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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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# EXPRESSION AND CHARACTERIZATION OF A SERINE PROTEINASE HOMOLOGUE FROM BLACK TIGER SHRIMP *Penaeus monodon*

**Miss Rungrat Jitvaropas**



**A Dissertation Submitted in Partial Fulfillment of the Requirements**
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**Department of Biochemistry**
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รุ่งรัตน์ จิตวโรภาส: การแสดงออกและลักษณะสมบัติของซีรีนโปรติเนสโฮโมลอกในกุ้งกุลาดำ (EXPRESSION AND CHARACTERIZATION OF A SERINE PROTEINASE HOMOLOGUE FROM BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษา: ศ.ดร. อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม: ผศ.ดร. Paul S. Gross, 188 หน้า.

การหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของยีนซีรีนโปรติเนสโฮโมลอกจากกุ้งกุลาดำ (*Pm*MasSPH) และกุ้งขาว (*Lv*MasSPH) ด้วยเทคนิค Rapid Amplification cDNA End (RACE) พบว่ายีน *Pm*MasSPH ที่สมบูรณ์มีขนาด 1,958 คู่เบส โดยมี open reading frame (ORF) ขนาด 1,572 คู่เบส ซึ่งถูกแปลรหัสเป็นโพลีเปปไทด์ขนาด 523 กรดอะมิโน ในขณะที่ลำดับนิวคลีโอไทด์ที่สมบูรณ์ของ *Lv*MasSPH มีขนาด 1,955 คู่เบส โดยมี ORF ขนาด 1,536 คู่เบส ซึ่งถูกแปลรหัสเป็นโพลีเปปไทด์ขนาด 511 กรดอะมิโน โครงสร้างของซีรีนโปรติเนสโฮโมลอกจากกุ้งกุลาดำและกุ้งขาวประกอบด้วยบริเวณที่มีไกลซีนจำนวนมากและคลิปไดเมนทางด้านปลายอะมิโน และ serine protainase-like domain ด้านปลายคาร์บอกซิล จากการวิเคราะห์เปรียบเทียบลำดับกรดอะมิโนของ *Pm*MasSPH พบว่ามีความเหมือนกับลำดับกรดอะมิโนของโพรฟีนอลออกซิเดสแอคติเวติงแฟกเตอร์ของปู ผึ้งและหนอนไหม 58% 55% และ 53% ตามลำดับ ส่วนลำดับกรดอะมิโนของ *Lv*MasSPH มีความเหมือนกับลำดับกรดอะมิโนของ *Pm*MasSPH 93% และจากการวิเคราะห์ phylogenetic tree พบว่า *Pm*MasSPH และ *Lv*MasSPH มีความสัมพันธ์ใกล้ชิดกับซีรีนโปรติเนสโฮโมลอกมากกว่าเอนไซม์ซีรีนโปรติเนส เมื่อศึกษาการแสดงออกของยีนซีรีนโปรติเนสโฮโมลอกของกุ้งกุลาดำด้วยเทคนิค *in situ* hybridization พบว่ายีนนี้มีการแสดงออกในเซลล์เม็ดเลือด และมีการแสดงออกเพิ่มขึ้นอย่างมีนัยสำคัญในเม็ดเลือดของกุ้งที่ติดเชื้อวิบริโอ 6 ชั่วโมง นอกจากนี้ได้ศึกษาการแสดงออกของยีน *Lv*MasSPH ในเม็ดเลือดกุ้งขาวที่ติดเชื้อวิบริโอด้วยเทคนิค Real-time RT-PCR พบว่ายีน *Lv*MasSPH ถูกเหนี่ยวนำให้มีการแสดงออกเพิ่มขึ้นเพื่อตอบสนองต่อการติดเชื้อวิบริโอ ในการศึกษาลักษณะสมบัติของโปรตีน *Pm*MasSPH โดยผลิตรีคอมบิแนนท์โปรตีน serine proteinase-like domain ในแบคทีเรีย *E. coli* สายพันธุ์ Rosetta (DE3) และทำรีคอมบิแนนท์โปรตีนให้บริสุทธิ์ด้วยคอลัมน์นิเกล เมื่อนำรีคอมบิแนนท์โปรตีนที่บริสุทธิ์มาทดสอบหน้าที่ต่างๆ พบว่ารีคอมบิแนนท์โปรตีนไม่มีความสามารถในการย่อยโปรตีน แต่สามารถยึดจับเม็ดเลือด และเชื้อแบคทีเรียแกรมลบและบวก รวมทั้งส่วนประกอบของผนังเชื้อจุลชีพได้ แสดงว่าโปรตีน *Pm*MasSPH อาจทำหน้าที่เป็นโมเลกุลจดจำสิ่งแปลกปลอม จากผลการทดลองทั้งหมดนี้แสดงว่า *Pm*MasSPH มีบทบาทสำคัญในระบบภูมิคุ้มกันของกุ้งกุลาดำ

สาขาวิชา ชีวเคมี ลายมือชื่อนิสิต รุ่งรัตน์ จิตวโรภาส

สาขาวิชา ชีวเคมี ลายมือชื่ออาจารย์ที่ปรึกษา อัญชลี ทัศนาขจร

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KEY WORD: SERINE PROTEINASE HOMOLOGUE/ *Penaeus monodon*/ RACE-PCR/ PO ACTIVITY/ INNATE IMMUNITY

RUNGRAT JITVAROPAS: EXPRESSION AND CHARACTERIZATION OF A SERINE PROTEINASE HOMOLOGUE FROM BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS COADVISOR: ASST.PROF. PAUL S. GROSS, Ph.D., 188 p.

The full-length cDNAs of the serine proteinase homologue from *Penaeus monodon* (*Pm*MasSPH) and *Litopenaeus vannamei* (*Lv*MasSPH) were identified by rapid amplification cDNA end (RACE) method. The complete cDNA sequence of *Pm*MasSPH (1,958 bp) contained an open reading frame (ORF) of 1,572 bp encoding a 523 amino acid protein. A full-length cDNA of *Lv*MasSPH (1,955 bp) consists of an ORF of 1,536 bp encoding for a polypeptide of 511 amino acids. Both of the shrimp SPHs contained a glycine-rich repeated region, a clip domain at the N-terminus and a SP-like domain at the C terminus. Sequence comparison showed that the deduced amino acid of *Pm*MasSPH had a sequence identity of 58%, 55% and 53% to those of *Callinectes sapidus* PPAF, *Apis mellifera* PPAF and *Bombyx mori* masquerade-like, respectively and the deduced amino acid of *Lv*MasSPH showed 93% identity to that of *Pm*MasSPH. A phylogenetic tree clearly revealed that *Pm*MasSPH and *Lv*MasSPH were more closely related to non-catalytic SPHs than to the active SP. *In situ* hybridization analysis showed that *Pm*MasSPH expressed in hemocytes and was up-regulated within 6 h after *Vibrio harveyi* injection. Moreover, Real-time RT-PCR analyses showed that the transcript of *Lv*MasSPH was induced in response to *V. harveyi* infection. To further characterize the functions of *Pm*MasSPH protein, the C-terminal SP-like domain was expressed in *Escherichia coli* Rosetta (DE3) and purified by a Ni NTA column. The refolded recombinant SP-like domain protein was then assayed for various biological functions. The refolded recombinant SP-like domain protein lacks proteolytic activity but mediates hemocyte adhesion, displays binding activity to Gram-negative and Gram-positive bacteria and cell wall components, suggesting that *Pm*MasSPH may act as a pattern recognition molecule. These results suggested that *Pm*MasSPH plays an important role in shrimp defense.

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Academic year: 2007

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| A | absorbance |
| bp | base pair |
| CFU | colony forming units |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytosine triphosphate |
| DEPC | diethylpyrocarbonate |
| dGTP | deoxyguanosine triphosphate |
| DNA | deoxyribonucleic acid |
| dTTP | deoxythymidine triphosphate |
| EST | expressed sequence taq |
| EtBr | ethidium bromide |
| h | hour |
| HLS | hemocyte lysate supernatant |
| kb | kilobase |
| kDa | kilodalton |
| L-DOPA | L-3, 4-dihydroxyphenylalanine |
| LPS | lipopolysaccharide |
| *Lv*MasSPH | serine proteinase homologue of *Litopenaeus vannamei* |
| M | molar |
| mg | milligram |
| min | minute |
| ml | millilitre |
| mM | millimolar |
| ng | nanogram |
| nm | nanometer |
| O.D. | optical density |
| $^{o}$C | degree celcius |
| ORF | open reading frame |

| | |
|---|---|
| PAGE | polyacrylamide gel electrophoresis |
| PAP, PPAE, ppA | prophenoloxidase activating enzyme |
| PCR | polymerase chain reaction |
| PG | peptidoglycan |
| *Pm*MasSPH | serine proteinase homologue of *Penaeus monodon* |
| PO | phenoloxidase |
| PPAF | prophenoloxidase activating factor |
| proPO | prophenoloxidase |
| proPO | prophenoloxidase |
| RACE | rapid amplification cdna end |
| RNA | ribonucleic acid |
| RT | reverse transcription |
| sec | second |
| SP | serine protease, serine proteinase |
| SPH | serine proteinase homologue |
| SP-like domain | serine proteinase-like domain |
| UTR | untranslated region |
| WSSV | white spot syndrome virus |
| YHV | yellow head virus |
| μg | microgram |
| μl | microlitre |
| μM | micromolar |

# CHAPTER I

# INTRODUCTION

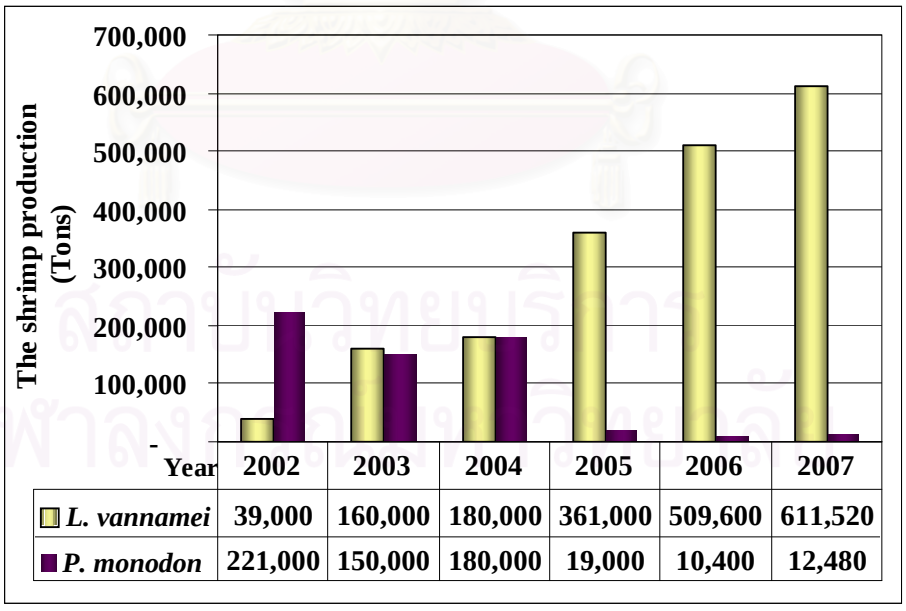
## 1.1 General introduction

Thailand has been the world's leading exporter of cultured shrimp since 1992. Shrimp farming has enhanced an economic growth and also employment distribution. The most important cultured shrimp in Thailand are the black tiger shrimp, *Penaeus monodon* and the white shrimp, *Litopenaeus vannamai* with a total production of 624,000 tons in 2007.

In Thailand, the black tiger shrimp farms and hatcheries are dispersed along the coastal areas. Southern provinces such as Nakon Sri Thammarat and Surat Thani yield the majority of harvests, whereas east and central provinces such as Samut Sakhon and Samut Songkhram yield the minority in terms of number. The shrimp cultivation in Thailand has rapidly generated large annual production with several advantages, including appropriate farming areas without serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils and terrain for pond construction. Culture of *P. monodon* increases the national revenue, therefore, making this penaeid shrimp species an economically important species in Thailand (Source: FAO Fishstat 2006).

However, since 2003 production of the black tiger shrimp has decreased due to several problems: mainly the outbreaks of bacterial and viral diseases including and also the unfavorable weather, the lack of high-quality broodstock and water pollution. Accordingly, *P. monodon* farming has been replaced with *L. vannamei*. (Figure 1.1)

*L. vannamei* has several advantages over the *P. monodon*, including the rapid growth rate, availability of specific pathogen free (SPF) stocks, disease resistance and high survival rate during larval rearing. Since *L. vannamei* is a nonnative species, its broodstock has to be imported mainly from Hawaii and possibly acts as a carrier of various new pathogens to the culture areas. Consequently, maintaining the production of native species *P. monodon* by domestication and genetic improvement is urgently required. Domestication will provide captive broodstock while genetic selection will provide high-quality traits with required properties such as disease resistance, rapid growth rate, tolerance of high stocking density, tolerance of low salinity and temperature, lower protein requirements, high survival during larval rearing as well as some other marketing advantages.




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

**Figure 1.1** The black tiger shrimp and white shrimp production in Thailand from 2002 to 2004 and the predicted production for 2007 (Source: FAO Fishstat 2006)

## 1.2 Taxonomy of *Penaeus monodon*

Penaeid shrimp 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 subphylum Crustacea is made up of 42,000, predominantly aquatic species, which belong to the 10 classes. Within the class Malacostraca; shrimp, crayfish, lobster and crab belong to the order Decapoda. The taxonomic definition of the black tiger shrimp, *P. monodon* is as follows (Bailey-Brock and Moss, 1992):

**Phylum** Arthropoda

**Subphylum** Crustacea

**Class** Malacostraca

**Subclass** Eumalacostraca

**Order** Decapoda

**Suborder** Natantia

**Infraorder** Penaeidea

**Superfamily** Penaeoidea

**Family** Penaeidae Rafinesque, 1985

**Genus** Penaeus Fabricius, 1798

**Subgenus** Penaeus

**Species** monodon

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

**Common name:** Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost prawn (Hong Kong), Jinga (India, Bombay region), Udang windu(Indonesia), Ushi-

ebi (Japan), Kamba ndogo (Kenya), Kalri (Pakistan), Sugpo (Phillipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Tim sa (Vietnam).

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

## 1.3 Morphology

The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmented abdomen (Figure 1.2). The thorax is covered by a single, immobile carapace, which protects internal organs and supports muscle origins. Most organs—such as gills, digestive system and heart—are located in the cephalothorax, while muscles concentrate on the abdomen. Appendages of the cephalothorax vary in appearance and function. In the head region, antennules and antennae perform sensory functions. Mandibles and two pairs of maxillae form jaw-like structures involved in food uptake (Solis, 1988). In the thorax region, maxillipeds are the first three pairs of appendages modified for food handling. The remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen (Bell and Lightner, 1988; Baily-Brock and Moss, 1992). A tail fan comprises a telson, which bears the anus, and two uropods attach to the last abdominal segment. The telson has deep medication groove without dorso-lateral spines. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawn (Anderson, 1993).

Secreted by an epidermal cell layer, the cuticle consists of chitin and protein in which calcium carbonate and calcium phosphate have been deposited. Epidermis detaches from the inner cuticle layer and begins to secrete a new cuticle, while the old

cuticle is moulted. After moulting the new cuticle is soft and is stretched to accommodate the increase size of the shrimp.

The black tiger shrimp has the following characteristic coloration: generally dark colored with carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow blackish waters or when shrimp are cultured in ponds, the color changes to dark and, often, to blackish brown (Motoh, 1981; Solis, 1988).

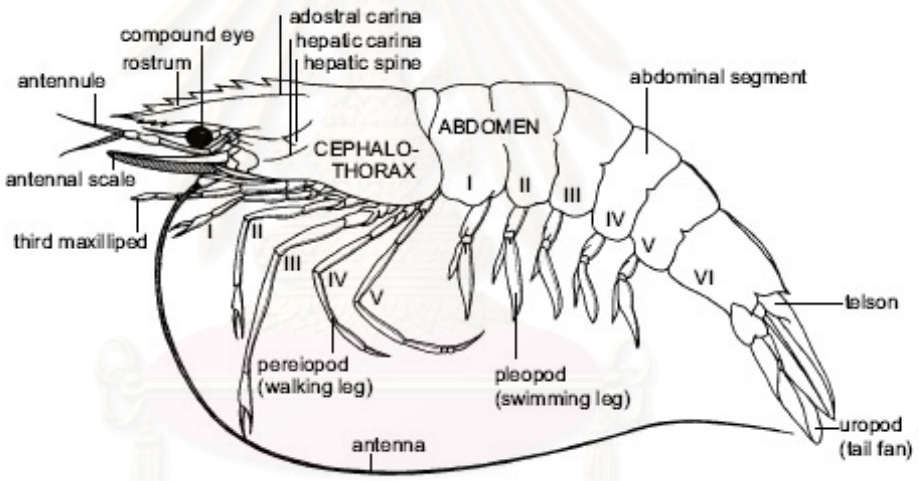



**Figure 1.2** Lateral view of the external morphology of *P. monodon* (Primavera, 1990)

The internal morphology of penaeid shrimp is outlined in Figure 1.3. Penaeids and other arthropods have an open circulatory system, and the blood and the blood cells are therefore called hemolymph and hemocytes, respectively. Crustaceans have a muscular heart that is dorsally located in cephalothorax. The valved hemolymph vessels leave the heart and branch several times before the hemolymph arrives at the sinuses, where exchange of substances takes place, scattering throughout the body.

After passing the gill, the hemolymph returns to the heart by means of three wide non-valved openings (Bauchau, 1981). A large part of cephalothorax in penaeid shrimp is occupied by the hepatopancreas. This digestive gland consists of diverticula of the intestine. Spaces between these hepatopancreatic tubules are hemolymph sinuses. The main functions of the hepatopancreas are absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the hemolymph vessels leaves the heart ends in the lymphoid organ where the hemolymph is filtered. This organ is located ventro-anteriorly to the hepatopancreas. The hemocytes are produced in hematopoietic tissue. This organ is dispersed in the cephalothorax, but mainly presented around the stomach and in the onset of the maxillipeds. Lymphoid organ and hematopoietic tissue are not shown in Figure 1.2. Gills are responsible for respiration process. The nervous system consists of two ventral nerve cords: a dorsal brain, and a pair of ganglia for each somite.

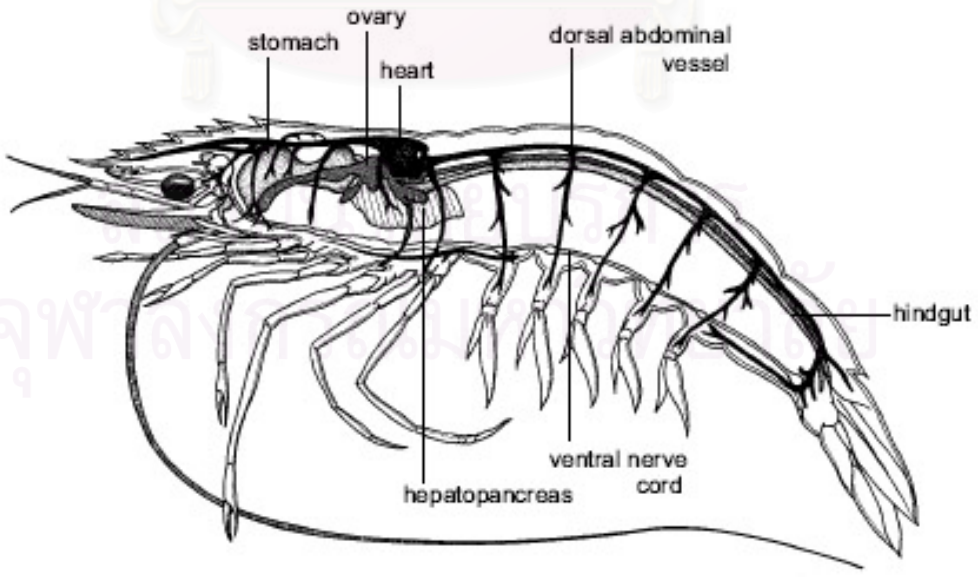



**Figure 1.3** Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990)

## 1.4 Distribution and life cycle

The black tiger shrimps are widely distributed throughout the greater part of the Indo-Pacific region, ranging northward to Japan and Taiwan, eastward to Tahiti, southward to Australia and westward to Africa. The penaeid life cycle includes several distinct stages found in various habitats (Figure 1.4). Juveniles prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinity, where mating and reproduction takes place. Females reproduce between 50,000-100,000 eggs per spawning (Rosenberry, 1997). The development of penaeid shrimp is complex. This complex life cycle begins with a larva hatching from the fertilized egg to the first stage, nauplius, metamorphoses to protozoa, mysis and post larval stages, sequentially (Figure 1.3). These stages require the developmental period of about 1-5 days, 5 days, 4-5 days, and 6-15 days, respectively (Solis, 1988). Shrimp larvae exhibit planktonic behavior. Swimming can be done using antennae in nauplii, antennae and thoracic appendages in protozoa and thoracic appendages in mysis larvae. The adult swims using the pleopods (abdominal appendages). Nauplii sustain on yolk granules within their bodies while the feeding starts in protozoea and mysis. The protozoea feed on algae and metamorphose into myses. The myses feed on algae and zooplankton, have many characteristics of adult shrimp, and develop into megalopas, the stage commonly called post-larvae (PLs). After these stages, the postlarvae will develop into juvenile and sub-adults, tolerating the variant physio-chemical environment. Sub-adults migrate back to sea where they finally mature to mate and spawn. The life-span of penaeid shrimp are approximately 2 years (Solis, 1998: Anderson, 1993).

