTCGAACTGGCCGTAATTCGACTGGGCGAATGGGACCTCTCCACGAAATGGACT  
 R E L E L A V I R L G E W D L S T E M D  
 GCACCAACACCAGCAATGGAAGCCGGTCTGTGCTCCTCCGGTCCAGGATTTTGACTTTG  
 C T N T S N G S R F C A P P V Q D F D F  
 AGGAAGTTATTGAACACCCATCCTACGACAACCGGACGCTCTTCTCAGATGACATCGCTC  
 E E V I E H P S Y D N R T L F S D D I A  
 TGATTGCACTGAGCAAACCAATCAACTTCTGACATCAGCAGGTTTCATCCAACCCGTGT  
 L I R L S K P I N F L T S A G F I Q P V  
 GCCTCCCGCCCGCCGATTTATCCCTGAGCGCCGAGGCCAGGAGCCAAGGAGCGATCGTGG  
 C L P P A D L S L S A E A R S Q G A I V  
 CCGGCTGGGGCGTCACCGAGAAGGGAATCCAGAGCGACAGGCTGCAGCATCTCATCCTGC  
 A G W G V T E K G I Q S D R L Q H L I L  
 CCTTCGTCGAGAACAAGAGTGCAACGAGAGGTACAGAGGCAACCTGGTCGCGGAACAGA  
 P F V E N K E C N E R Y R G N L V A E Q  
 TCTGCATGGGAGGGGAAGCCGGCAAGGACTCCCGCAGGGGGGATTCGGGAGGCCCTCTGA  
 I C M G G E A G K D S R R G D S G G P L  
 TCATGAAAGCAGGATCCGAAAGAGAGGTATCGATGCAAATTTGGGATCGTATCCTACGGTC  
 I M K A G S E R E V S M Q I G I V S Y G  
 CTACGAGTTGCGGCCAGAAAGGATTCCTGGCGTCTATACTTCCGTCAGCCATTATAGGT  
 P T S C G Q K G F P G V Y T S V S H Y R  
 CCTGGGTCGAGGAAACTCTTAGGCCGTAGATAAAGAAAACGGAGAATGGAGGTAAGAAAA  
 S W V E E T L R P \*

**2. Amino acid sequence of recombinant mature *PmClipSP2*, underline showed His tag sequence for using purification.**

MSQGTGCHRDECTLLTDCPKLLDLLKNPTLDSIRELQAATCFINKRQPWCCPAPVTEPPKV  
IKESLLPPNCGLVGDVGVVGGEDAPIDAYPWKAVLGYRIGGLPEIHFECCGGSVINERYIMTA  
AHCVNANILNERELELAVIRLGEWDLSTEMDCTNTSNGSRFCAPPVQDFDFEEVIEHPSYDN  
RTLFSDDIALIRLSKPINFLTSAGFIQPVCLPPADLSLSAEARSQGAIVAGWGVTEKGIQSD  
RLQHLILPFVENKECNERYRGNLVAEQICMGGEAGKDSCRGDSSGGLIMKAGSEREVSMQIG  
IVSYGPTSCGQKGFPGVYTSVSHYRSWVEETLRPHHHHH

**APPENDIX B**

## 1. Synthesis and purification of double strand RNA of *PmClipSP2* and GFP gene



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

Lane 1 : *PmClipSP2* dsRNA before treat with Dnase

Lane 2 : GFP dsRNA before treat with Dnase

Lane 3 : *PmClipSP2* dsRNA after treat with Dnase

Lane 4 : GFP dsRNA after treat with Dnase

Lane 5 : *PmClipSP2* dsRNA after precipitate

Lane 6 : GFP dsRNA after precipitate

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

group	OD <sub>490</sub>	PO activity ( x 10 <sup>-2</sup> )	Average	SD
<i>PmClipSP2</i> dsRNA	0.1095	0.1825	0.1861	0.0295
	0.0954	0.1586		
	0.1302	0.2172		
GFP dsRNA	0.2320	0.3867	0.3955	0.0561
	0.2065	0.3442		
	0.2733	0.4555		
NaCl	0.2160	0.3597	0.3950	0.0756
	0.2061	0.3435		
	0.2890	0.4818		

## **BIOGRAPHY**

Miss Kanyanat promrungreang was born on August 18, 1986 in Prachinburi. She graduated with the degree of Bachelor of Science from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2009. She has studied for the degree of Master of Science at program in Biochemistry, Chulalongkorn University since 2009.