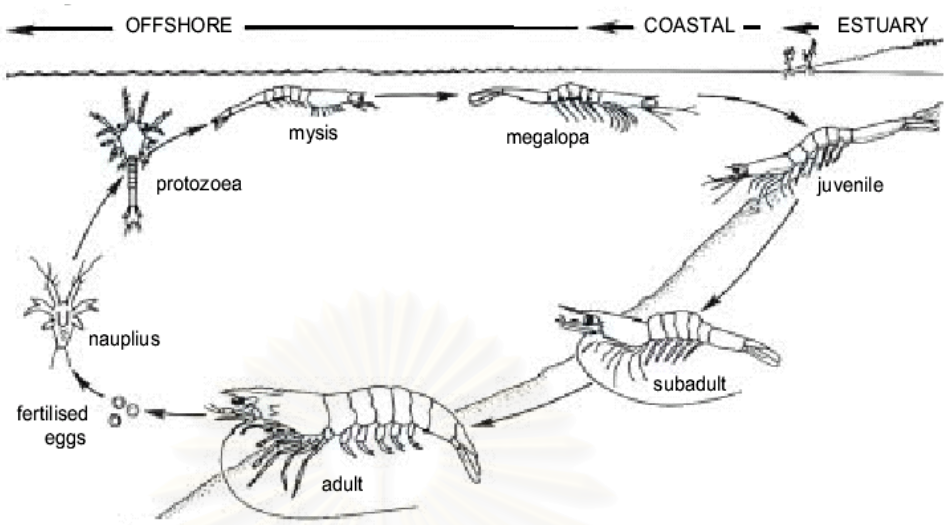



**Figure 1.4** The life cycle of *P. monodon* (Baily-Brook and Moss, 1992)

## 1.5 Shrimp diseases

Infectious diseases become serious problems in shrimp industry in many countries. This is because of an increase in shrimp farming and lack of proper knowledge involving shrimp biology, farm management, and diseases. Moreover, shrimp aquaculture is presently based on wild animals which are not completely acclimatized to the artificial conditions of shrimp hatcheries and farms where water quality, microbiological flora and nutrition are vastly different from those in the natural habitat. These intensive artificial conditions lead to physiological disturbances and immunodeficiencies that increase sensitivity to the pathogens.

The diseases of *P. monodon* are mainly caused by virus and bacteria. In Thailand, the outbreaks of infectious disease have become the most serious problem since 1993. Yellow head disease spread in central and southern Thailand during 1993 to 1994 (Hasson et al., 1995), white spot disease during 1994 to 1996 (Flegel, 1997).

Nowadays, luminous bacteria disease is the most serious problem for the shrimp culture. These pathogens cause serious diseases of larval and post-larval stages of *P. monodon* and lead to stock mortality. They also lead to the overfishing of wild shrimp larvae and an overexploitation of bloodstock.

### 1.5.1 Viral disease

The shrimp farming industry in Thailand encountered a severe problem from viral infectious diseases for over a decade. The important virus species that have been reported in *P. monodon* are white spot syndrome virus (WSSV) and yellow-head virus (YHV) which cause white spot syndrome (WSS) and yellow-head disease (YH), respectively (Boonyaratpalin et al., 1993; Wongteerasupaya et al., 1995). The outbreak of these virus diseases causes great losses in the shrimp industry in several producing countries including Thailand.

### 1.5.2 White spot syndrome (WSS)

White spot syndrome (WSS) is a viral disease affecting most of the commercially globally cultivated marine shrimp species (Chou et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Span and Lester, 1997). Lightner (1996) has called this virus white spot syndrome baculovirus (WSSV) because its morphological characteristics are similar to insect baculovirus. However, phylogenetic analysis of ribonucleotide reductase and protein kinase genes revealed that WSSV does not share a common ancestor with baculovirus (Hulten et al., 2000; Hulten and Valk, 2001).

This virus is an enveloped DNA virus of bacilliform to cylindrical morphology with an average size of 120 x 275 ± 22 nm and has a tail-like projection at one end of the particle (Kasornchandra et al., 1995; Wongteerasupaya et al., 1995). The viral genome contains double-stranded DNA of about 292 to 305 kb in length (Hulten et al., 2001; Yang et al., 2001).

White spots on the exoskeleton and epidermis are the most commonly observed clinical sign of WSS in infected shrimp. However, the presence of white spots does not always mean that the condition is terminal. For instance, under non-stressful conditions, infected shrimp that have white spots may survive indefinitely. If the white spots appear together with lethargy, a pink to reddish-brown coloration, the gathering of affected shrimp around the edges of ponds throughout the day, and a rapid reduction in food consumption, a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of the signs.

WSS can cause up to 100% mortality, causing a correspondingly devastating economic impact. WSSV is extremely virulent and has a wide host range (Lo et al., 1996b). Diseases caused by viruses especially by white spot syndrome virus (WSSV) are the greatest challenge to the worldwide shrimp aquaculture. The innate immunity of shrimp has attracted extensive attention, but no factor involved in the virus resistance has been reported until 2003. A differential pmAV cDNA cloned from WSSV virus-resistant shrimp *P. monodon* was found to have an open reading frame (ORF) encoding a 170 amino acid peptide with a C-type lectin-like domain (CTLD) displaying a strong antiviral activity in inhibiting virus-induced cytopathic effect in fish cell *in vitro* (Lau et al., 2003). It had been reported by Zhang et al. in 2004 that hemocyanin isolated from *P. monodon* had non-specific antiviral properties.

Moreover, interferon-like protein from the hemocytes of virus-resistant *Marsupenaeus japonicus* could inhibit grouper iridovirus (SGIV) in grouper (GP) cells (He et al., 2005).

### 1.5.3 Yellow-head (YH) disease

YH disease was known to infect and cause mass mortality in shrimp farming operations throughout South East Asian countries. In Thailand, the disease was first reported in 1990. YH disease occurs in the juvenile to sub-adult stages of shrimp, especially at 50-70 days of grow-out (Lightner, 1996). YHV is a pleomorphic, enveloped virus with single stranded RNA of positive polarity primarily localized in the cytoplasm of infected cells (Cowley et al., 1999). It may belong to the family coronaviruses.

YHV infected shrimp often exhibits light yellow coloration of the dorsal cephalothorax area and have a generally pale or bleached appearance (Limsuwan, 1991). At the onset of YHD, food consumption of shrimp is at an abnormally high rate for several days, then abruptly ceases feeding. A few moribund shrimp appear swimming slowly near the surface at the pond edges. After infection, mortality may reach as high as 100% of affected populations within 3-5 days from the onset of disease. In the black tiger shrimp, typical signs of YH disease include characteristic yellowing of the hepatopancreas and gill. 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).

### 1.5.4 Bacterial disease

*Vibrio* species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). However, the bacteria causing the most serious diseases of the larval and postlarval stages of *P. monodon* are of the genus *Vibrio* which behave more like true pathogens than opportunist (Johnson, 1978; Lightner, 1983, 1988, 1996).

The luminescent bacterium, *Vibrio harveyi*, frequently related to the outbreaks of luminous vibriosis in cultured *P. monodon* in hatcheries in many countries such as Australia, China, India, Indonesia, Thailand, Philippines, and Taiwan (Vandenberghe et al., 1998). In Thailand, vibriosis is the main cause of production loss in penaeid shrimp farms (Nash et al., 1992). This bacterial disease causes mortality up to nearly 100% of affected populations: larvae, post-larvae, juveniles, sub-adults and adults (Lightner, 1983). Luminescent vibriosis bacteria, *V. harveyi*, were claimed to be the most causative agent associated with shrimp mortality. *V. harveyi* is a rod shape, Gram-negative bacterium with 0.5-0.8 μm width and 1.4-2.6 μm in length. The bacteria are able to emit a blue-green color light by a reaction catalyzed by luciferase. The substrates are reduced flavin mononucleotide ($FMNH_2$), a long chain aldehyde (RCHO), and oxygen which react according to the following reaction:

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} FMN + HO + RCOOH + light$$

The disease caused by *V. harveyi* is widely known as luminous disease. The gross signs of localized infection in the cuticle or sub-cuticle are called shell disease or black or brown spot disease. These superficial infections can develop into systemic

infections under some circumstances. These systemic infections can cause mortality. Other gross features of the infected shrimp are milky white body and appendages, weakness, disoriented swimming, lethargy and loss of appetite, eventually leading to death.

Presumptive diagnosis is made on the basis of clinical signs and culture of the suspensions of hepatopancreas or blood on trytic plate supplemented with 2% (w/v) NaCl. After incubation at 30 °C overnight, colonies of *V. harveyi* show strong luminescence in dim light.

To avoid drug resistance from use of antibiotic in the control of bacteria, utilization of probiotics such as *Pseudomonas* I-2 and *Bacillus subtilis* BT23—which has been reported to be capable of producing compounds with inhibitory effects on the growth of *V. harveyi*—is an interesting alternative strategy to control shrimp pathogenic *Vibrio* in cultured system (Chythanya et al., 2002; Vaseeharan and Ramasamy, 2003).

## 1.6 The immune responses in invertebrates

All living organisms have developed immune system for defending themselves against microbial invasion or other foreign substances. Immune system can be evolutionarily classified into two types: adaptive (acquired) and innate (natural) immunity. Vertebrates possess both adaptive and innate immune systems, whereas invertebrates have only innate immune system. The adaptive immune system functions by producing highly specific recognition molecules, known as antibodies,

which can memorize foreign molecules after the first exposure. The innate immune system involves a large number of generalized effector molecules.

Innate immune system is a more phylogenetically ancient defense mechanism found in all multicellular microorganisms. This first line of defense helps to limit infection at an early stage and relies on germline-encoded receptors recognizing conserved molecular patterns that are present on the microorganisms (Janeway, 1998). Whereas adaptive immune system is a more sophisticated and complicated mechanism including immunological memory (Lee and Söderhäll, 2001).

## 1.7 Crustacean immune system

Crustacean immune system is innate immune system based on cellular and humoral components of the circulatory system (Figure 1.5). The hard cuticle covering all external surfaces of crustaceans is the first line of defense between them and the environment. Moreover, the innate immune system can respond rapidly if microorganisms invade the animals. Major defense systems are carried out in the hemolymph containing cells called hemocytes. Hemocytes and plasma protein recognize large groups of pathogens by means of common molecular patterns of particular microbes. Hemocytes as effectors of the cellular immune response are also involved in synthesizing the majority of humoral effectors. Cellular response, the actions with direct participation of blood cells, includes phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting (Jiravanichpaisal et al., 2006). On the contrary, humoral factors act in the defense without direct involvement of the cells even though many of the factors are originally synthesized and stored in the blood cells. These factors include enzyme and proteins involved in prophenoloxidase (proPO) system,

clotting proteins, agglutinins, hydrolytic enzymes, proteinase inhibitors, and antimicrobial peptides.

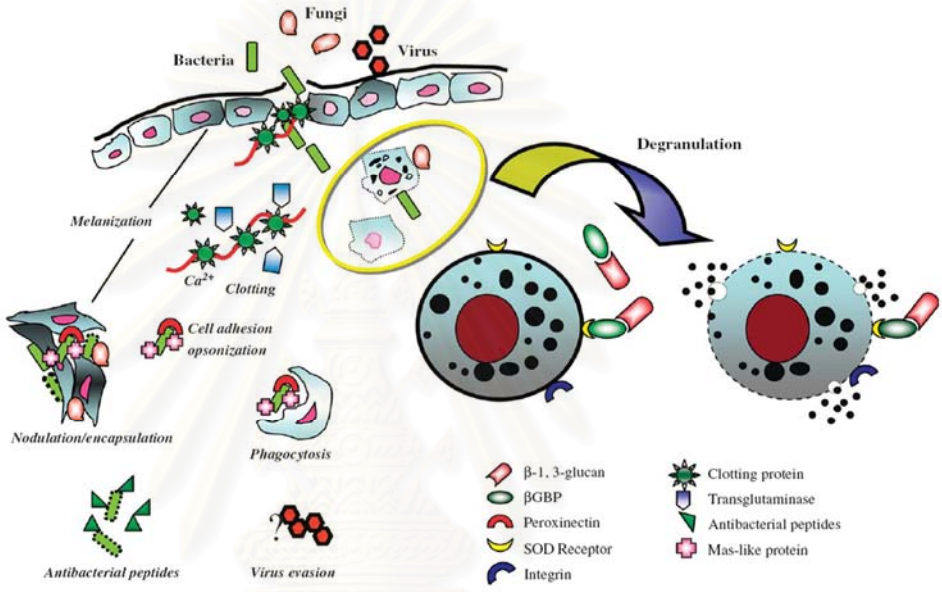



**Figure 1.5** Schematic overview of the innate immune response (Jiravanichpaisal et al., 2006)



### 1.7.1 Blood cells

Crustacean circulating hemocytes important in immune system, which can be morphologically grouped into three subpopulations: hyaline cells, semigranular cells, and granular cells. Hyaline cells (5-10% of the circulating hemocytes)—the smallest of the hemocytes lacking cytoplasmic granules—are involved in phagocytosis and coagulation (Söderhäll et al., 1986). Semigranular cells—the most abundant type of

hemocytes (75% of all hemocytes)—contain a variable number (1-40) of small (S) granules (0.4 μm diameter) which display some phagocytic capacities. Semigranular cells specialize in particle encapsulation, and by degranulation, they appear to be the most sensitive and the first to react during an immune response. Granular cells (10-20% of the hemocytes) contain a large number of secretory large (L) granules (0.8 μm diameter). Both granular cells and semigranular cells store the components of prophenoloxidase activating system and cytotoxic reaction (Smith and Söderhäll 1983b; Söderhäll et al., 1985).

### 1.7.2 Pattern recognition proteins

When foreign substances attack animals, the first immune process is recognition of a broad spectrum of factors that are released or are present on the surface of invading microorganisms. This process is mediated by the hemocytes and by plasmatic protein. There is little knowledge about the molecular mechanisms that mediate the recognition; however, in crustaceans, several types of modulator proteins identified recognize cell wall components of pathogens. The target recognition of innate immunity, so-called “pattern recognition molecules (PRMs)”, is shared among groups of pathogens. Host organisms have developed the response to these PRMs by a set of receptors referred to as “pattern recognition proteins or receptors (PRPs or PRRs)”. These patterns include the lipopolysaccharides (LPS) of Gram negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram positive bacteria, the mannans of yeasts, the β-1,3-glucan of fungi, and double-stranded RNA of viruses (Hoffmann et al., 1999). Most current research has emphasized the possible

roles of non-self recognition molecules in the vertebrate and the invertebrate immune system.

Carbohydrate recognition is important because carbohydrates are common constituents of microbial cell wall, and microbial carbohydrates have distinct structures from those of carbohydrates of eukaryotic cells. Therefore, LPS or/and β-1,3-glucan binding proteins (LBP, βGBP, or LGBP), peptidoglycan recognition protein (PGRP), several kinds of lectins, and hemolin have been identified in a variety of invertebrates with different biological functions proposed following their binding to their targets (Lee et al., 2002).

In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin that, besides its bacterial agglutination ability, increases phagocytic rate (Vargas-Albores, 1995). A second protein involved in the recognition of microbial products and the activation of cellular functions is the β-glucan binding protein. It is apparently monovalent and dose not induce agglutination, but activates degranulation and the proPO system. Thus, these recognition proteins are capable of activating cellular activities only after reacting with the microbial carbohydrates (LPS peptidogycan or glucan) (Vargas-Albores et al., 2000). In 2002, Roux et al. proposed that shrimp LGBP is an inducible acute-phase protein that may play a critical role in shrimp WSSV interaction and that the WSSV infection regulated the activation and/or activity of the proPO cascade in a novel way.

### 1.7.3 Cell-mediated defense reactions

Cellular defense reactions include such processes as phagocytosis, encapsulation, and nodule formation (Millar and Ratcliffe, 1994). Phagocytosis—a common phenomenon in all organisms—includes foreign body attachment, ingestion and destruction. Encapsulation, a process wherein layers of cells surround the foreign material, occurs when a parasite is too large to be ingested by phagocytosis. Nodule formation, which appears to be similar to capsule formation, occurs when the number of invading bacteria is high. These structures, capsules and nodules are always melanized in arthropods.

### 1.7.4 Hemocyte adhesion molecules

In invertebrate immunity, cell adhesion is essential for the cellular immune responses of encapsulation and nodule formation. Blood cells of the crayfish, *Pacifastacus leniusculus*, can release a cell-adhesive and opsonic peroxidase called peroxinectin. A site containing the motif, KGD, appears to be adhesive by binding to a transmembrane receptor of the integrin family on the blood cells. Peroxinectin also binds to a peripheral blood cell surface CuZn-superoxide dismutase. The peroxidase-integrin interaction appears to have evolved early and seems conserved; human myeloperoxidase supports cell adhesion via the αMβ2 integrin. There is evidence for peroxinectin-like proteins in other arthropods. Effects by RGD peptides indicate that integrins mediate blood cell adhesion and cellular immunity in diverse invertebrate species. Other blood cell molecules proposed to be involved in cell adhesion in invertebrates include the insect plasmatocyte-spreading peptide, as well as soluble and

transmembrane proteins which show some similarity to vertebrate adhesive or extracellular matrix molecules. Proteins such as the Ig family member hemolin or proteins found in insect hosts for parasitic wasps, inhibit cell adhesion and may regulate or block cellular immunity.

### 1.7.5 The prophenoloxidase (proPO) system

The proPO activating system is composed of several proteins involved in melanin production, cell adhesion, encapsulation, and phagocytosis (Söderhäll et al., 1998; Sritunyalucksana and Söderhäll., 2000).

*In vitro* studies have shown that phenoloxidase (PO) exists as an inactive precursor—prophenoloxidase (proPO)—which is activated by a stepwise process involving serine proteases activated by microbial cell wall components such as low quantities of lipopolysaccharides or peptidoglycans from bacteria, and β-1,3-glucans from fungi through pattern-recognition proteins (PRPs) (Ariki et al., 2004). An enzyme capable of activating the proPO *in vivo* is called prophenoloxidase activating enzyme (factor) (ppA, PPAE, PPAF)**.** In crayfish, ppA is a trypsin-like proteinase present as an inactive form in the hemocyte granules. After degranulation, the enzyme is released together with proPO and becomes an active form in the presence of microbial elicitors. The active ppA will convert proPO to an active form, phenoloxidase (PO) (Aspán and Söderhäll, 1991; Aspán et al., 1995). PO is a copper-containing protein and a key enzyme in melanin synthesis (Söderhäll and Cerenius. 1998; Shiao et al., 2001). It both catalyses o-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can non-enzymatically

polymerise to melanin (Figure 1.6). PO is a sticky protein and can adhere to the surface of parasites leading to melanisation of the pathogens. Melanisation is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites such as crayfish plague fungus, *Aphanomyces astaci* (Söderhäll and Ajaxon, 1982). The production of forming insoluble melanin deposits involving in the process of sclerotisation, wound healing, and encapsulation of foreign materials (Theopold et al., 2004). To prevent excessive activation of the proPO cascade, it is needed to be regulated by proteinase inhibitors.

The prophenoloxidase activating enzyme (PPA) is a zymogenic protein (proppA). The C-terminal half of the proppA comprises a typical serine proteinase domain with a sequence similar to other invertebrate and vertebrate serine proteinases. The N-terminal half consists of a cationic glycine-rich domain, a cationic proline-rich domain, and a clip-domain, in which the disulfide-bonding pattern is likely to be identical to those of the horseshoe crab big defensin and mammalian β-defensins. The clip-domains in proppAs may function as antibacterial peptides (Wang et al., 2001a).

In penaeid shrimp, enzymes in the proPO system are localized in the semigranular and granular cells (Perazzolo and Barracco, 1997). This is in accordance with a recent study showing that *P. monodon* proPO mRNA is expressed only in the hemocytes (Sritunyalucksana et al., 2000).

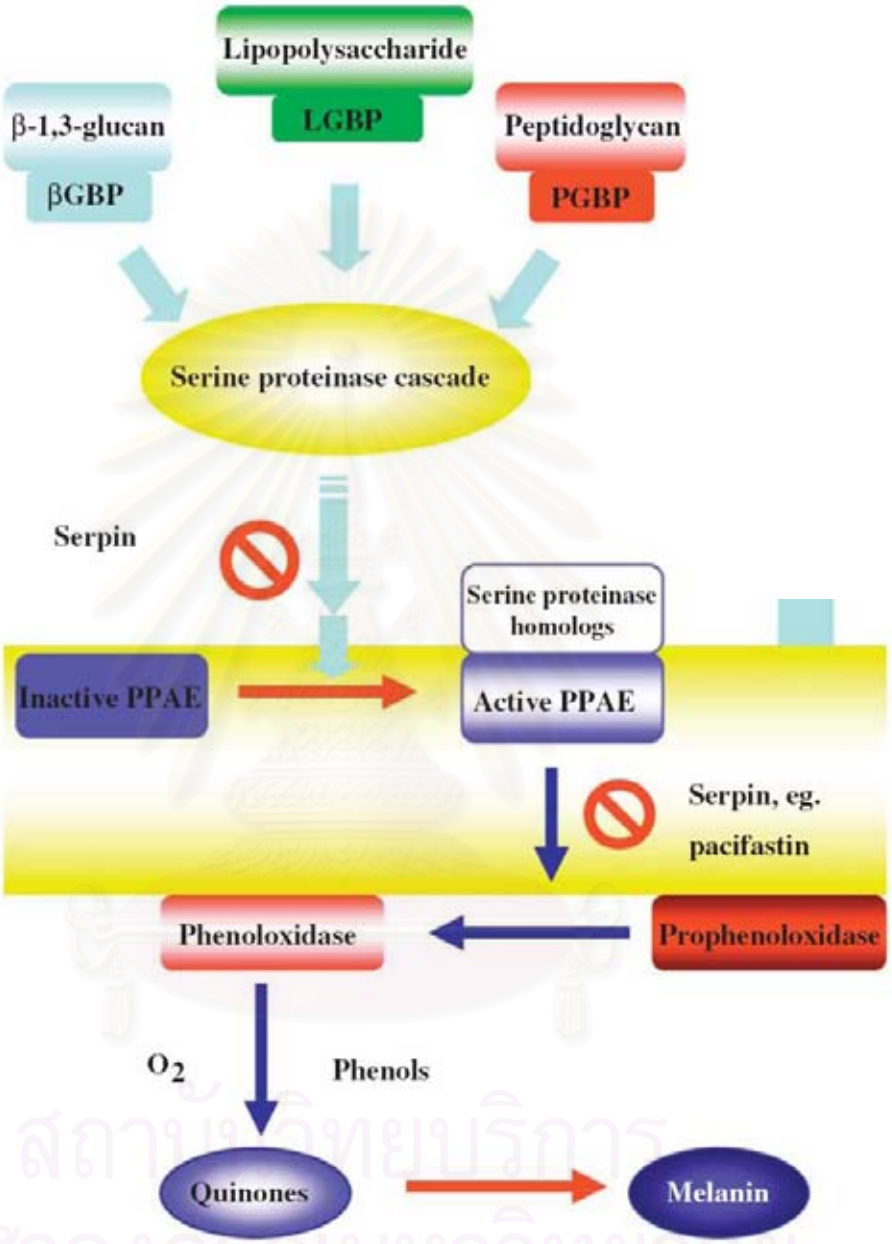



**Figure 1.6** Overview of the arthropod prophenoloxidase (proPO) system (modified from Jiravanichpaisal et al., 2006)

### 1.7.6 The coagulation system/ the clotting system

Hemolymph coagulation is a defensive response of crustaceans preventing both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al., 1991). It is a proteolytic cascade activated by microbial cell wall components. The coagulation system involves a plasma-clotting protein (CP) and a hemocyte-derived transglutaminase (TG) (Kopäcek et al., 1993a; Yeh et al., 1998).

Clotting has been most studied in two non-insect arthropod species with significantly different clotting reactions: freshwater crayfish and horseshoe crab. The clotting system in crayfish depends on the direct tranglutaminase (TGase)-mediated cross linking of a specific plasma protein, whereas the process in horseshoe crab is regulated by a proteolytic cascade activated by bacterial elicitors through specific recognition proteins.

In crayfish, clotting occurs through polymerization of a clotting protein in plasma. The crayfish CP is a dimeric protein of which subunit has both free lysine and glutamine. They are recognized and become covalently linked to each other by a calcium ion dependent TGases (Hall et al., 1999; Wang et al., 2001b; Yeh et al., 1998) (Figure 1.7).

CPs are synthesized in the hepatopancreas and released to hemolymph. In crustaceans, CPs were found in several species: the freshwater crayfish (Kopacek et al., 1993b), *P. monodon* (Yeh et al., 1998), and the lobster *Panulirus interruptus* (Doolittle and Fuller, 1972).

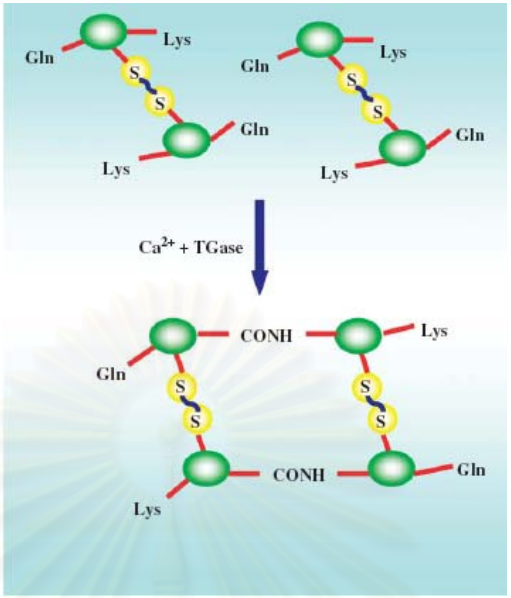



**Figure 1.7** The clotting system of crayfish and shrimp (Jiravanichpaisal et al., 2006)

### 1.7.7 Antimicrobial peptides (AMPs)

Animal peptide antibiotics are defined as anti-microbial agents made by an animal including humans with a function important for the innate immunity of that animal. Most of the AMPs are small in size, generally less than 150-200 amino acid residues, with amphipathic structure and cationic property. However, the anionic peptides also exist. Their small size makes them easy to be synthesized without dedicated cells or tissues, and therefore rapidly diffuse to the point of infection. For many of these peptides, there is evidence that one of the targets for the peptide is the lipid bilayer of the membrane. This is because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. With

regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the organism by rupture of the membrane or that it perturbs the membrane lipid bilayer, allowing for leakage of certain cellular components as well as dissipating the electrical potential of the membrane.

AMPs are active against a large spectrum of microorganisms: bacteria and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Hancock and Diamond, 2000; Murakami et al., 1991; pan et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991).

There are few reports on antimicrobial peptides in shrimp. Penaeidins-a new family of antimicrobial peptides acting against Gram positive bacteria and fungi-had been reported in penaeid shrimp *L. vannamei* (Destoumieux et al., 1997). cDNA clones of penaeidin isoform were also isolated from the hemocytes of *L. vannamei*, *P. setferus* (Gross et al., 2001), and *P. monodon* (Supungul et al., 2004). Crustins, an antimicrobial peptide, were identified from 2 species of *Penaeid* shrimp: *L. vannamei* and *L. setiferus*. Several isoforms of crustins were observed in both shrimp species. The 11.5 kDa antibacterial protein from *Carcinus maenas*, crustins from shrimp showed no homology with other known antibacterial peptides, but possessed sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Peptides derived from the hemocyanin of *L. vannamei*, *P. stylirostris*, and *P. monodon* possessing antiviral activity has also been identified (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004). Recently, histones and histone derived peptides of *L. vannamei* has been reported as innate immune effectors because they can inhibit growth of Gram-positive bacteria (Patat et al., 2004).

### 1.7.8 Proteinase inhibitors

Proteinase inhibitors, also produced by the hemocytes, are necessary to protect host from microbial proteinases and regulate the proteinase cascades (the proPO and coagulation system). Function of proteinases in many pathogenic fungi is help the fungi in penetrating the cuticle of their arthropod hosts. Proteinases also contribute to the virulence of bacterial pathogens. Some of the proteinase inhibitors in hemolymph may defend the host against such microbial proteinases. For instance, the silk worm (*Bombyx mori*) serine proteinase inhibitor is active against proteinases from fungal pathogens (Eguchi et al., 1993). Several of *Manduca sexta* serpin gene-1 variants inhibit bacterial and fungal serine proteinases (Jiang et al., 1998). Proteinase inhibitors in the cuticle or at the surface of the integument might also function in protection against fungal infection. An external secretion from grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity (Polanowski et al., 1997).

In vertebrates, injury and microbial infection lead to activation of the blood coagulation and proPO systems. Both of these systems employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides), resulting in rapid and efficient responses to the threats to health (Whaley et al., 1993; O'Brien et al., 1993). Blood clotting and phenoloxidase activation can also be harmful to the host if they are not limited as local and transient reactions. For this reason, the proteinases in these systems are tightly regulated by proteinase inhibitors.

Like blood clotting, phenoloxidase activation is normally regulated *in vivo* as a local reaction with brief duration. Also comparable to blood clotting, the regulation may be partly due to serine proteinase inhibitors in plasma (Kanost et. al., 1996). Pacifastin and, to the lesser degree, α-macroglobulin inhibit crayfish PPO activation (Aspan et al., 1990). Among the low molecular weight inhibitors from insect hemolymph, Kunitz family inhibitors from *M. sexta, Sarcophaga bullata,* and *B. mori* (Sugumaran et al., 1985; Saul et al., 1986 and Aso et al., 1994) and the 4 kDa locust inhibitors (Boigegrain et al., 1992) can interfere with PPO activation. Serpin-1J from hemolymph of *M. sexta* inhibits the activity of a serine proteinase linked to prophenoloxidase activation (Jiang et al., 1997). Recently, the *M. sexta* serpin-6 was isolated from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3) (Wang and Jiang, 2004). In addition, its structure and function were further characterized by cloning and expression in *E. coli* expression system (Zou and Jiang, 2005) The results indicated that serpin-6 plays important roles in the regulation of immune proteinases in the hemolymph. It is likely that each proteinase in the PPO cascade is regulated by one or more specific inhibitors present in plasma or in hemocyte granules.

Interestingly, the WAP domain (InterPro code IPR002221 ) is a 50-residue protein with four disulfide bonds, conforming to a conserved tightly packed structure named four disulfide core (4-DSC) (Grutter et al., 1988; Ranganathan et al., 1999; Tsunemi et al., 1996). Many of the proteins containing a 4-DSC domain bind to membrane-bound receptors and have proteinase inhibitor activities (Ranganathan et al., 1991).

### 1.7.9 Apoptotic and tumor proteins

Apoptosis or programmed cell death plays a major role in differentiation, development, tissue homeostasis and cell-mediated immunity as well as defense against environmental insults including pathogen attack (Kerr et al., 1977; Thompson et al., 1995). Recent studies have shown that many pathogens exert control on the processes regulating apoptosis in the host. The induction of apoptosis upon infection results from a complex interaction of parasite proteins with cellular host proteins. In the infected cells, induction of apoptosis significantly imparts protection to the host from the pathogen (Hasnain et al., 2003). However, if over apoptosis, it will be implicated in shrimp death, so apoptosis inhibitor is necessary. For example, both survivin and P109 protein are involved in apoptosis inhibition (Liston et al., 1996; Uren et al., 1996; Tambunan et al., 1998). Besides, survivin is involved in regulation of cell division during HIV-1 infection (Zhu et al., 2003).

### 1.7.10 Other immune molecules

Cyclophilins are highly conserved proteins first identified as main binding proteins for cyclosporin A (CsA), an immunosuppressive (Fischer et al., 1989). They were later identified as peptidyl-prolyl cis/trans isomerases (PPIase) and have been proposed to be involved in protein folding (Galat, 1993). Cyclophilins are involved in cellular processes and have many clinical applications such as cell signaling (Mattila et al., 1990), apoptosis (Montague et al., 1997), oxidative stress (Jaschke et al., 1998), heat shock, and hypoxia (Andreeva et al., 1997). For example, CyPA is predominantly in cytosol but can be secreted by macrophages in response to

stimulation with bacterial endotoxin (Sherry et al., 1992) or by vascular smooth muscle cells in response to oxidative stress (Jin et al., 2000). CyPB is found within the endoplasmic reticulum and secreted into milk and plasma (Spik et al., 1991; Allain et al., 1995; Arber et al., 1992). CyPB has been shown to enhance platelet adhesion to collagen (Allain et al., 1999) whereas Cyclophilin A shown to help protect cells from oxidative stress (Jaschke et al., 1998). A shrimp cyclophilin was also identified from *L. vannamei and L. setiferus* (Gross et al., 2001). Cyclophilins have diverse regulatory functions in mammalian cells, but it is noteworthy that they can be involved in viral attachment to cells (Saphire et al., 1999) and in the stress response to oxygen depletion (Santos et al., 2000).

Lectins are sugar-binding proteins that agglutinate cells and/or precipitate glycoconjugate molecules with a carbohydrate portion such as polysaccharide, glycoproteins, glycolipids, and others. There are many different lectins, including tachylectins from hemolymph plasma of the horseshoe crab *Tachypleus tridentatus* (Gokudan et al., 1999). They are involved in a variety of processes, including the innate immune response critical for the detection and elimination of infectious microorganisms (Weis et al., 1998; Kilpatrick, 2000).

Generally, they recognize sugar or carbohydrate structures on the surfaces of pathogens that are not present on host cell. It has been reported these lectins have an LPS- binding property (Koizumi et al., 1999 and Jomori and Natori., 1992). these LPS binding proteins have biological function of a bacterial clearance activity and an opsonic effect. Lectins are responsible for promoting phagocytosis and stimulating the proPO system (Yu et al., 1999; Yu and Kanost, 2000). Tachylectins were found to

have hemagglutinating and antibacterial activities which are important in the immune system (Kawabata and Iwanaga, 1999).

## 1.8 Clip-domain family of serine proteinase and serine proteinase homologue in arthropods

The proteinase systems in arthropods involve in diverse biological processes, including immune responses and embryonic development. These serine proteinases share a common feature: one or more disulfide-bridged structure called a clip domain at amino terminus and the presence of three conserved amino acid residues, His, Asp, and Ser, within the active site of serine proteinase domain (Perona and Craik, 1995).

The first arthropod clip domain to be identified in proclotting enzyme was from the horseshoe crab *Tachypleus tridentatus*. Biochemical and molecular biological data indicate that proclotting enzyme and other members of this protein family comprise two parts: a regulatory amino-terminal clip domain and a catalytic serine proteinase domain at the carboxyl terminus. The two domains are connected with a linking sequence, varying in length between 23 and 101 amino acid residues. Clip domains with the length of 37 to 55 amino acid residues are in compact structure maintained by three disulfide bonds (Jiang and Kanost, 2000) . The proteinases in the clip domain family synthesized as zymogens can be activated by a specific proteolytic cleavage at the activation site located between the clip domain and the catalytic domain. When cleaved, the two domains of the enzyme are linked with an additional pair of cysteine residues, so the catalytic heavy chain remains covalently connected by a disulfide bond to the clip domain-containing light chain. Located at the end of a clotting pathway, the proclotting enzyme is cleaved to form active clotting enzyme in

the presence of microbial cell surface molecules such as bacterial lipopolysaccharides and β-1,3-glucan-a fungal cell wall component. The active clotting enzyme then cleaves coagulogen to form insoluble coagulin, which polymerizes to form a clot.

*Drosophila melanogaster* snake and easter had been identified as the clip-domain proteinases. They participate in an embryo-developing pathway, resulting in stimulating development of ventral structures in the embryo (Belvin and Anderson, 1996). Not only in embryonic development, but they also take part in immune response upon fungal challenge as a mediator through spätzle protein (Lemaitre et al., 1996).

Moreover, in 1998 Ashida and Brey reported that prophenoloxidase (proPO) activation, a biological process related to insect immunity, involves a serine proteinase cascade. Phenoloxidase (PO) catalyzes the production of quinones, precursors for cuticle sclerotization and melanin synthesis. All arthropod POs synthesized as inactive precursors (proPO) become enzymatically active upon activation by limited proteolysis. The serine proteinase that performs the proPO activation has been called in different names including the proPO-activating protein or enzyme. Hence, the term proPO-activating enzyme (ppA, PAP, PPAE) will be used for this serine proteinase. Some ppAs have been found in several insects and crustacean such as the proPO activating enzymes from *Bombyx mori* (Satoh et al., 1999), *M. sexta* (Jiang et al., 1998; Jiang et al., 2003), crayfish blood cells (Wang et al., 2001) and from plasma of *Holotrichia diomphalia* (Lee et al., 1998). All these characteristics include they are all produced as zymogens which become proteolytically active in the presence of elicitors such as microbial products (Figure 1.8) and additional protein components of the proPO system, they have sequence

similarities with *Drosophila easter*, and they contain clip-like domains at the amino terminus (Söderhäll and Cerenius, 1998).

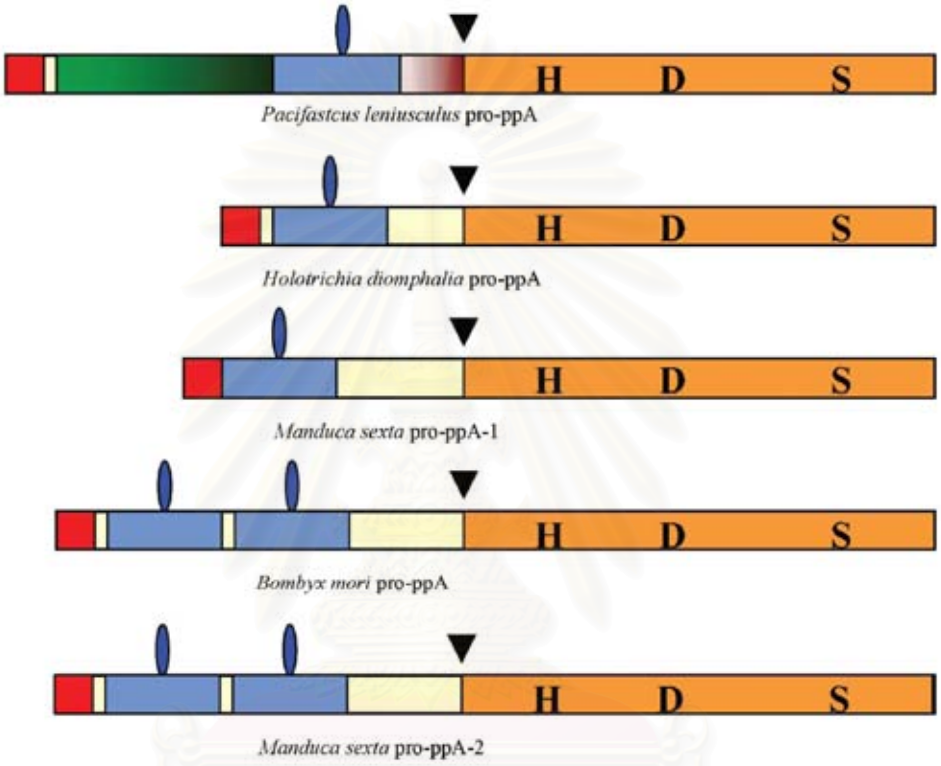



**Figure 1.8** Domain organizations of the zymogens of the arthropod prophenoloxidase-activating enzymes (pro-ppAs). The red, orange and blue squares represent the hydrophobic signal peptide, a C-terminal serine proteinase domain and at least one clip domains in N-terminus, respectively. The cleavage sites are indicated by arrows. The *P. leniusculus* pro-ppA contains two additional domains that area glycine-rich region (green), and a proline-rich region (brown). (Cerenius, 2004)

In 1995, Murugasu-Oei et al reported the discovery of a clip domain serine proteinase homologue (c-SPH)—so called masquerade (mas)—from *D. melanogaster*, which was shown to stabilize muscle attachment in the *Drosophila* embryo. C-SPH proteins share some common structural features: disulfide knotted clip domain(s) at the amino terminus and a catalytically inactive serine proteinase-like domain at the carboxyl terminus. Clip-domain structure is stabilized by three disulfide bonds. It had been reported that the clip domain may be involved in protein-protein interactions, regulation of the protease activity, and antimicrobial activity (Jiang and Kanost, 2000). The serine proteinase-like domains in c-SPHs are catalytically inactive due to the substitution of Gly for the catalytic Ser residue. The c-SPHs synthesized as precursors require a proteolytic activation (Figure 1.9). After the activation, N- and C-terminal domains are linked by an additional inter-domain disulfide bond. In crayfish *Pacifastacus Leniusculus*, the masquerade-like protein has not been experimentally determined for the exact position of the disulfide bonds linking between subunits. It was produced as a precursor (Huang et al., 2000) and processed by proteolytic cleavage at the three indicated sites upon binding to bacteria or yeast (Lee and Soderhall, 2001). Some insects, *H. diomphalia* prophenoloxidase activating factor II (PPAFII) is processed by the PPAFIII (SP) (Kim et al., 2002). The *M. sexta* SPHs are cleaved by unknown proteinases in the hemolymph (Yu et al., 2003). In comparison to catalytically active clip domain serine proteinase (c-SP), the c-SPHs have three unique structural features, including four signature regions (I-IV) and one calcium-binding loop.

Serine proteinase homologues identified in vertebrates and invertebrates have different biological functions: antimicrobial activities, as a growth factor, an adhesion molecule, or as an immune molecule (Huang et al., 2000). In *Pacifastacus*

*leniusculus*, a mas-like protein from hemocytes was shown to be involved in granulocyte adhesion, pattern recognition, and opsonization but not shown to be directly involved in proPO activation (Huang et al., 2000; Lee and Soderhall, 2001). In *H. diomphalia, Tenebrio molitor, M. sexta, Cotesia rubecula,* and *Callinectes sapidus*, their PPAF as cofactors take part in the regulation of the proPO activation (Lin et al., 2006). But in *B. mori*, PPAE can be directly involved in the activation of proPO in the absence of the SPH (Satoh et al., 1999).

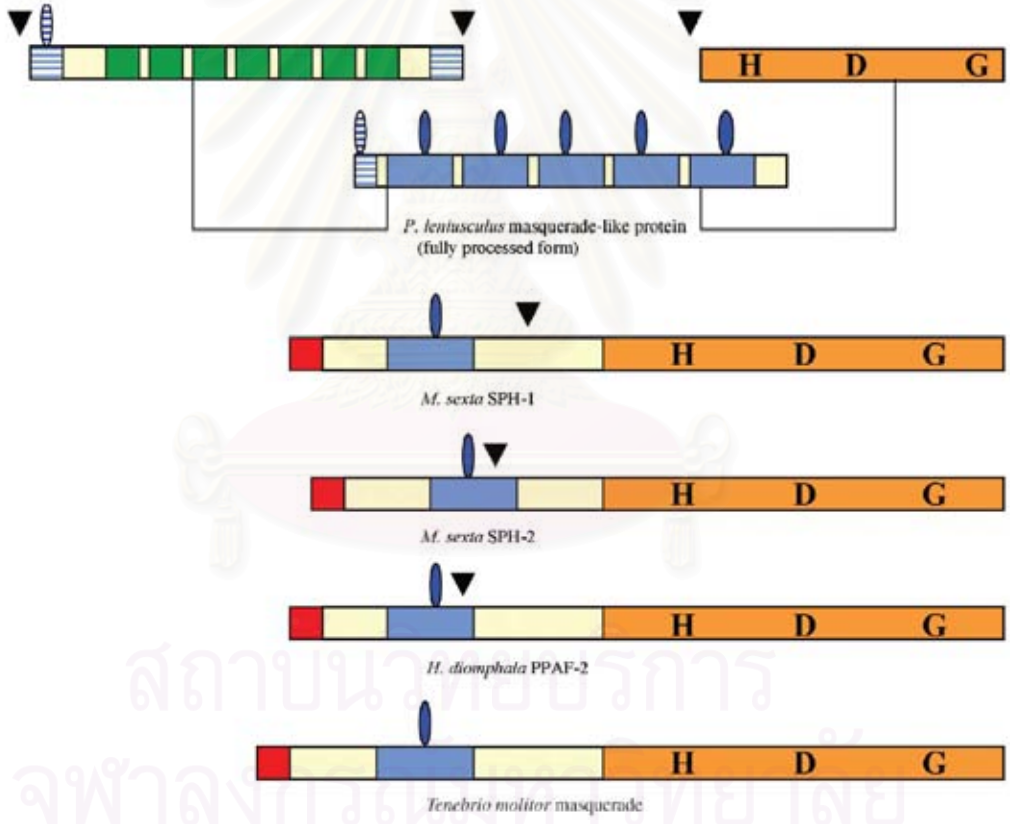



**Figure 1.9** Schematic representations of arthropod serine proteinase homologues known to involve in innate immune response. They all possess a C-terminal serine proteinase domain (orange) and one or several clip domains (blue). The striped areas, arrow heads and red box indicate the proteolytically processed clip domains, cleavage sites determined experimentally and hydrophobic signal sequences. The green areas show the repeated glycine-rich motifs in crayfish masquerade-like protein. (Cerenius, 2004)

## 1.9 Previous studies

From the *P. monodon* EST project (http://pmonodon.biotec.or.th), we identified a putative *Pm*MasSPH cDNA from the *Vibrio harveyi*-challenged hemocyte cDNA library of *P. monodon*. A partial sequence of the cDNA (accession number BI784455) showed the highest similarity to a PPAF of *C. sapidus* (e-value of $6x10^{-18}$). In addition, the expression of *Pm*MasSPH in hemocytes was induced upon *V. harveyi* and WSSV infection (Pulsook, 2005). The results suggested that *Pm*MasSPH might play an important role in shrimp immune response.

## 1.10 Objectives

The aims of the present study are to identify and clone a full-length sequence of a serine proteinase homologue cDNA from *P. monodon* (*Pm*MasSPH) using the RACE-PCR method and to evaluate its mRNA expression and localization in hemocytes of *Vibrio*-infected shrimp using *in situ* hybridization. Moreover, a complete sequence of a serine proteinase homologue of *L. vannamei* (*Lv*MasSPH) will be also identified, and its expression in response to the bacterial challenge will be examined. Furthermore, the biological function(s) of *Pm*MasSPH will be investigated by recombinantly expression of C-terminal SP-like domain and characterized for various biological functions including serine proteinase activity, cell adhesion activity, bacterial binding, and phenoloxidase (PO) activity. This will lead to a better understanding of shrimp immunity, and the comprehension of shrimp immune mechanisms will be applicable to the disease control in shrimp aquaculture.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Equipments

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

Amicon Ultra-4 concentrators (Millipore).

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

Balance: Satorius 1702 (Scientific Promotion Co.)

CX31 Biological Microscope (Olympus)

Evaporator (CentriVap Concentration Labconco)

Gel documentation (SYNGENE)

Gene Pulser (Bio-RAD)

Hybridization oven (Hybrid)

iCycler iQ™ Real-Time Detection System (Bio-Rad)

Incubator (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets (NuAire, Inc.)

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

Microtiter plate reader (Beckman Coulter AD200)

PCR Mastercycler (Eppendorf AG)

PD-10 column (GE Healthcare)

pH meter Model # SA720 (Orion)

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

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen)

Spectrophotometer (eppendorf)

Sterring hot plate (Fisher Scientific)

Touch mixer Model # 232 (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

## 2.2 Chemicals, Reagents and Biological substance

0.4% Trypan blue solution (Sigma)

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

2-Mercaptoethanol, $C_2H_6OS$ (Fluka)

3-(N-morpholino) propanesulfonic acid (MOPS) (USB)

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

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

Absolute methanol, CH3OH (Scharlau)

Acetic anhydride (Sigma)

Acrylamide (Plus one)

Agarose (Sekem)

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

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

Amplicillin (BioBasic)

Anti-Digoxigenin-AP Fab fragment (Roche)

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, $BH_3O_3$ (MERCK)

Bovine serum albumin (Fluka)

Bromophenol blue (MERCK)

Calcium chloride ($CaCl_2$) (MERCK)

Chloramphenicol (Sigma)

Chloroform, $CHCl_3$ (MERCK)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

Dextran sulfate (BioBasic Inc.)

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

di-Sodium hydrogen orthophosphate anhydrous, $Na_2HPO_4$ (Carlo Erba)

Dithiothreitol (Pharmacia)

Ethidium bromide (Sigma)

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

Formaldehyde, $CH_2O$ (BDH)

Formamide deionized (Sigma)

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

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

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

Glycerol, $C_3H_8O_3$ (Scharlau)

Glycine, $NH_2CH_2COOH$ (Scharlau)

Hydrochloric acid (HCl) (MERCK)

IQ SYBR Green Supermix (Bio-Rad)

Imidazole (Fluka)

Isopropanol, $C_3H_7OH$ (MERCK)

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

Kanamycin (BIO BASIC Inc.)

Laminarin from Laminaria (Sigma)

Levamisole (Sigma)

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

Magnesium chloride ($MgCl_2$) (MERCK)

Methanol, $CH_3OH$ (MERCK)

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

N, N', methylenebisacrylamide (Fluka)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Normal sheep serum (Roche)

Nytrans® super charge nylon membrane (Schleicher&Schuell)

Paraformaldehyde (Sigma)

Peptidoglycan from Staphylococcus aureus (Sigma)

pET28b(+) vector (Novagen)

Phenol, saturated (MERCK)

Prestained protein molecular weight marker (Fermentas)

RNA markers (Promega)

Salmon sperm DNA (Sigma)

Skim milk powder (Mission)

Sodium acetate, $CH_3COONa$ (Carlo Erba)

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

Sodium chloride, NaCl (BDH)

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

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

Sodium hydroxide, NaOH (Eka Nobel)

Triethanolamine (Unilab)

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

Triton® X-100 (MERCK)

Trizol reagent (Gibco BRL)

Tryptic soy broth (Difco)

Tween™-20 (Flula)

Urea (Fluka, Switzerland)

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



## 2.3 Kits and enzymes

ImProm-II™ Reverse Transcription system kit (Promega)

Mini Quick Spin RNA Columns (Roche Applied Science)

NucleoSpin® Extract II Kits (MACHEREY-NAGEL)

pGEM®-T Easy Vector Systems (Promega)

QIAprep® Miniprep kits (QIAGEN)

QuickPrep® Micro mRNA Purification Kit (GE Healthcare)

SMART™ RACE cDNA Amplification Kit (Clontech)

*Bam*HI (Biolabs)

DyNazyme II DNA polymerase (Finnzymes)

*Eco*RI (Biolabs)

Elastase (Sigma)

*Hin*dIII (Biolabs)

*Nco*I (Biolabs)

*Not*I (Biolabs)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

*Sac*I (Biolabs)

*Sal*I (Biolabs)

Subtilisin (Sigma)

T3 & T7 RNA polymerase (Roche)

T4 DNA ligase

Trypsin (Sigma)

α-Chymotrypsin (Sigma)



## 2.4 Microorganisms

*Escherichia coli* strain Rosetta (DE3)

*Escherichia coli* strain XL-I blue

*Escherichia coli* 363

*Vibrio harveyi* 1526

*Staphylococcus aureus*

## 2.5 Software

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

Clustal X (Thompson, 1997)

GENETYX (Software Development Inc.)

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

PHYLIP (Felsenstein, 1993)

SECentral (Scientific & Educational Software)

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

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

## 2.6 Animals

Healthy black tiger shrimp (*Penaeus monodon)* and white shrimp (*Litopenaeus vannamei*) sub-adults (20-25 g of body weight) were purchased from commercial farms in Thailand and acclimated in an aquarium under laboratory condition (15 ppt salinity artificial seawater with air-pumped circulating and at the ambient temperature; 28 ± 4 $^{o}$C) at least 3 days before the experiment. They were divided into three separate tanks that were normal shrimp, shrimp injected with 0.85% (w/v) NaCl and *Vibrio harveyi* 1526-challenged shrimp, respectively.

## 2.7 Preparation of *V. harveyi* challenged shrimps and diagnosis of *V. harveyi* infection

*V. harveyi* 1526 was kindly provided by Charoenpokphand Company and activated on tryptic soy agar (TSA) plate. A single colony of *V. harveyi* was inoculated in tryptic soy broth (TSB) medium supplemented with 2% (w/v) NaCl at 30 °C overnight. The starter was cultured in new TSB medium by shaking at 30 °C until an optical density at 600 nm ($OD_{600}$) reached 0.6. The cultured bacteria in mid-logarithmic phase growth were then diluted 1:100 with a sterile normal saline solution (0.85% NaCl, w/v) and plated on TSA for colony counting (colony forming units; CFU) (Wood, 1988). One hundred microliters of the $10^6$ CFU/ml diluted cultures was intramuscularly injected into the $4^{th}$ abdominal segment, whereas the control group was injected with 100 μl of 0.85% NaCl (w/v).

*V. harveyi* infection of shrimp was assayed by streaking the suspension of hepatopancreas fluid on TSA plates and incubating at 30 °C overnight as described by Ruangpan (1999). Colonies of *V. harveyi* 1526 from infected shrimps revealed green luminescence in the dark.

## 2.8 Total RNA extraction

Hemolymphs were taken from the ventral-sinus cavity of shrimp and withdrawn into the 1 ml syringe with a 26 gauge-needle containing one-forth volume of the pre-cooled anticoagulant (10% sodium citrate) (Figure 2.1). Hemocytes were immediately harvested from individual shrimp by centrifugation at 500xg for 10 min at 4 °C to separate the plasma from hemocytes. Total RNA from hemocytes of the black tiger shrimp and the white shrimp were extracted using Trizol reagent (Gibco

BRL). RNA and DNA were separated by the selective partition after adjusting the pH of a phenolic lysate as previously described (Chomczynski and Sacchi, 1987). The RNA was recovered in the aqueous phase at acidic pH whilst DNA in the sample was retained in the interface and organic phase. The hemocyte pellets were resuspended and homogenized in 1 ml of Trizol reagent. The homogenates were incubated at room temperature for 5 min to serve complete segregation of nucleoprotein complexes. Then, 200 μl of chloroform were added and vigorous shaken for 15 seconds. After incubation at room temperature for 5 min, the mixtures were centrifuged at 12,000x*g* for 15 min at 4 °C. Following transfer of the RNA-containing aqueous phase to a fresh tube, total RNA was precipitated by addition of one volume of absolute isopropanol, then incubating at -20 °C for 15 min. The supernatant was removed by centrifugation as above and the total RNA pellets were washed with 500 μl of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water. The total RNA was stored in 70% (v/v) ethanol at -80 °C until used. The ethanol supernatant was completely removed by centrifugation at 12,000x*g* for 15 min at 4 °C. The RNA pellets were dried at room temperature for 10-15 min and dissolved in an appropriate amount of DEPC-treated water.

Acclimatized shrimps Challenged shrimps Hemolymph collection

**Figure 2.1** Overview of hemolymph sample collections

## 2.9 Determination of the quantity and quality of RNA samples

The quantity and quality of total RNA was spectrophotometrically measured at 260 nm and analyzed by formaldehyde-agarose gel electrophoresis, respectively.

The concentration of total RNA could be determined in ng/μl using the following formular; [RNA] = $A_{260}$ x dilution factor x 40[†]

[†] An OD unit at 260 nm corresponds to approximately 40 ng/μl of RNA (Sambrook et al., 1989). The relative purity of RNA samples was examined by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at the wavelength of 280 nm. The good quality of RNA sample should have an $A_{260/280}$ ratio above 1.7.

The quality of the extracted RNA was analyzed by formaldehyde-agarose gel electrophoresis as following described. A 1.2% (w/v) formaldehyde agarose gel was prepared in 1x MOPS buffer (final concentration of 0.2 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0). The gel slurry was heated until completely dissolving and placed to cool down at room temp before formaldehyde (0.66 M final concentration) was added. Then, the melted formaldehyde-agarose gel was poured into a chamber set and applied the comb. The RNA marker and RNA samples were prepared under the denaturing condition. The RNA sample in DECP-treated water, 7.4 M of formamide, 1.64 M of formaldehyde, 1x MOPS and DECP-treated water to a final volume of 12 μl were heated at 70 °C for 10 min and the mixtures were immediately chilled on ice. After that, three micro litters of the 5x RNA loading dye buffer containing 50% (v/v) glycerol, 1mM EDTA, pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 0.025% (w/v) ethidium bromide was added to each sample and loaded to formaldehyde-agarose gel. Electrophoresis was run in 1x MOPS buffer at 100 volts

for 50 min. Sizes of RNA were visualized under a UV transiluminator by comparing with a standard RNA marker (Promega).

## 2.10 DNase treatment of total RNA samples

The obtained total RNA was further treated with RQ1 RNase-free DNase (Promega, 1 units/5 μg of the total RNA) at 37 °C for 30 min to remove the contaminating chromosomal DNA. Then, the RNA pellets were purified by phenol/chloroform extraction following by ethanol precipitation. Briefly, the reaction volume was adjusted to 40 μl with DEPC-treated water, 250 μl of Trizol reagent were added and vortex for 10 sec. Two hundred microliters of chloroform was then added and vigorously shaken for 15 sec. The resulting mixture was stored at room temperature for 2-5 min and centrifuged at 12,000xg for 15 min at 4 °C. The RNA in upper phase was precipitated by isopropanol and washed 70% (v/v) ethanol. After that, RNA pellet was briefly air-dried and dissolved with an appropriate amount of DEPC-treated water. The concentration of DNA-free total RNA was determined as described in 2.9



## 2.11 mRNA purification

The QuickPrep® Micro mRNA Purification kit (Pharmacia Biotech) was provided for rapid mRNA isolation that is essentially free of contaminating proteins, nucleic acids and carbohydrates. The concept of this method base on the poly(A)$^+$ mRNA attached to oligo(dT)-cellulose and eluted with low ionic strength buffer. The mRNA was purified from total RNA following the manufacturer's instructions The 1

ml oligo(dT)-cellulose was homogeneously swirled and immediately pipetted into a 1.5 ml microcentrifuge tube. After centrifugation for 1 min, the buffer was removed. The total RNA was applied onto the oligo (dT)-cellulose and gently mixed by inverting for 3 min. The mixture was centrifuged at the max speed for 1 min. The supernatant was removed and resin was washed with the high-salt buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA, 0.5 M NaCl) five times followed by three times with the low-salt buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA, 0.1 M NaCl). Next, the resin was resuspended with 300 μl of the low salt buffer and transferred to a microspin column placed on a microcentrifuge tube. After spin down, the column was added with 500 μl of the low-salt buffer three times and centrifuged at the full speed for 1 min. The flow through in collection tube was discarded. After that, the column was placed in a fresh 1.5 ml microcentrifuge tube. Two hundred microliters of pre-warmed (65 °C) elution buffer (10 mM Tris-HCl; pH 7.5, 1 mM EDTA) was added to the top of the resin bed. The eluted mRNA was collected by centrifugation at the full speed for 1 min. The eluted mRNA was kept at -80 °C until used.

## 2.12 Identification of the full-length cDNA of *Pm*MasSPH and *Lv*MasSPH using the Rapid Amplification of the 5’ and 3’ cDNA Ends (5’ and 3’ RACEs) technique

RACE technique was used to identify the complete cDNA sequences as described below.

### 2.12.1 First strand RACE-cDNA synthesis

The first strand RACE-cDNA was synthesized according to the manufacturer’s instructions (Figure 2.2). Briefly, mRNA (3 μg) obtained from shrimp

hemocyte were mixed with SMART II™ A oilgonucleotide and 5'-RACE CDS primer (for 5'RACE cDNA library) or 3'-RACE CDS primer (for 3'RACE cDNA library). The mixtures were pre-heated for 10 min at 80 $^{o}$C and immediately cooled on iced water for 10 min. The first strand cDNA was synthesized using PowerScript reverse transcriptase at 42 $^{o}$C for 2 h in a hot-lid thermal cycle. The reaction contains 2 µl of 5x First-Strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl and 30 mM $MgCl_2$), 1 µl of 20 mM DTT, 1 µl of 10 mM dNTP Mix and 1 µl of BD PowerScript™ Reverse Transcriptase. Finally, the RACE cDNA were diluted with 250 µl of Tricine-EDTA buffer (10 mM Tricine-KOH, pH 8.5 and 1 mM EDTA and heated at 72$^{o}$C for 10 min. The obtained 5' and 3'- RACE cDNA were kept at -20 $^{o}$C until used.

### 2.12.2 Primer designation

For *Pm*MasSPH, the 5'gene-specific primer (PmRACE) was designed from EST sequence (accession number BI784455) of a serine proteinase homologue from the hemocyte cDNA library of *P. monodon* (Supungul et al., 2002) using SECentral program (Scientific & Educational Software). The full-length sequence of *Lv*MasSPH cDNA was identified by 5' and 3' RACE technique using firstly primer base on the *Pm*MasSPH sequence.

Primer sequences for RACE-cDNA synthesis and RACE-PCR were listed in Table 2.1. The gene-specific primers designed here produce overlapping RACE products as shown in Figure 2.3.

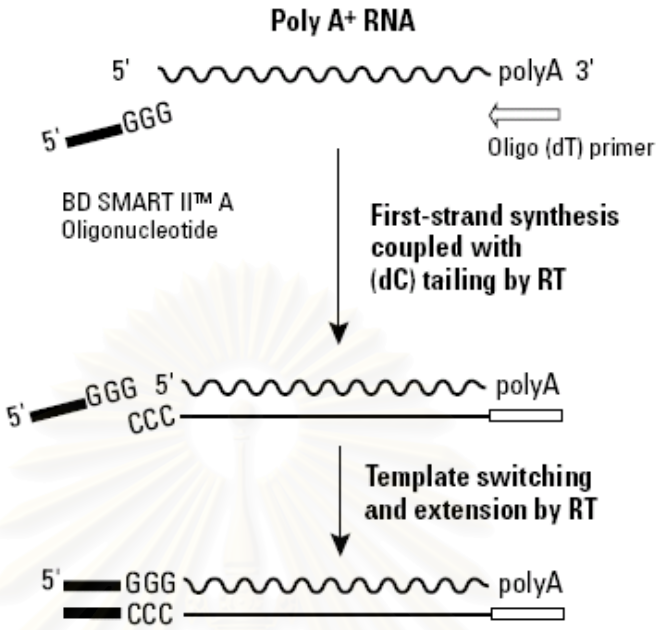



**Figure 2.2** Principle of SMART™ RACE cDNA synthesis (Clontech) First-strand cDNA was synthesized using a modified oligo(dT) primer. The BD PowerScript™ reverse transcriptase exhibits terminal transferase activity after it reaches the end of the mRNA template, adding several dC residues to the 3' end of the first-strand cDNA. The BD SMART II A Oligonucleotide contains a terminal stretch of G residues that anneal to the dC-rich cDNA tail and serves as an extended template for BD PowerScript Reverse Transcriptase.

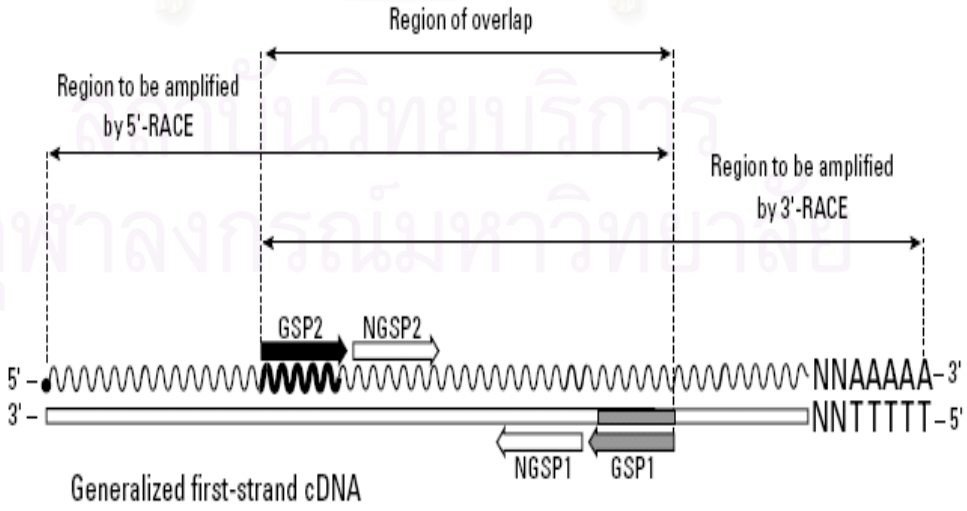



**Figure 2.3** The location of gene specific primer within gene (Clontech)

**Table 2.1** Primer sequences for RACE-cDNA synthesis and RACE-PCR

| Species | Primer name | Sequence | Tm (°C) |
|---|---|---|---|
| *P. monodon* | **Full length& 5'RACE** | | |
| | 5PmRACE | 5' TTCTCCCTTCCGAAAGCATCACTGGT 3' | 64 |
| | 5UTR-PmMas | 5' TGGAGAGAGAGGAAGAGAGAAGGTCGCT 3' | 63 |
| *L. vannamei* | | | |
| | PmMas-F | 5' TGCTTCTTTTGGAAGGGAGAATGC 3' | 70 |
| | PmMas-R | 5' ACGTGTCGATCCCCTGCTGGC 3' | 70 |
| | **5'RACE** | | |
| | 5LvRACE1 | 5' AACAATTCGTTCATCATCGCT 3' | 53 |
| | 5LvRACE2 | 5' TGTATCGTTGCATCCTCCCTT 3' | 56 |
| | **3'RACE** | | |
| | 3LvRACE3 | 5' GGAGAGTGGGACACCCAG 3' | 57 |
| | 3LvRACE4 | 5' CAAGGAAGTGGCTCTCCCTGT 3' | 59 |
| | **Full length** | | |
| | 5UTR-LvMas | 5' AGCGAGAGGGAAGGACGAATTGTG 3' | 68 |
| | 3UTR-LvMas | 5' TGTATCTCCTGCTCCGCCCAATG 3' | 68 |
| **Kits** | | | |
| **RACE-cDNA synthesis** | | | |
| SMART II™ A Oligonucleotide | | 5'AAGCAGTGGTATCAACGCAGAGTACGCGGG 3' | |
| 3'-RACE CDS | | 5'AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN* 3' | |
| 5'-RACE CDS | | 5' $(T)_{25}$V N* 3' | |
| **RACE-PCR** | | | |
| 10x Universal Primer A (UPM) | | Long : 5' CTAATACGACTCACTATAGGGCAAGCAGTG GTATCAACGCAGAGT 3' | |
| | | Short : 5' CTAATACGACTCACTATAGGGC 3' | |
| Nested Universal Primer (NUP) | | 5' AAGCAGTGGTATCAACGCAGAGT 3' | |

* N = A, C, G, or T; V = A, G, or C

### 2.12.3 Rapid amplification of cDNA ends (RACE)-PCR

For *P. monodon*, 5PmRACE and UPM primers were used for 5' RACE-PCR. The amplification reaction for GC-rich amplicon was performed using DyNAzyme II DNA Polymerase (Finnzymes) in the presence of 2.5% DMSO. 5'RACE-PCR of *Pm*MasSPH was performed in a 50 µl reaction volume containing 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% gelatin), 1.5 mM of $MgCl_2$, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 2.5% of DMSO, 0.4 µM of a RACE gene specific primer (GSP), 2.0 unit of DyNAzyme II DNA Polymerase (Finnzymes), 5 µl of 10x UPM and 2.5 µl of 5' RACE-Ready cDNA template. PCR conditions were as follows: five cycles consisting of 94 $^{o}$C for 45 sec, 68 $^{o}$C for 45 sec and 72 $^{o}$C for 2 min; and 25 cycles consisting of 94 $^{o}$C for 45 sec, 55 $^{o}$C for 45 sec and 72 $^{o}$C for 2 min. The final extension was carried out at 72 $^{o}$C for 10 min.

No sequence information was available for the cDNA of *Lv*MasSPH from *L. vannamei* so the first ampicon was amplified using the PmMas-F and PmMas-R primers which were designed from the sequence of *Pm*MasSPH. The 5' and 3' RACE-PCR of *Lv*MasSPH were firstly amplified using UPM and GSP primers (5LvRACE1 and 3LvRACE3 primers, respectively). The RACE-PCR reaction and conditions were performed the same as RACE-PCR of *Pm*MasSPH except the addition of DMSO. The nested PCR strategy was employed to increase specificity using NUP and nested GSP primers (5LvRACE2 and 3LvRACE4 primers for the second round of 5' and 3' RACE-PCR, respectively) as described in Table 2.1. Five microliters of the 50 fold diluted PCR product was used as the template for nested PCR under the condition similar to the first PCR. The PCR products of the first and nested PCR were assessed by electrophoresis using 1x TBE buffer.

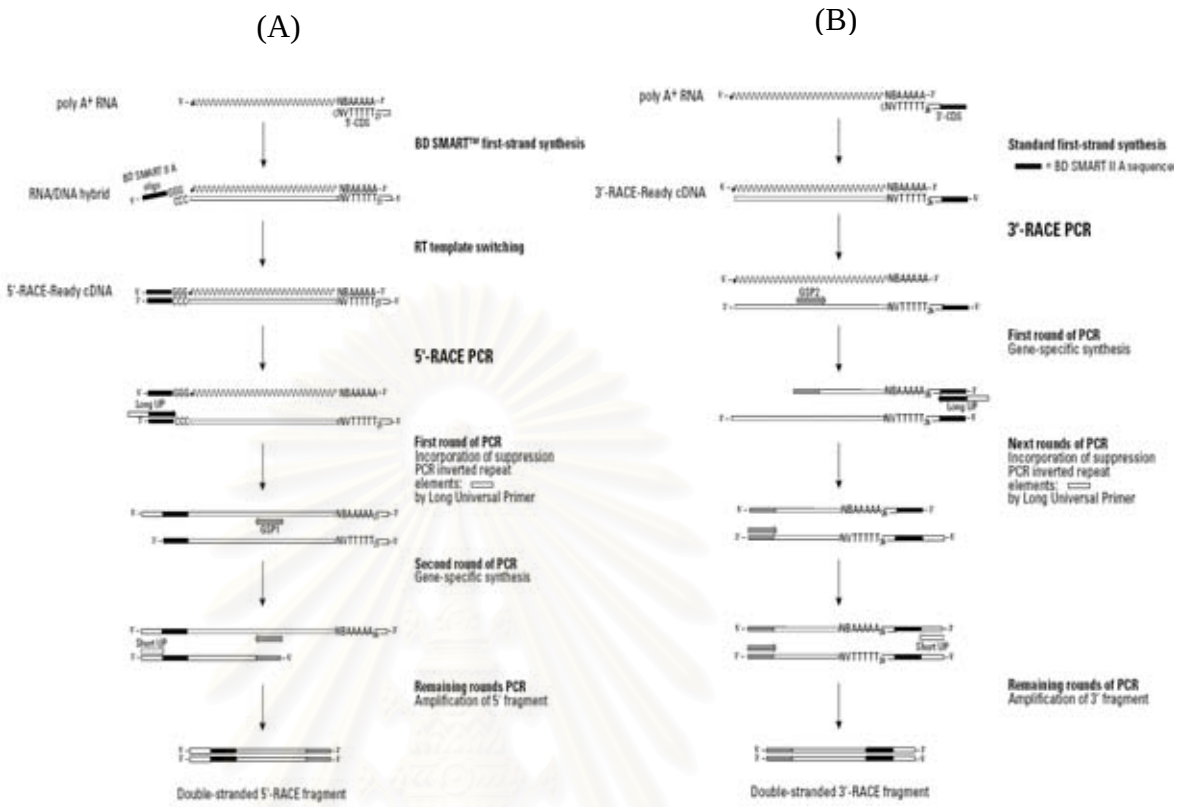



**Figure 2.4** Overview of the BD SMART™ RACE procedure (Clontech)
A and B represent the mechanism of the 5'-RACE and 3'-RACE reactions, respectively.

### 2.12.4 Amplification of the complete sequence of the *Pm*MasSPH and *Lv*MasSPH

Amplification of a single fragment of the *Pm*MasSPH and *Lv*MasSPH were carried out using the 5'and 3' untranslated primers: 5PmRACE, 5UTR-PmMas, 5UTR-LvMas and 3UTR-LvMas, respectively (Table 2.1). The amplification reaction was performed using BD Advantage 2 polymerase mix under the following conditions: pre-denaturated at 94 $^{o}$C for 3 min followed by 30 cycles of a 94 $^{o}$C denaturation step for 1 min, a 68 $^{o}$C annealing step for 1 min and a 72 $^{o}$C extension step for 2 min. The final extension was carried out at 72 $^{o}$C for 10 min.

### 2.12.5 Agarose gel electrophoresis

The PCR products were electrophoretically analysed by agarose gel which was prepared by melting the slurry 1.2% (W/V) agarose gel in 1x TBE (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) using the microwave oven. After the gel solution was cool down at 55 °C pouring into a tray and applied the well comb. Before loading into the gel, the PCR products were combined with 1/10 volumes of the 10x loading dye (0.25% bromophenol blue, 0.25% Xylene cyanol FF, and 25% Ficoll in water). The GeneRuler™ 100 bp and 1 kb DNA Ladder plus (fementas) were used for identifying of the DNA size. The agarose gel electrophoresis was performed in 1x TBE buffer at 100 volts until the lower bromophenol blue dye migrated about 3/4 of the gel length. After that, the gel was stained in a 2.5 μg/ml ethidium bromide (EtBr) solution for 30 sec and destained by submerged in distilled water for 10 min. Ethidium bromide-stained PCR products were visualized under the UV transilluminator.

### 2.12.6 Purification of PCR product from agarose gel

The expected bands were purified from agarose gel by NucleoSpin® Extract II Kits (MACHEREY-NAGEL) as describe below: The expected product was excised from the gel using a clean sharp scalpel, and then the weight of the gel slice was determined. The gel slice was completely dissolved in three volumes of NT buffer containing chaotropic salt at 60$^{o}$C. The sample was then loaded into the column and centrifuged at 12,000x g for 1 min to remove the supernatant. The column was washed with 500 μl of NT2 buffer and centrifuged as described above. Six hundred microliters of NT3 buffer were added into the column and centrifuged. The additional

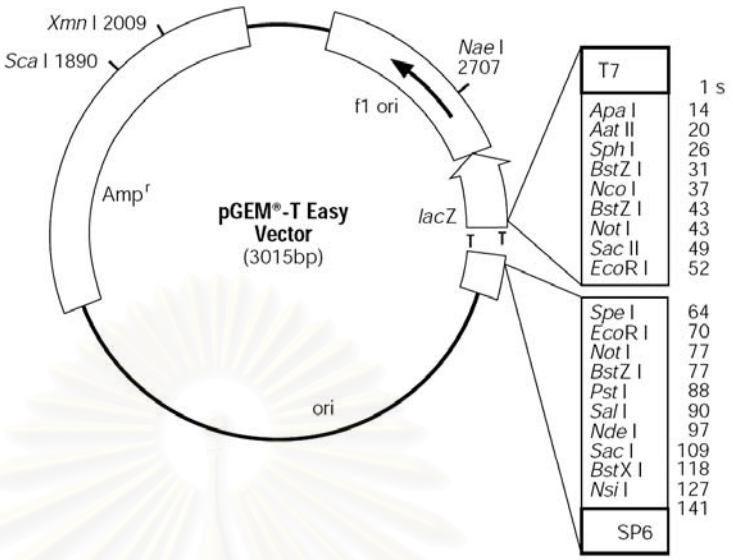
centrifugation was used for completely removal of the NT3 buffer containing ethanol. The column was placed into a clean 1.5 ml microcentrifuge tube. The DNA was eluted with 40 µl of elution NE buffer (5 mM Tris-Cl, pH 8.5) and stood at room temperature for 1 min before centrifugation. The eluted DNA was then stored at -20 °C until used.

### 2.12.7 Cloning of DNA fragment into pGEM-T Easy

The DNA fragment was ligated into pGEM-T Easy vector (Figure 2.5). The pGEM-T Easy vector was 3,015 bp in length and had unique restriction sites in the multiple cloning region flanked by T7 and SP6 RNA promoters, therefore T7 and SP6 primers can be used to identify the recombinant clone. The reaction was composed of 5µl of 2x Rapid ligation buffer, 1µl of pGEM®-T Easy Vector (50ng), proper amount of PCR product, 1µl of T4 DNA ligase (3 Weiss units/µl), and deionized water to a final volume of 10 µl. The reactions were mixed by pipetting, briefly spun and incubated at 4 °C overnight. The appropriate amount of insert in the ligation reaction was calculated following equation:

$$\text{ng of insert} = \left( \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \right) \times \text{insert:vector molar ratio}$$

(A)




(B)

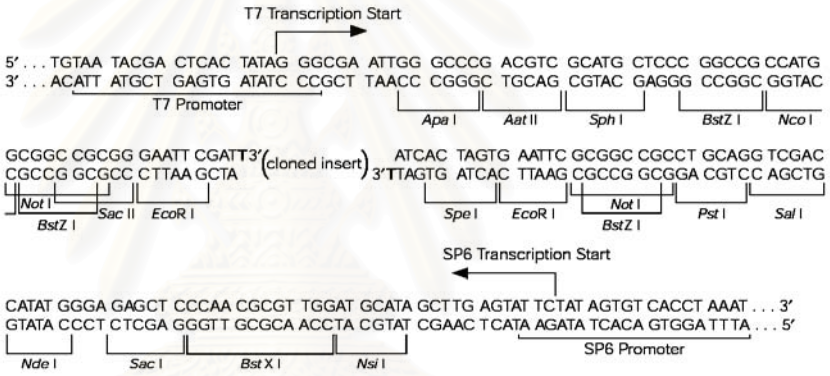



**Figure 2.5** pGEM®-T easy vector map (A) and multiple cloning site sequences (B) (Promega)

### 2.12.8 Competent cells preparation

The starter of *E. coli* strain XL-1 blue was prepared from a single colony cultured in 10 ml of LB broth (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl) and cultured at 37 $^{o}$C with shaking at 250 rpm overnight. One percent of starter was inoculated into 1 L of LB broth and incubated at 37 $^{o}$C with vigorous shaking for 3-5 h until $OD_{600}$ of the cells reached 0.5-0.7. Cells were then chilled on ice for 15-30 min and harvested by centrifugation at 5,000x g for 10 min at 4 $^{o}$C. The supernatant was removed as much as possible. The cell pellet was

washed twice time with cold sterilized water and followed by cold sterilized 10% (v/v) glycerol with gently mixing and centrifugation. The pellet was suspended in cold sterilized 10% (v/v) glycerol to a final volume of 2-3 ml. This cell suspension was divided into 40 µl aliquots and stored at -80 °C until used.

### 2.12.9 Electrotransformation

The ligation reaction was transformed to *Escherichia coli* XL-1 Blue. The competent cells were gently thawed on ice, mixed with 1 µl of ligation mixture and then placed on ice for 1 min. The mixture was transformed by electroporation in a cold 0.2 cm cuvette with setting the apparatus as follows: 25 µF of the Gene pulser, 200 Ω of the pulse controller unit, and 2.50 kV of the Gene pulser apparatus (Bio-RAD). After electroporation, SOC medium (2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose) was immediately added to the cuvette and quickly resuspended cells. The cell suspension was transferred to a new tube and incubated at 37 °C with shaking at 250 rpm for an hour. One hundred microliters of transformant was then spread onto a LB agar plate containing 100 µg/ml of amplicillin, 20 µg/ml of X-gal and 30 µg/ml of IPTG and then incubated at 37$^{o}$C for overnight. After incubation, the recombinant clone was identified by colony PCR using universal T7 and SP6 primers.

### 2.12.10 Screening of transformant by colony PCR

White colonies were picked and screened for the inserts by colony PCR. The amplification was carried out in a 20 µl reaction volume containing 1x PCR buffer, 200 µM of dNTP mix, 0.25 µM of T7 and SP6 primers, 2.5 units of DyNAzyme™ II

DNA polymerase. The single colony was diluted in 10 µl sterilized water. One microliter of colony suspension was employed as the template in the PCR reaction. The PCR profile was performed at 94 °C for 3 min, 30 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis.

### 2.12.11 Plasmid DNA Extraction using QIAprep® Miniprep kit

The plasmid was isolated from the positive clones by a QIAprep® Miniprep kits described in Qiagen's handbook. The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane under high salt condition. Firstly, bacterial cells were harvested by centrifugation and resuspend in 250 µl P1 buffer containing RNase A. Next, the 250 µl P2 buffer was added and mix thoroughly by inverting the tube 4–6 times for cell lyses. The cell lysate was neutralized by adding 350 µl N3 buffer. After maximum speed centrifugation for 10 min, the supernatant containing the plasmid was applied to column by pipetting. The column was centrifuged for 30–60 s, and then the flow-through was discarded. The QIAprep spin column was washed twice by adding 0.5 ml Buffer PB and 0.75 ml Buffer PE, respectively, and then centrifuged to remove residual ethanol from PE Buffer. Finally, the QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl EB buffer (10 mM Tris-HCl, pH 8.5) to the center of each column. After incubation at room temperature for 1 min, the eluted fraction was collected by centrifugation for 1 min.

**2.12.12 Detection of the recombinant plasmid**

The recombinant plasmid contained interested gene was examined with restriction enzyme digestion using *Eco*RI. The digested plasmid was analyzed by agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (100 bp and 1kb ladder marker). The recombinant plasmid was sequenced by an automatic DNA sequencer at the Macrogen Inc.

**2.12.13 DNA sequence analysis**

DNA sequences were edited and translated using the GENETYX software program (Software Development Inc.). The sequences were further compared with data in the GenBank (http://www.ncbi.nlm.nih.gov) using the BlastX program (Altschul et al., 1997). The significant probabilities and identity were considered from E-values < $10^{-4}$ and the match included > 10 amino acid residues for BlastX (Supungul et al., 2004). Putative motifs and domains were investigated using SMART program. Related sequences were searched in GenBank and aligned using Clustal X program (Thompson et al., 1997). The potential cleavage site of the signal peptide and putative N-Glycosylation sites were predicted by SignalP software (http://cbs.dtu.dk/ services/SignalP/) and NetNglyc software (http://cbs.dtu.dk/ services/NetNGlyc), respectively. Aligned sequences were bootstrapped 1000 times using Seqboot. Sequence divergence between different serine protease (SP) and serine proteinase homologue (SPH) was calculated based on the two-parameter method using PRODIST (Kimura, 1980). Boostrapped neighbour-joining trees were constructed using Neighbour and Consense. All phylogenetic reconstruction programs are routine

in PHYLIP (Felsenstein, 1993). Trees were appropriately illustrated using TreeView (http://taxonomy.zoology.gla.ac.uk/rod.html).

## 2.13 Analysis of the expression and localization of *Pm*MasSPH gene using *in situ* hybridization

*In situ* hybridization technique allows specific nucleic acid sequences to be detected in morphologically preserved cells or tissue sections.

### 2.13.1 Hemocyte preparation

Shrimp were divided into two groups. One group was the *V. harveyi* challenged shrimp and another group was the control shrimp, which were injected with normal saline. The hemolymph was collected at 0, 6, 24, 48, and 72 h after injection using Modified Alsever Solution, MAS (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, and 9 mM EDTA, pH 7.0) as an anticoagulant. The harvested hemocytes were resuspended and fixed in freshly prepared ice-cold 4% paraformaldehyde in MAS for 10 min. After centrifugation as above, the plasma proteins were removed from hemocyte by washing twice with MAS. Then, the hemocytes were resuspended in MAS and the hemocyte concentration was determined using a hemocytometer counting under microscope. The hemocytes ($2x10^5$ cells) were coated onto the poly-L-lysine coated slide using the cytospin at 1,000xg for 5 min. The hemocytes coated slides were dried at room temperature for few minutes and stored at 4 $^{o}$C before used.

### 2.13.2 Digoxigenin (DIG)-labeled riboprobe preparation

The recombinant plasmid containing the *Pm*MasSPH gene was obtained from the PCR amplification using Pmsitu_F primer: 5' ATAAGAATGCG GCCGCAAATAAATCTTCCTAGTCCC 3' and Pmsitu_R primer: 5' TACGTACT CATTGATATCAGGTTTGG 3' under standard conditions. The amplicon was cloned into pGEM-T easy vector and the sequence correction was determined by sequencing. The recombinant plasmid was linearized by restriction enzyme digestion: *Bam*HI and *Sac*II to produce the sense and antisense probes, respectively. The linearized plasmids were precipitated by ethanol precipitation and quantified on agarose gel electrophoresis using GeneRuler™ 1kb DNA ladder (Fermentas) as standard DNA marker.

Digoxigenin (DIG)-labeled probes were transcribed by *in vitro* transcription using T7 polymerase for sense probe and Sp6 polymerase for antisense probe. The reaction volume of 20 µl contains 1 µg of purified linearized plasmid, 2 µl of 10 x concentrated transcription buffer (400 mM Tris-HCl, pH 8.0, 60 mM MgCl2, 100 mM Dithiothreitol (DTT), and 20 mM spermidine), 2 µl of 10x concentrated DIG RNA labeling mix (10 mM each of ATP, CTP, and GTP, 6.5 mM UTP, 3.5 mM DIG-UTP; pH 7.5), 40 units of T7 or Sp6 RNA polymerase. The components were mixed and incubated at 37 $^{o}$C for 2 h. The transcripts were then treated with two units of *RNa*se-free *DNa*se I incubating at 37 $^{o}$C for 15 min. The polymerase reactions were terminated by adding 1 µl of 0.5 M EDTA, pH 8.0. The labeled RNA transcripts were purified by Mini Quick Spin RNA Columns (Roche) to remove unincorporated nucleotides from labeled RNA. The mini Quick Spin Columns contains the G-50 Sephadex in 1x STE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 100 mM

NaCl). The column was prepared to resuspend column matrix. The column was removed the top cap, and then snapped off bottom tip, and placed into sterile microcentrifuge tube. Next, column was spun at 1000xg for 1 min to pack the column and remove the buffer. The sample was carefully applied to center of bed, centrifuged again for 4 min. The eluate containing the nucleic acids was recovered and stored at -20°C until used. Each labeled RNA transcript was quantified by agrose gel electrophoresis.

**2.13.3 Negative controls**

Two negative control experiments were treated with the sense riboprobe and RNase-treatment antisense riboprobe, respectively. For RNase-treatment antisense riboprobe, 1:500 diluted of 1 mg/ml of RNase A was added onto the hemocytes coated slides and incubated at 37°C for 30 min. The slides were then washed three times with TBS (0.1 mM Tris-HCl pH 7.4 and 0.9% NaCl) for 5 min before proceeding to the prehybridization step.

**2.13.4 Pre-hybridization treatments**

The hemocyte coated slides were washed twice with 0.2 M Tris-HCl, pH 7.4 for 5 min each and 0.2 M Tris-HCl, pH 7.4 containing 0.1 M glycine for 10 min, respectively. The cells were rinsed with PBS (0.1 M phosphate buffer, pH 7.4 containing 0.9% NaCl) for 5 min. After fixation in 4% paraformaldehyde in PBS buffer containing 5 mM MgCl2 for 15 min, the slides were rinsed again with PBS buffer for 5 min and incubated in 0.1 M triethanolamine, pH 8.0 containing 0.25% acetic anhydride for 10 min. The coated slides were then dehydrated in 30%, 70%,

and 100% ethanol for 5 min each. The cells were dried at room temperature for at least 2 h.

### 2.13.5 Riboprobe hybridization

The hybridization solution containing the DIG-labeled riboprobes (40-100 ng per slide) in 2x SSC buffer (diluted from 20x SSC; 300 mM sodium citrate pH 7.0 and 3 M NaCl), 0.5 mg/ml salmon sperm DNA, 10% dextran sulfate, 10x denhart's solution, 50% formamide, 0.5 mg/ml tRNA from *E.coli* and 100 mM dithiothreitol was prepared and denatured at 55 °C for 10 min. The mixture was immediately chilled on ice for 5 min, then dropped onto the slides and covered the glass slip. The hemocyte coated slides were incubated at 55 °C overnight in adequate humid chamber (1x SSC and 30% formamide). The slides were washed twice with 2x SSC for 15 min each shaking in a platform shaker and then treated with 20 μg/ml RNase A in 2x SSC at 37°C for 30 min. After that, the slides were sequentially rinsed with 1x SSC, 0.5x SSC and 0.1x SSC supplemented with 0.07% 2-mercaptoethanol at room temperature for 10 min each. Subsequently, they were additional washed twice with 0.1x SSC containing 0.07% 2-mercaptoethanol at 55°C for 30 min. The slides in the last solution were placed on ice approximately an hour.

### 2.13.6 Riboprobe detection using alkaline phosphatase-conjugated sheep anti-DIG antibody

Before the detection step, the slides were washed to remove the mercaptoethanol with 0.1x SSC for 5 min and rinsed twice with TBS containing 0.05% of Triton X-100 for 5 min. The slides were pre-incubated in blocking solution

(TBS supplemented with 1% normal sheep serum and 0.05% Triton X-100) for 20 min. Next, 500 µl of antibody solution (1:1000 diluted alkaline phosphatase-conjugated sheep anti-DIG antibody in blocking buffer) were added to each slide and incubated overnight in a humid chamber. All steps were done at the room temperature. After incubation, the slides were washed three times in TBS for 10 min each and 2 times in detection buffer (0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, and 0.9% NaCl) for 5 min each. Five hundred microliters of substrate solution (375 µg/ml of NBT; nitroblue tetrazolium dissolved in 70% dimethyl formamide, 188 µg/ml of BCIP and 1 mM levamisole in detection buffer) were overlaid onto the slides and incubated in the dark condition. After the optimal development, the reactions were terminated by washing in TBS for 10 min and briefly rinsed with distilled water. The slides were mounted with a glycerol solution (10% glycerol in TBS) and kept at 4°C. Positive cells were counted under microscope and the percentage of positive cells was calculated from the number of purple stained cells divided by total cells. The statistical calculations were analyzed by ANOVA and DUNCAN.

## 2.14 Investigation of the *Lv*MasSPH gene expression using quantitative Real-Time RT-PCR

Quantitative Real-Time RT-PCR is based on amplify and simultaneously quantify a targeted DNA. The key feature is that the quantity of amplified DNA after each amplification cycle was detected as it accumulates in the reaction in real-time. The basic method of quantification is the use of fluorescent dye labeled nucleotide that intercalate with double-stranded DNA.

### 2.14.1 Challenge experiment and preparation of the first-stranded cDNA from *L. vannamei* hemocytes

The *V. harveyi* 1526 challenged white shrimps and the normal saline injected shrimps (the control) were prepared as described in 2.7. The total RNA samples were individually extracted from hemocytes at different time 0, 6, 24, 48 and 74 h post-injection as described in 2.8. Each time point, the total RNA from three individual shrimps were pooled in each tube and prepared in triplicate reactions. Then, the total RNAs were subsequently treated with RQ1 RNase-free DNase (Promega) and determined the quantity as well as quality of RNA samples as mention above.

DNA-free total RNAs from each time point were used as the template for first strand cDNA synthesis. The first-strand cDNAs were synthesized using the ImProm-II™ Reverse Transcriptase System kit (Promega) according to the manufacturer's protocol. The reactions consisting of 1 μg of total RNA and 0.5 μg of oligo ($dT_{18}$) primer were heated at 70 °C for 5 min and immediately chilled on ice for 5 min. After that, 4 μl of 5x reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of dNTP Mix (10 mM each), 20 units of Ribonuclease inhibitor and 1 μl of ImProm-II reverse transcriptase were added and mixed by pipetting. The reactions were annealed by incubating at 25 °C for 5 min and at 42 °C for 60 min for extension. Then, the reverse transcriptase activity was inactivated by heating at 70 °C for 15 min. The constructed cDNAs were stored at –20°C until use.

### 2.14.2 Primer design

The expression pattern of a serine proteinase homologue from the white shrimp was examined using the primers LvRT-F and LvRT-R. The β-actin gene was used as an internal control for real-time PCR. The designed primers and real-time PCR conditions were shown in Table 2.2.

**Table 2.2** Primer sequence and real-time PCR conditions.

| Primer name | Sequence | Primer conc. (μM) | Denaturing (°C/sec) | Annealing (°C/sec) | Elongation (°C/sec) |
|---|---|---|---|---|---|
| LvRT-F<br>LvRT-R | 5' GTGGAGGGAGTCGGCGAGAAG 3'<br>5' AAACGGGCCTTGAGTGATCTTGC 3' | 0.2<br>0.2 | 95/10 | 55/15 | 72/20 |
| β-actin-F<br>β-actin-R | 5' GAACCTCTCGTTGCCGATGGTG 3'<br>5' GAAGCTGTGCTACGTGGCTCTG 3' | 0.1<br>0.1 | 95/10 | 55/15 | 72/20 |

### 2.14.3 Real-time RT-PCR

Real-time RT-PCR assay was accomplished by SYBR Green I dye detection using the iCycler iQ™ Real-Time detection system (Bio-Rad). The amplified reactions were done in a 96-well plate containing 10 μl of 2xSYBR Green supermix (Bio-rad), 5 μl of 1:50 diluted cDNA, the appropriate amount of each forward and reverse primer represented in Table 2.2 and adjusted the 20 μl final volume with sterilize water. The SYBR Green real-time RT-PCR condition was 95 $^{o}$C for 8 min followed by 40 cycles of denaturation, annealing and extension as shown in Table 2.2. Each experiment was carried out in triplicate and fluorescent data were monitored at the end of each extension step. The reactions containing sterile water instead of cDNA template were used as the negative control. At the end of amplification, the specificity of each primer pair was verified by assessing the melting curve of the PCR product.

The reactions were analyzed at 95 $^{o}$C for 1 min and afterwards 50 $^{o}$C for 1 min, followed by heating 80 cycles of staring at 50 $^{o}$C with 0.5 $^{o}$C increments for 10 sec each cycle.

### 2.14.4 Determination of PCR efficiency

Each gene was amplified by different specific primers, so it might be revealed the different PCR efficiency that was determined by constructing a standard curve. The standard curve of each gene was prepared by the same cDNA which was serially diluted in five fold dilution from 1:5 to 1: 3,125. Each amplified reactions were executed in triplicate including a negative control. After finishing the amplification, the data were analyzed the amplification plots and the standard curve by software of the iCycler iQ™ Real-Time detection system. The standard curve graphs of reference and target genes were plotted the Ct values against copy number of product (log scale). The linear graph should be obtained the correlation coefficient certainly more than 0.99 and PCR efficiency equal to $10^{-1/slope}$ in each graph. These PCR efficiency values were employed to calculate in relative expression ratio.

### 2.14.5 Data analysis of real-time RT-PCR

The fluorescent signals of the amplified products were analyzed by the data analysis software of the of iCycler iQ™ Real-Time Detection system (Bio-Rad) using the PCR baseline Subtracted curve fit method. The relative expression ratio was calculated using the value of the threshold cycles or Ct value. The threshold was a reference line that used for distinguish the gene amplified signal from background. The relative expression ratio analyses the amount of target transcript relative to an

internal standard (β-actin) in the same cDNA. The Ct values of the *V. harveyi*-injected samples at each time point were normalized with the Ct values of the saline-injected samples. A mathematical model in previous reported by Pfaffl (2001) was used for determine the relative expression ratio following the formula:

$$\text{Relative expression ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta Ct_{\text{ref}}(\text{control-sample})}}$$

$E_{target}$ is the real-time PCR efficiency of target gene transcript;

$E_{ref}$ is the real-time PCR efficiency of reference gene transcript;

$\Delta Ct_{target}$ is the Ct deviation of control (saline-injected) - sample (*V. harveyi*-injected) of the target gene transcript;

$\Delta Ct_{ref}$ is the Ct deviation of control (saline-injected) - sample (*V. harveyi*-injected) of the reference gene transcript.

The significantly different expression levels were examined by One Way analysis of variance (ANOVA) following by a post hoc test (Duncan's new multiple range test). Significant differences were indicated at $p < 0.05$.

## 2.15 Cloning of a serine proteinase-like (SP-like) domain of *Pm*MasSPH

### 2.15.1 Preparation of an inserted SP-like domain fragment

The SP-like domain (849 bp in size) of *Pm*MasSPH was amplified by PCR using upstream and downstream primers containing the indicated 5' flanking restriction sequence and an in frame six histidine encoding tag (SP6HF_**NcoI**: 5' CAT G**CC ATG G**GC CAT CAT CAT CAT CAT CAT CGC ATC ACT GGA TTC

AAG GAT 3' and SPR_**HindIII**: 5' CCC **AAG CTT** TTA AAT AAA TCT TCC GTA GTC CCA 3'). Primers were designed from the previously reported *Pm*MasSPH sequence (DQ455050) (Amparyup et al., 2007). Polymerase chain reaction (PCR) was performed using Advantage 2 polymerase mix (Clontech) with an initial 94 °C for 3 min stage followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The amplicons were purified from 1.2% (w/v) TBE agarose gels using NucleoSpin® Extract II Kits (MACHEREY-NAGEL) and ligated into pGEM-T-easy cloning vector (Promega). The recombinant plasmid was transformed into *E. coli* (K12) strain XL-1 Blue and selected on LB agar plate containing ampicillin/X-Gal/IPTG for propagation of the recombinant plasmid. The recombinant plasmids were screened with colony PCR before DNA sequencing. Nucleotide sequencing was used to confirm the sequence correction of the vector and inserted DNA junction. The recombinant plasmid consisting of correct insert was digested with *Nco*I and *Hin*dIII and purified from gel by NucleoSpin® Extract II Kits.

### 2.15.2 pET-28b(+) expression vector preparation

The pET-28b(+) was used as an *E. coli* expression vector that comprised T7 promoter, His Tag coding sequence, T7 Tag coding sequence, multiple cloning sites, *lacI* coding sequence, pBR322 origin and Kan coding sequence. The restriction sites used for cloning the SP-like domain were *Nco*I and *Hin*dIII as presented in Figure 2.6. At first, a colony containing pET-28b(+) was cultured in 10 ml of LB broth containing 30 μg/ml kanamycin at 37 °C with orbital shaking at 250 rpm an overnight. The plasmid was extracted by QIAprep® Miniprep kits (QIAGEN). The extracted

pET-28b(+) was cut with *Nco*I-*Hin*dIII and then purified from gel by NucleoSpin® Extract II Kits.

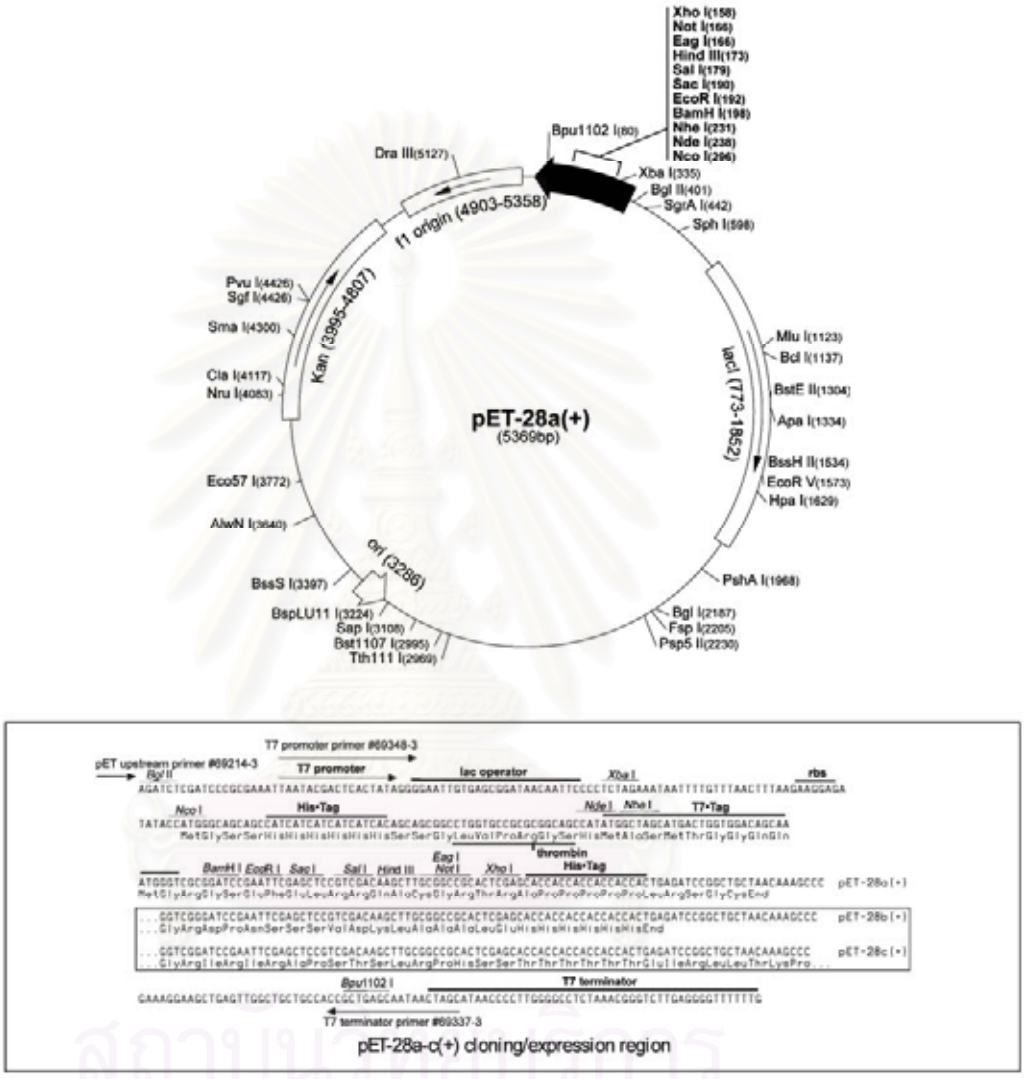



**Figure 2.6** pET-28b(+) vector map and sequences in and around the multiple cloning sites (Novagen)

### 2.15.3 Ligation and electrotransformation into *E. coli* strain Rosetta (DE3)

The constructed plasmids carrying the correct insert were then directionally sub-cloned, following *Nco*I-*Hin*dIII excision, into the multiple cloning site of pET-28b(+) (Novagen). The ligation reaction was composed of 15 µl of 2x Rapid Ligation

buffer, 2 µl of T4 ligase, 5 µl of pET-28b(+) and 5 µl of interested gene which were previously digested with *Nco*I/*Hin*dIII. The steriled water was added to adjust to 30 µl final volume. The mixture was then mixed, briefly spun and incubated at 4 $^{o}$C for overnight. The ligation mixture was transformed into *E. coli* strain Rosetta (DE3).

### 2.15.4 Isolation and detection of the construct plasmid

The transformants were screened on the kanamycin agar plate and re-checked by colony PCR. The selected clones were cultured and extracted the recombinant plasmid using QIAprep® Miniprep kits (QIAGEN). And then, the recombinant plasmids were detected by restriction enzyme digestion with *Nco*I/*Hin*dIII. The digested plasmid was subjected into agarose gel electrophoresis staining with ethidium bromide. The size of DNA fragment was compared with 1 kb standard DNA ladder. DNA sequencing was used to confirm the correct and in-frame sequence of each clone.

## 2.16 Overexpression of SP-like domain in the *E. coli* system

The selected clones were inoculated in Luria–Bertani (LB) medium containing 30 µg/ml kanamycin and 34 µg/ml chloramphenicol at 37 °C overnight with agitation at 250 rpm. The cultures were diluted 1:100 into fresh LB broth supplement with antibiotics and grown until an $OD_{600}$ of the cultures reached 0.6. Just former to induction, the split cultures will serve as an uninduced control. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (final concentration of 1 mM IPTG). The cells at 1, 2, 3, 4, 5 and 6 h. after induction were collected, harvested by centrifugation at 10,000xg for 10 min at 4 °C, washed and resuspended in 20 mM

phosphate buffer pH 7.4. The cell suspensions were disrupted by at least 3 rounds of freeze/thaw and sonication. After centrifugation at 12,000xg for 10 min, the inclusion bodies were washed twice with 20 mM phosphate buffer pH 7.4 supplement with 2% Triton X-100 and 20 mM phosphate buffer pH 7.4, respectively. The recombinant proteins, expressed as inclusion bodies, were solubilized under denature condition with binding buffer (20 mM phosphate buffer pH 7.4 containing 8 M urea and 20 mM imidazole) shaking at room temperature overnight. The solubilized proteins were clarified by centrifugation at 10,000xg for 10 min at 4 °C before column loading.

## 2.17 Purification of recombinant SP-like domain

The inclusion bodies were purified under denaturing conditions in 8 M urea using nickel affinity chromatography (GE Healthcare). Ni-NTA agarose was packed into the PD-10 column and washed twice with distilled water and binding buffer, respectively. The clarified recombinant protein was mixed with the prepared resin in column by shaking at room temperature for an hour. Then, the flow through was collected at a gravity flow. The column was washed with binding buffer followed by the wash buffer (20 mM phosphate buffer pH 7.4 containing 8M urea and 20 mM imidazole). After washing, the purified protein was eluted with elution buffer (20 mM phosphate buffer pH 7.4 containing 8M urea and 500 mM imidazole). The purified proteins were subsequently allowed to refold by three dialysis steps, first against a buffer containing 20 mM Tris–HCl, pH 8.0 plus 2M urea, then twice with the same buffer without urea. Each dialysis step was performed for at least 10 h at 4 °C. The refolding proteins were concentrated by ultrafiltration through 30 kDa cut off Amicon Ultra-4 concentrators (Millipore). The purified recombinant C-terminal SP-like

domain was used for preparation of a specific polyclonal antibody by the commercial service of the AMS Clinical Services Center, Chiang Mai University.

## 2.18 Protein detection

### 2.18.1 Polyacrylamide gel electrophoresis under denaturing and nondenaturing conditions

Analysis of the purified SP-like domain by SDS–PAGE was subjected in 12% polyacrylamide gels following Laemmli method (Laemmli, 1970). This method separates proteins based principally on their molecular weights. The 12% separating gel was prepared by mixing 3.3 ml of deionized water, 4 ml of 30% acrylamide mix solution (29% (w/v) of acrylamide and 1% (w/v) of N,N', methylenebisacrylamide), 2.5 ml of 1.5 M Tris-HCl, pH 8.8, 100 µl of 0.4% (w/v) of SDS, 100 µl of 10% (w/v) ammonium persulfate, and 40 µl of TEMED. The gels were placed in a vertical position at room temperature for complete polymerization. The 5% stacking gel was prepared by mixing 3.4 ml of deionized water, 830 µl of 30 % acrylamide mix solution, 630 µl of 1 M Tris-HCl, pH 6.8, 50 µl of 10 % (w/v) SDS, 50 µl of 10% (w/v) ammonium persulfate, and 5 µl of TEMED. The mixture was poured directly on the top of polymerized separating gel and the comb was carefully inserted on the top of the stacking gel. The SDS-PAGE was run in 1x Tris-glycine electrophoresis buffer pH 8.3 (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS) at 20 mA/gel for 1.40 h. The prestained protein ladder (Fermentas) were used as the standard protein marker. Protein samples were denatured by boiling for 10 min in 5 x SDS loading buffer (60 mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 10% (w/v) SDS, 14.4 mM 2-mercaptoethanol and 1% (w/v) bromophenol blue) and then the gel was visualized by

coomassie brilliant blue staining. The coomassie gel staining solution contains 0.25% (w/v) coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) glacial acetic acid. After staining for at least 4 h, the gels were de-stained by 30% (v/v) methanol and 10% (v/v) glacial acetic acid with gentle shaking.

Non-denaturing gel electrophoresis, also called native gel electrophoresis, separates proteins based primarily on their size and charge properties. All reagents were prepared the same as SDS-PAGE analysis except SDS and 2-mercaptoethanol adding and the sample preparation was not boiled.

### 2.18.2 Amount of protein determination

The protein content was measured according to Bradford method (Bradford, 1976) using bovine serum albumin (Fluka) as the standard (Appendix C). This method is base on the binding of coomassie brilliant blue G 250 dye to proteins. When the dye binds to proteins, the red form of dye is converted to the blue color. One hundread microlitters of diluted sample solution was mixed with Bradford working buffer and left for 2 min before the absorbance at 595 nm was measured. The Bradford working buffer (100 ml) was a mixture of 6 ml Bradford stock solution (350 g Coomassie blue G250, 100 ml 95% ethanol and 200 ml 85% phosphoric acid), 3 ml 95% ethanol, 6 ml 85% phosphoric acid and 85 ml distilled water.

### 2.18.3 Immunoblotting analyses

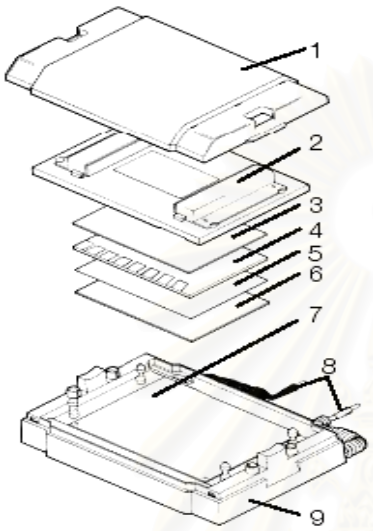
For immunoblotting analysis, separated proteins on SDS-PAGE were electrotransferred to a nitrocellulose membrane in a transfer buffer containing 48 mM Tris-HCl pH 9.2, 39 mM glycine, 20% methanol using Trans-Blot® SD (Bio-Rad) at

100 mA for 1 h. The membrane, gel and filter paper were soaked in transfer buffer before they were consequently laid on Trans-Blot® SD. The filter paper was placed on platform, followed by membrane, gel and filter paper, respectively as the model shown in Figure 2.7. The membrane was blocked by immersion in 5% skimmed milk in phosphate buffer saline pH 7.4 containing 0.05% Tween20 (PBS-Tween buffer) at room temperature overnight with orbital shaking, washed in PBS-Tween buffer, then incubated with the mouse anti-His antiserum (GE Healthcare) (1:3000 dilution in 1% skimmed milk in PBS-Tween buffer) at 37 °C for an hour. After washing in the same buffer, bound primary antibody was amplified by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:5000 dilution, Jackson ImmunoResearch Laboratories, Inc.) for an hour and after washing (as above) the bound antibody was detected by color development using NBT/BCIP (Fermentas) as substrate dissolving in 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$ pH 9.5.

## 2.19 Determination of the proteolytic cleavage site of *Pm*MasSPH

To detect the intact (non-processed) and processed forms of the *Pm*MasSPH, hemocytes lysate (HLS) was prepared in AC-1 anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) followed by centrifugation, and the cells were washed and homogenized in 0.15 M NaCl containing 2 mM EDTA to yield HLS1. Alternatively, anticoagulant (10% sodium citrate) was used to collect the blood, and the cells were washed and homogenized in 0.15 M NaCl without EDTA; this preparation was called HLS2. The HLS2 was activated by laminarin for an hour to produce the processed form. The HLSs were subjected to 12% SDS-PAGE under reducing conditions. The anti-SP-like Ab was

used for immunoblotting to compare the intact *Pm*MasSPH in HLS1 with the processed form of the *Pm*MasSPH in HLS2 after laminarin activation.




1. Safety lid
2. Cathode assembly with latches
3. Filter paper
4. Gel
5. Membrane
6. Filter paper
7. Spring-loaded anode platform mounted on 4 guideposts
8. Power cables
9. Base

**Figure 2.7** Exploded view of the Trans-Blot® SD (Bio-Rad).

## 2.20 Characterization of recombinant protein

### 2.20.1 MALDI-TOF mass spectrometry

The molecular mass of the purified recombinant SP-like domain protein was analyzed by Matrix Assisted Llaser Desorption-Iionization Time of Flight (MALDI-TOF) mass spectrometry by the commercial service of the Proteomic Service Center, Bioservice Unit (BIOTEC, Thailand).

### 2.20.2 Proteinase activity assay

The proteinase activity of the SP-like domain was assayed using the chromogenic p-nitroanilide substrates, 293.6 µM N-benzoyl–Phe–Val–Arg–p-

nitroanilide (Sigma), 147.3 µM N-succinyl–Ala–Ala–Pro–Phe–p-nitroanilide (Sigma) and 443.1 µM N-succinyl–Ala–Ala–Ala–p-nitroanilide (Sigma) as substrates for trypsin, chymotrypsin and elastase, respectively. The proteinase enzymes (trypsin, chymotrypsin and elastase) were prepared in 50 mM Tris-HCl buffer pH 8.0 and used as positive controls in each assay. An aliquot of 60 µl of the recombinant SP-like domain (final concentration of 0.404 µM and 4.04 µM) or positive control enzymes (final concentration of 0.005 µM trypsin, 0.003 µM chymotrypsin and 0.039 µM elastase) was pre-incubated with 10 µl chromogenic peptide in a 96 well plate. After incubation at 30 °C for 10 min, the reaction was stopped by the addition of 20 µl of 50% (v/v) acetic acid and the increase in p-nitroaniline production was recorded by measuring the absorbance at 405 nm. Assays were carried out in duplicate.

**2.20.3 Cell adhesion assay**

The cell adhesion assay was carried out as described by Johansson and Söderhäll (1988) with a slight modification. The sterile glass coverslips (22 × 22 mm, Chance Proper, Ltd.) were laid in 6-well flat bottom plates, coated with 200 µl of 10 µg/ml purified recombinant SP-like domain at 20 °C for an hour, washed by three washes with sterile water and dry at 40 °C and subsequently blocked with 0.1% (w/v) BSA to protect residual non-specific protein binding sites on the coverslip. Coverslips coated with BSA only were used as the control. Hemocyte suspensions from subadult *P. monodon* shrimps (approximately 20 g) were prepared in 0.45 M NaCl and comprised of ~90% living cells as determined by standard trypan blue exclusion assays. Trypan blue (0.4% trypan blue, Sigma) is a negative charged chromopore that can interact with the non-viable cell (blue stained cell) whereas the intact cell is

excluded the dye (unstained cell). The density of hemocyte suspension was assessed in a hemocytometer using a light microscope, adjusted to 2 x$10^4$ cells/ml, and 180 µl of suspension was overlaid onto each coated coverslip together with 20 µl of 100 mM $CaCl_2$, and incubated at 20 °C for 1 h. The unbound hemocytes were then washed off by three washes with Penaeid Shrimp Solution (PSS; 0.45 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 7.6) and fixed in 3.7% (w/v) formaldehyde. The number of attached cells was counted at 200× magnification under an inverted microscope, covering at least 10% of the area initially covered by the cell suspension. Cell adhesion activity (%) was calculated in comparison with the number of initial hemocytes (3,600 cells). The assays were performed in triplicate from each individual shrimp.

**2.20.4 Assay of binding activity to bacteria, LPS, laminarin and peptidoglycan**

The SP-like domain was investigated for binding to the Gram-negative bacteria *Escherichia coli* 363 and *Vibrio harveyi* 1526, and to the Gram-positive bacteria *Staphylococcus aureus*, using a method described by Lee and Söderhäll (2001) with modification. Briefly, the cultured bacteria in mid-log logarithmic phase growth ($OD_{600}$ of 0.6) were plated on nutrient agar for colony counting (colony forming units; CFU, Appendix C). Then, the starters of *E. coli* and *S. aureus* 363 were cultured in LB medium by shaking at 37 °C or 30 °C according to the strains whereas *V. harveyi* 1526 was grown in tryptic soy broth (TSB) medium at 30 °C until an $OD_{600}$ reached 0.6. The cells were fixed in 37% (w/v) formaldehyde by gently shaking at 37 °C for an hour to terminate the enzymatic activity of bacteria. The purified proteins (10 µg of the SP-like domain) were incubated with 100 µl of *E. coli* 363, *V. harveyi* or *S.*

*aureus* (approximately $2.8 \times 10^8$, $3.2 \times 10^8$ and $4 \times 10^{10}$ cells, respectively) with gentle rocking at 4 °C for 35 min. After centrifugation at 2,000 *g* for 10 min, the supernatant containing unbound protein was removed. The bacteria pellets were resuspended and washed two more times with PSS buffer in this manner. Bound proteins were finally eluted from the bacteria by 1× SDS-PAGE sample loading buffer. Bacteria treated with PSS buffer only were used as controls. The resulting supernatant (unbound protein), washed (unbound/weakly bound protein), and eluted (bound protein) fractions were run on 12% (w/v) SDS-PAGE under reducing conditions, transferred to nitrocellulose and subjected to immunoblotting using the anti-His antibody as described above

In another method, the LPS from *E. coli* serotype 0111:B4 (Sigma), laminarin which consists of a β-1,3-glucan chain from *Laminaria digitata* (Sigma), and peptidoglycan from *Staphylococcus aureus* (Sigma) were used for testing the binding activity to the SP-like domain. The purified proteins (10 µg of the SP-like domain) were mixed with 200 µg of LPS, laminarin or peptidoglycan in PSS buffer for 1 h at 4 °C and then centrifuged at 16,000*g* at 4 °C for 15 min. The washed and eluted steps were operated as described above. The obtained supernatant, washed fraction and bound protein were subsequently analyzed by SDS-PAGE and Western blot as described above.



### 2.20.5 Assay for phenoloxidase activity

The hemocyte lysate supernatant (HLS) were prepared. First of all, hemolymph was withdrawn from the ventral sinus using a 1 ml syringe with a 26 gauge-needle containing pre-cooled anticoagulant using an equal volume of MAS to

hemolymph. The hemocytes from three individual shrimps were immediately harvested, centrifuged at 500g for 5 min and the pellet was resuspended and lysed in 10 mM cacodylate buffer pH 7.0 containing 100 mM $CaCl_2$ (CAC; 0.01 M sodium cacodylate, 0.45 M NaCl, 100 mM $CaCl_2$, 26 mM $MgCl_2$, pH 7.0). The homogenates were centrifuged at 25,000g for 20 min at 4 °C and the resulting supernatant was harvested and is referred to as hemocyte lysate supernatant (HLS), and was immediately used for phenoloxidase activity assay. The protein concentration of the HLS was determined by the Bradford method.

To determine the involvement of the SP-like domains of *Pm*MasSPH in the shrimp proPO system, PO activity was examined according to the method of Söderhäll and Smith (1983). In this experiment, 50 µg of laminarin (positive control), 5 µg SP-like domain and BSA (non-specific protein control) were added separately or in combination to 30 µl of 1 mg/ml HLS in the wells of a flat bottom 96-well plate. The mixture volume was adjusted to 200 µl with CAC buffer containing 100 mM $CaCl_2$ and incubated for 10 min at room temperature. The background PO activity was assayed as the control reaction that consisted of HLS without protein addition. Subsequently, 50 µl of 3 mg/ml L-3, 4-dihydroxyphenylalanine (L-DOPA) were added to each well as substrate and PO activity was spectrophotometrically measured at 490 nm every 5 min using a microtiter plate reader (Beckman Coulter AD200). Specific PO activity was defined as the increase in OD490/min/mg protein of HLS (Leonard et al., 1985). Each assay was repeated three times.

# CHAPTER III

# RESULTS

## 3.1 Total RNA and mRNA extraction from *V. harveyi*-challenged shrimps

Total RNA was extracted from hemocytes of the black tiger shrimp and the white shrimp injected with *Vibrio harveyi*. The hemocytes were collected and total RNA was extracted using Trizol reagent. The total RNA from ten individual shrimps were pooled in a tube and subsequently treated with RQ1 RNase-free DNase for further mRNA preparation. The quantity as well as quality of the total RNA samples were determined as described in Material and Method chapter. The amount of pooled total RNA from infected *P. monodon* and *L. vannamei* were 113 μg and 98 μg, respectively. The quality of the extracted RNA was analyzed by 1.2% formaldehyde-agarose gel electrophoresis. Smeared band and a major band of 18S rRNA and 28S rRNA with size of 1.9 kb and 4.7 kb, respectively were detected as shown in Figure 3.1. The mRNA was prepared from total RNA using the QuickPrep® Micro mRNA Purification kit. The average quantity of mRNA from infected *P. monodon* and *L. vannamei* were 27 and 21 μg, respectively. Three micrograms of mRNA from each shrimp were used to synthesize the 5'RACE and 3'RACE cDNA libraries.

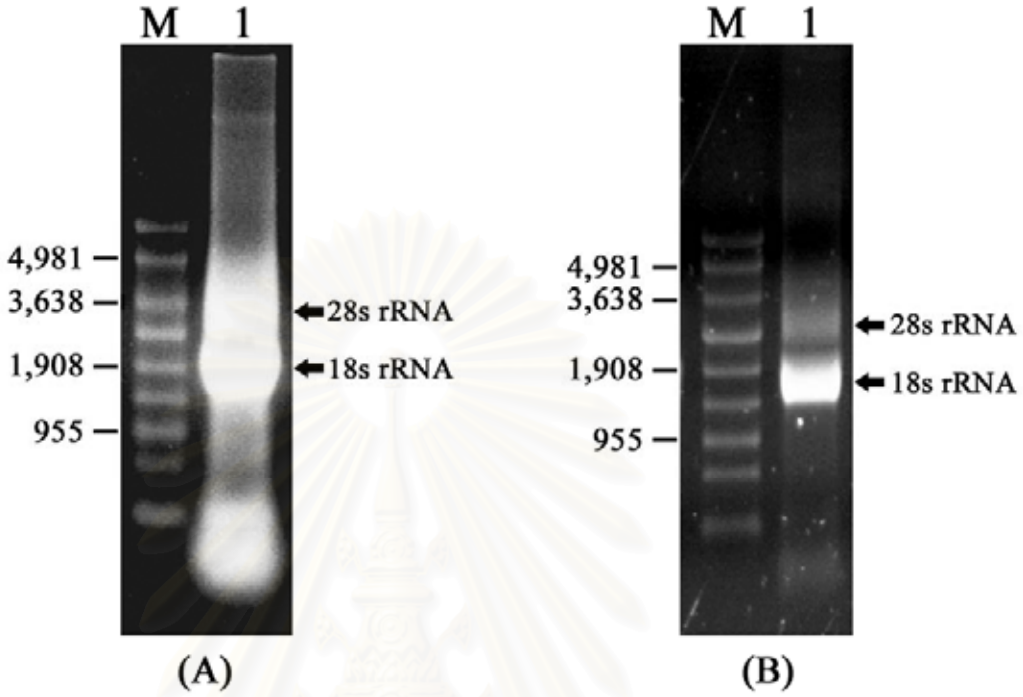



**Figure 3.1** Total RNA from hemocytes of 24 h post-challenged *P. monodon* (A) and *L. vannamei* (B) were separated on the 1.2% formaldehyde agarose gel. Lane M: RNA marker, Lanes 1-2: RNA samples from infected shrimp

## 3.2 Identification of a full-length cDNA of *Pm*MasSPH

From the *P. monodon* EST project (http://pmonodon.biotec.or.th), we identified a putative serine proteinase homologue (*Pm*MasSPH) cDNA with size of 570 bp (260 bp of ORF) from *Vibrio harveyi*-challenged hemocyte cDNA library. A partial sequence of the cDNA (accession number BI784455) showed the highest similarity to a prophenoloxidase activating factor (PPAF) of blue crab *Callinectes sapidus* with 64% homology and e-value of $6 \times 10^{-18}$. To obtain a complete sequence of this cDNA, the RACE-PCR technique was employed.

### 3.2.1 RACE-PCR of *Pm*MasSPH

A partial sequence of *Pm*MasSPH cDNA from EST library contains the stop codon and poly A tail suggesting that the 3' part of *Pm*MasSPH cDNA was obtained. The 5' RACE-PCR was performed to obtain the 5' part of this sequence and carried out according to the manufacturer's instruction (Clonetech) including the addition of DMSO. By 5'RACE-PCR, the PCR product amplified by the 5PmRACE (located in the 3'UTR) and UPM primers (Figure 3.2) was about 1,850 bp which contained an initial start codon for Met and 5' untranslated (5'UTR) region. Then, 5UTR-PmMas primer was designed based on the obtained 5'-RACE sequence, and used for amplification of the full-length cDNA. The PCR products that formed a perfect contig overlapped with homology alignment (Figure 3.2). A complete *Pm*MasSPH cDNA sequence was obtained.

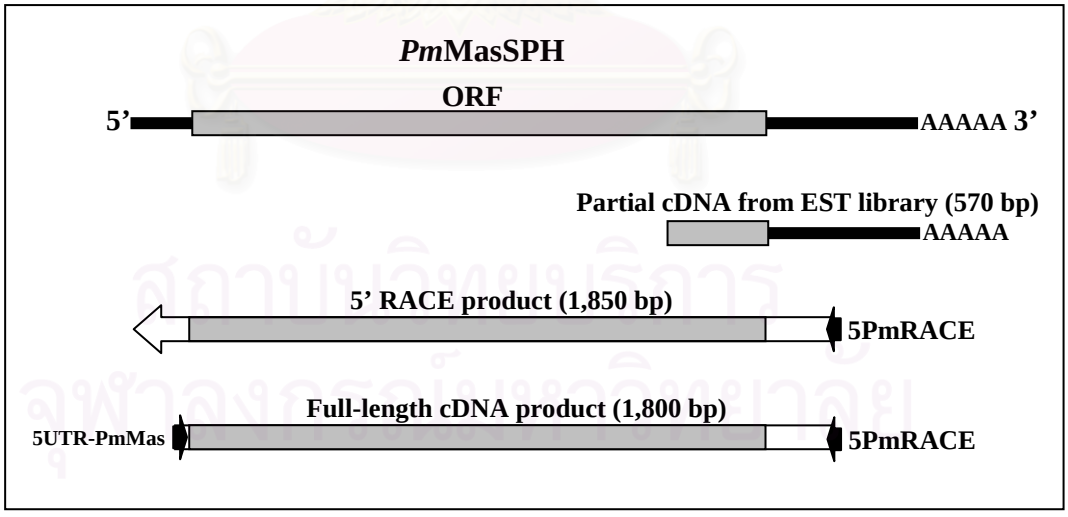



**Figure 3.2** Schematic representation of primer sites and PCR fragments of putative *Pm*MasSPH from *P. monodon* The gray box shows the ORF. The location of primers were indicated by the black arrows.

### 3.2.2 Amplification of the complete sequence of the *Pm*MasSPH

A single band was achieved in the amplification reaction of the *Pm*MasSPH using 5UTR-PmMas and 5PmRACE primers. The size of the amplicon was approximately 1,800 bp which corresponded to the expected size of the full-length cDNA of *Pm*MasSPH (Figure 3.3). The amplified product was then cloned and sequenced.

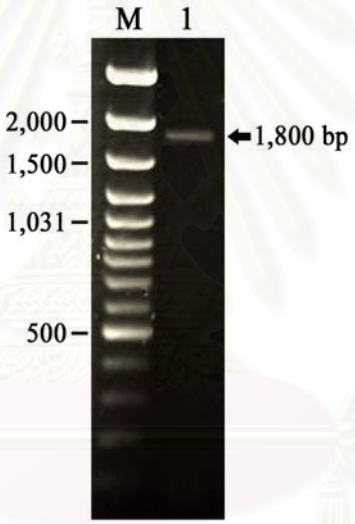



**Figure 3.3** Ethidium bromide stained 1.2% agarose gel of the full-length product of *Pm*MasSPH revealed a major band of approximately 1,800 bp. Lane M represents a 100 bp DNA ladder marker. The expected band was indicated by an arrow.

### 3.2.3 Sequence analysis of the *Pm*MasSPH from *P. monodon*

The putative cDNA and deduced amino acid sequence of a serine proteinase homologue from *P. monodon* (*Pm*MasSPH) have been submitted to the NCBI GenBank (accession number DQ455050) and are shown in Figure 3.4. The full-length

cDNA (1,958 bp) consists of a 79 bp 5'untranslated region (UTR), a 1,572 bp open reading frame (ORF) and a 307 bp 3'UTR with a poly(A) tail. The translation initiation sequence (AAGATGC) is in agreement with the Kozak consensus sequence of *C. sapidus* PPAF (AAGATGC) (Kozak, 1986). A polyadenylation signal (AATATA) is at 15 bp upstream of a poly(A) tail. The 5'-nucleotide sequence is a GC-rich region (70%) containing 11 repeats with a core sequence, CTCGG(G/A)GGTCAAGG(G/A)GG(C/A)GG(T/C).

The ORF encodes a polypeptide of 523 amino acids. Analysis of the SignalP program indicates the presence of a cleavage site between amino acids Gly19 and Cys20. The calculated molecular mass of the mature protein (504 amino acids) is 51.58 kDa with a predicted isoelectric point (pI) of 4.86. Two putative N-glycosylation sites—NDT (amino acid position 28) and NDT (amino acid position 203)—are found, suggesting that it is a glycoprotein (Appendix A). There are two putative proteolytic activation cleavage sites: one in the clip domain (between Arg195 and Phe196) and the other at the beginning of the SP-like domain (between Arg250 and Ile251). Moreover, the *Pm*MasSPH contains a putative integrin binding (KGD) motif at the C-terminal SP-like domain.

The N-terminal domain of the mature protein contains 11 glycine-rich repeats (LGGQGGG) and a clip domain whereas the C-terminal domain contains a serine proteinase-like (SP-like) domain from residues 250 to 498. The serine proteinase domain exhibits feature characteristic of a typical serine proteinase, including the conserved His301 and Asp351 except for Ser replaced by Gly452, suggesting that the protein is a non-catalytic serine proteinase.

# *Pm*MasSPH sequence

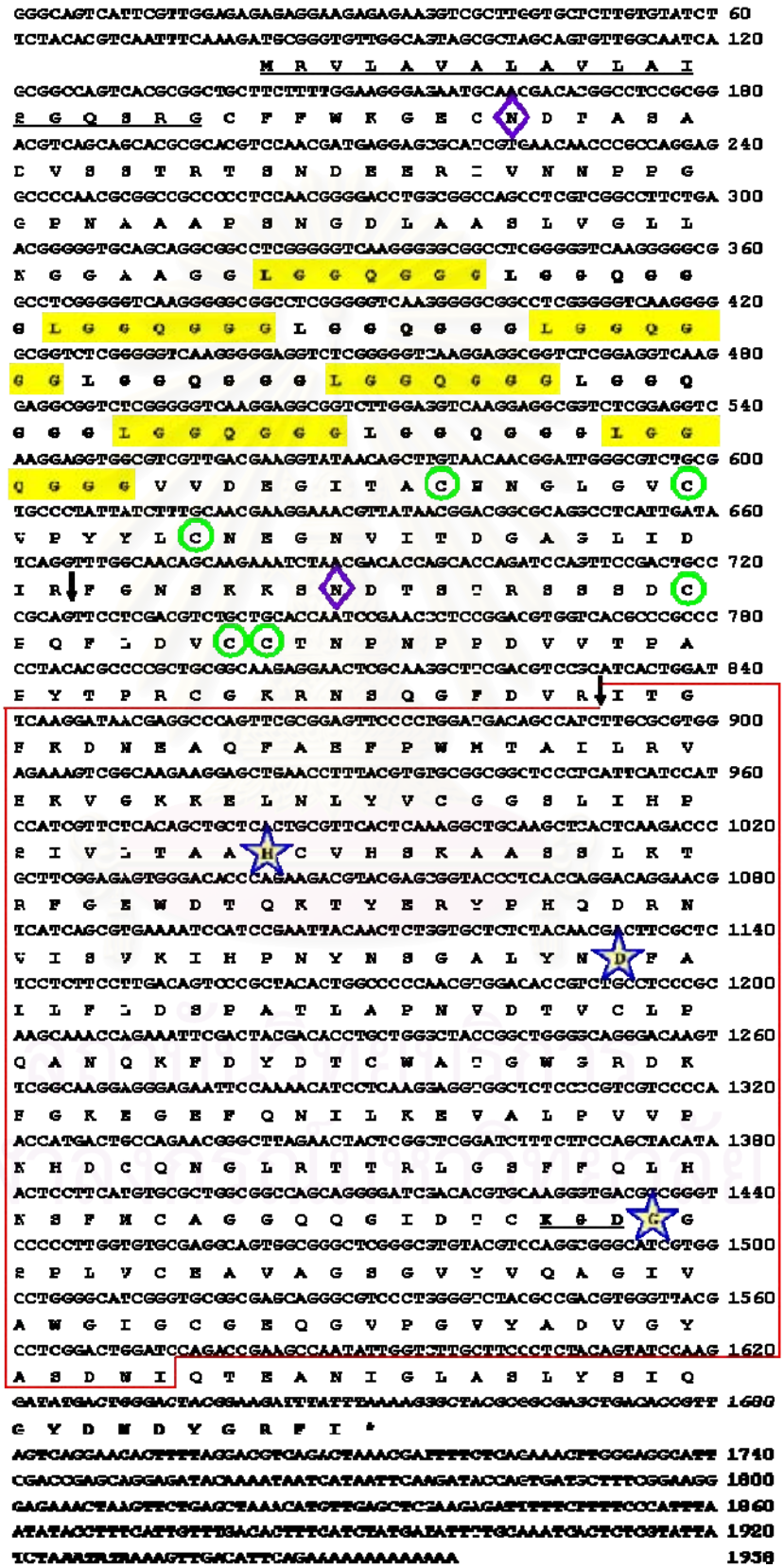


**Figure 3.4 (continued)**

**Figure 3.4** The full-length nucleotide and deduced amino acid sequence of the *Pm*MasSPH from the black tiger shrimp. The deduced amino acid sequence is shown below the nucleotide sequence. The putative polyadenylation signal is in bold and italicized. The putative signal peptide sequence is underlined. *Pm*MasSPH is composed of a signal peptide (underlined), glycine-rich regions (bold and shaded), cysteine residues in the clip domain (open circle) and a SP-like domain (box). The replacement catalytic triad (H, D, G) is marked as the star. The arrows and diamonds indicate the putative activation cleavage sites and putative N-linked glycosylation sites, respectively. The putative integrin-binding motifs (KGD) are represented in bold and underlined.

### 3.2.4 Sequence comparison of *Pm*MasSPH

Homology search of *Pm*MasSPH against the GenBank database using the BLASTX search program showed high similarity to a family of arthropod serine proteinase homologues (SPHs), masquerade-like serine proteinase homologues (mas-like SPHs), prophenoloxidase activation factors (PPAFs) and serine proteases (SPs) containing the N-terminal clip domain and a C-terminal SP-like domain. Comparison of the *Pm*MasSPH deduced amino acid sequence showed identity of 58%, 55%, 53%, 52% and 50% to those of *Callinectes sapidus* PPAF (accession no. AAS60227), *Apis mellifera* PPAF (accession no. XP_623150), *Bombyx mori* masquerade-like SPH (accession no. AAN77090), *Lonomia obliqua* PPAF1 (accession no. AAV91458) and *Tenebrio molitor* PPAF (accession no. CAC12696), respectively (Table 3.1).

**Table 3.1** The BLASTX results and percentages of identity of *Pm*MasSPH sequence to homologue sequences in the GenBank database

| Sequence homology | Closest species | % Identity to *Pm*MasSPH | Expect values | Score (bits) | Accession no. |
|---|---|---|---|---|---|
| PPAF | *C. sapidus* | 199/343 (58%) | 7e-110 | 400 | AAS60227 |
| PPAF | *A. mellifera* | 183/331 (55%) | 6e-106 | 387 | XP_623150 |
| MasSPH | *B. mori* | 185/343 (53%) | 1e-94 | 350 | AAN77090 |
| PPAFI | *L. obliqua* | 180/344 (52%) | 1e-93 | 347 | AAV91458 |
| PPAF | *T. molitor* | 171/342 (50%) | 1e-93 | 347 | CAC12696 |
| SPH-4 | *M. sexta* | 178/354 (50%) | 3e-92 | 342 | AAV91027 |
| SPH-1 | *M. sexta* | 167/351 (47%) | 2e-83 | 313 | AAM69352 |
| PPAF | *H. diomphalia* | 159/356 (44%) | 6e-82 | 308 | CAC12665 |
| MasSPH | *T. molitor* | 164/387 (42%) | 2e-77 | 293 | BAC15605 |

## 3.3 Identification of a full-length cDNA of *Lv*MasSPH

No sequence information was available for the cDNA of *Lv*MasSPH from *L. vannamei*. First, we tried to attain the complete *Lv*MasSPH sequence by PCR amplification using the 5UTR-PmMas and 5PmRACE primer based on the sequence at the 5’ and 3’ UTR of *Pm*MasSPH (see Figure 3.2). Unfortunately, the PCR product was not obtained. The first ampicon of 1,292 bp was successfully amplified using the PmMas-F and PmMas-R primers which located in the coding region of *Pm*MasSPH cDNA (Figure 3.5). Because this putative *Lv*MasSPH fragment contained an incomplete coding sequence, 5’and 3’RACE-PCR were performed to achieve the full-length sequence. 5LvRACE1, 5LvRACE2, 3LvRACE3 and 3LvRACE4 primers were designed from the 5’ and 3’ end of the obtained *Lv*MasSPH sequence, and used for the full-length cDNA identification.

### 3.3.1 RACE-PCR of *Lv*MasSPH

5LvRACE1 and UPM primers were used to perform the first round of 5'RACE-PCR, and 5LvRACE2 and NUP primers were used to perform the second round of PCR. A 227 bp fragment was amplified, which contained an initial start codon and 5'UTR. Subsequently, nested 3'RACE-PCR was performed using two sequence-specific primers (3LvRACE3 and 3LvRACE4). 3'RACE yielded a product of 678 bp that included the stop codon and poly(A) tail. The PCR products that formed a perfect contig overlapped with homology alignment (Figure 3.5). A complete *Lv*MasSPH cDNA sequence was obtained.

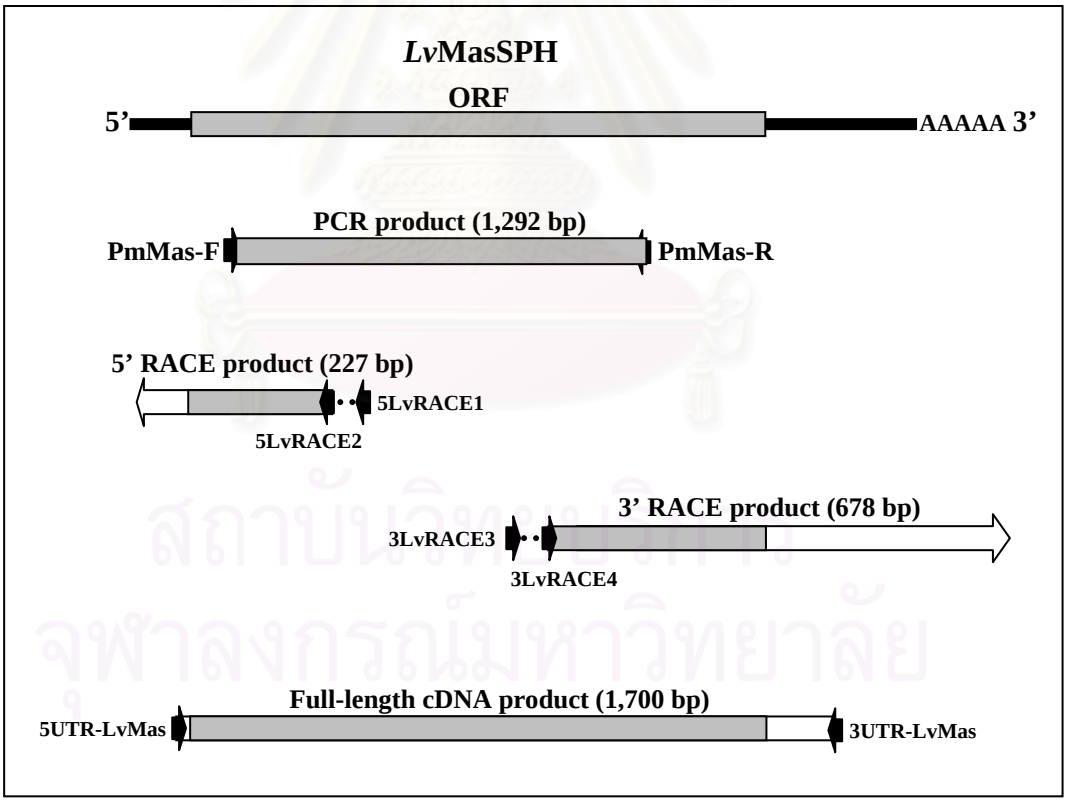



**Figure 3.5** Schematic representation of primer sites and PCR fragments of putative *Lv*MasSPH from *L. vannamei*. The gray box shows the ORF. The location of primers were indicated by the black arrows.

### 3.3.2 Amplification of the complete sequence of the *Lv*MasSPH

A single band was obtained in the amplification reaction of the *Lv*MasSPH using 5UTR-LvMas and 3UTR-LvMas primers. The size of the amplicon was approximately 1,700 bp which corresponded to the expected size of the full-length cDNA of *Lv*MasSPH (Figure 3.6). The amplified product was then cloned and sequenced.

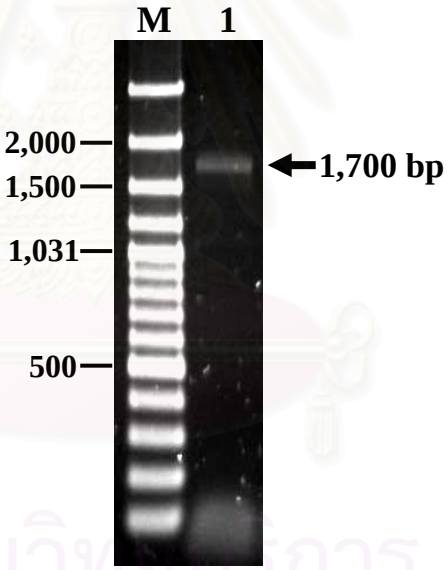



**Figure 3.6** Ethidium bromide stained 1.2% agarose gel of the full-length product of *Lv*MasSPH revealed a major band of approximately 1,700 bp. Lane M represents a 100 bp DNA ladder marker. The expected band was indicated by an arrow.

### 3.3.3 Sequence analysis of the *Lv*MasSPH from *L. vannamei*

The nucleotide and deduced amino acid sequence of a serine proteinase homologue from *L. vannamei* (*Lv*MasSPH) are shown in Figure 3.7. The obtained full-length cDNA sequence (1,955 bp) consisted of a 79 bp 5'UTR, an ORF of 1,536 bp coding for a polypeptide of 511 amino acids, and a 340 bp 3'UTR. The 3'UTR contains a stop codon (TAG), a potential polyadenylation sequence (AATAAA), and a poly(A) tail.

The deduced protein sequence contains 511 amino acid residues with a putative secretory signal peptide cleavage site between Gly19 and Cys20. The molecular weight of a putative mature protein is approximately 50.93 kDa and its estimated pI is 5.20. Two potential N-glycosylation sites are found at the Asn28 and Asn191 (Appendix A) and two putative proteolytic activation cleavage sites present in the clip domain (between Arg183 and Phe184) and another at the beginning of the SP-like domain (between Arg238 and Ile239). In addition, the *Lv*MasSPH also contains a putative integrin binding (KGD) motif at the C-terminal SP-like domain.

*Lv*MasSPH and *Pm*MasSPH have similar domain structures: an N-terminal glycine-rich repeated region, disulfide knotted clip domain and a SP-like domain at the C terminus. The replacement catalytic triad of SP-like domain has a Gly residue substitution for critical serine residue (His289, Asp339, and Gly440). The difference is that the *Lv*MasSPH contains 9 unique glycine-rich repeats (LGGQGGG) whereas *Pm*MasSPH contains 11 repeats.

# *Lv*MasSPH sequence

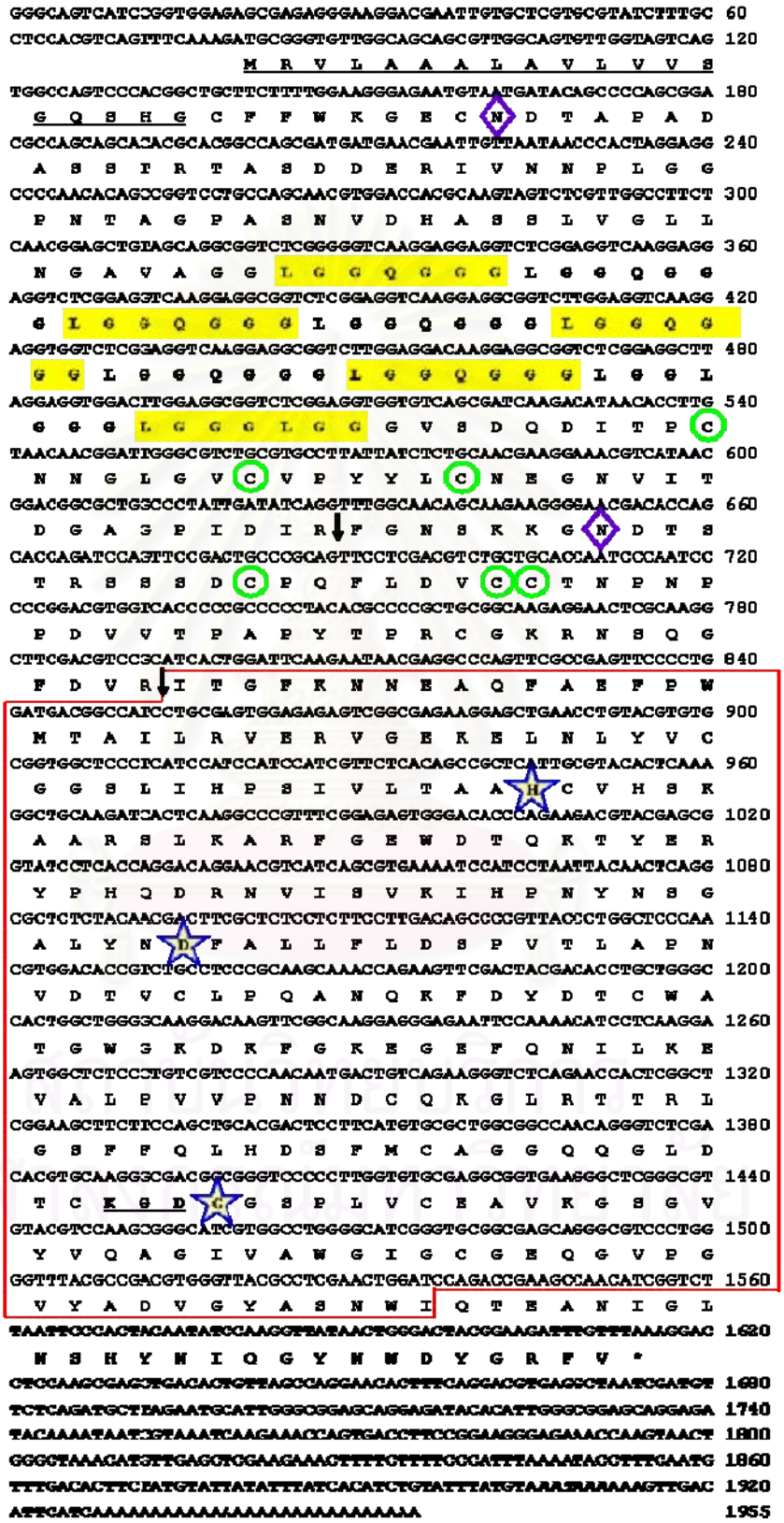


**Figure 3.7 (continued)**

**Figure 3.7** The full-length nucleotide and deduced amino acid sequence of the *Lv*MasSPH from the white shrimp. The deduced amino acid sequence is shown below the nucleotide sequence. The putative polyadenylation signal is in bold and italicized. The putative signal peptide sequence is underlined. *Lv*MasSPH is composed of a signal peptide (underlined), glycine-rich regions (bold and shaded), cysteine residues in the clip domain (open circle) and a SP-like domain (box). The replacement catalytic triad (H, D, G) is marked as the star. The arrows and diamonds indicate the putative activation cleavage sites and putative N-linked glycosylation sites, respectively. The putative integrin-binding motifs (KGD) are represented in bold and underlined.

### 3.3.4 Sequence comparison of *Lv*MasSPH

Homology search of *Lv*MasSPH against the GenBank database using the BLASTX search program showed high similarity to a family of arthropod serine proteinase homologues (SPHs), masquerade-like serine proteinase homologues (mas-like SPHs), prophenoloxidase activation factors (PPAFs) and serine proteases (SPs) containing the N-terminal clip domain and a C-terminal SP-like domain. Comparison of the *Lv*MasSPH deduced amino acid sequence showed identity of 93%, 92%, 92%, 57% and 57% to those of *P. monodon Pm*MasSPH (accession no. ABE03741), *P. monodon* SPH509 (accession no. ABI78947), *P. monodon* SPH516 (accession no. ABD62888), *C. sapidus* PPAF (accession no. AAS60227) and *Nasonia vitripennis* PPAF (accession no. XP_001600151), respectively. Summary of the blast results shown in Table 3.2.

**Table 3.2** The BLASTX results and percentages of identity of *Lv*MasSPH sequence to homologue sequences in the GenBank database

| Sequence homology | Closest species | % Identity to *Lv*MasSPH | Expect values | Score (bits) | Accession no. |
|---|---|---|---|---|---|
| *Pm*MasSPH | *P. monodon* | 342/366 (93%) | 0.0 | 734 | ABE03741 |
| SPH509 | *P. monodon* | 340/366 (92%) | 0.0 | 729 | ABI78947 |
| SPH516 | *P. monodon* | 339/366 (92%) | 0.0 | 728 | ABD62888 |
| PPAF | *C. sapidus* | 198/346 (57%) | 2e-109 | 400 | AAS60227 |
| PPAF | *N. vitripennis* | 198/346 (57%) | 5e-107 | 392 | XP_001600151 |
| SP | *A. aegypti* | 183/345 (53%) | 1e-101 | 374 | EAT46191.1 |
| MasSPH | *B. mori* | 187/343 (54%) | 3e-95 | 353 | AAN77090 |
| PPAFI | *L. obliqua* | 184/344 (53%) | 2e-93 | 347 | AAV91458 |
| PPAF | *T. molitor* | 174/357 (48%) | 4e-93 | 346 | CAC12696 |
| SPH-4 | *M. sexta* | 179/353 (50%) | 4e-92 | 342 | AAV91027 |

## 3.4 Multiple sequence alignment analysis

Multiple sequence alignment of the clip domains (Figure 3.8A) and the SP-like domains (Figure 3.8B) was compared among arthropod SPHs, PPAFs and prophenoloxidase activating proteinases (PAPs). Although, the overall sequence similarity between different clip domains is low, the six Cys residues (Cys1 to Cys5, Cys2 to Cys4 and Cys3 to Cys6) corresponding to the three disulfide bonds, which were determined experimentally, in horseshoe crab proclotting enzyme (Muta et al., 1990) are strictly conserved in all sequences.

The SP-like domains of arthropod SPHs and PPAFs include the highly conserved residues surrounding the replacement catalytic triad (His, Asp, Gly), whereas SP domains of the insect PAP and PPAFI exhibit the characteristic features of typical serine proteinases that contain the His, Asp and Ser in the catalytic triad. Based on the sequence alignment, the three amino acid residues (Asp, Gly, Gly) form the substrate specificity pocket in C-terminal domain (Perona and Craik, 1995). In

addition, four putative disulfide bonds in SP-like domain of *Pm*MasSPH are the same as those found in the shrimp and insect SPHs, whereas there are only three disulfide linkages in the SP-like domain of black tiger shrimp cSPH (AAT42131), SPH3 (ABO33174) and crayfish mas-like SPH (AAX55746). The multiple alignment of SP-like domains reveal that *Pm*MasSPH is apparently similar to the *Pm*SPH516 and *Pm*SPH509 from *P. monodon*, *Lv*MasSPH of *L. vannamei*, and blue crab PPAF, but less similar to *Pm*CSPH and *Pm*SPH3 from *P. monodon*, and crayfish mas-like SPH (Figure 3.8B).

## 3.5 Phylogenetic analysis

A phylogenetic analysis of the serine proteinase domain of SPHs and SPs in arthropods was performed at the amino acid level using PHYLIP by neighbour-joining (NJ) method (Figure 3.9). Based on NJ analysis, arthropod PPAFs, SPHs, PPAEs and SPs can be classified into three major groups: (1) PPAFs and SPHs, (2) PPAEs, and (3) SPs and a SPH. The first group can be classified into four subgroups; one subgroup containing the insect PPAFs (*Am*PPAF, *Tm*PPAF, *Dm*SPH35, *Bm*MasSPH, *Tm*MasSPH, *Hd*PPAFII); the second subgroup consisting of the crustacean MasSPH (*Pm*MasSPH, *Pm*SPH516, *Pm*SPH509, *Lv*MasSPH, *Cs*PPAF), the third subgroup containing *Pm*CSPH, *Pm*SPH3 and crayfish *Pl*MasSPH, and another subgroup containing crayfish masquerade-like protein (*Pl*Mas) and fruit fly mas (*Dm*Mas). The result suggested that the catalytically active serine proteases are distantly related to their inactive serine proteinase homologues except *Mj*SPH and the multi-clip domain SPHs, such as *Pl*Mas and *Dm*Mas, are apparently not similar to a single clip domain SPHs.

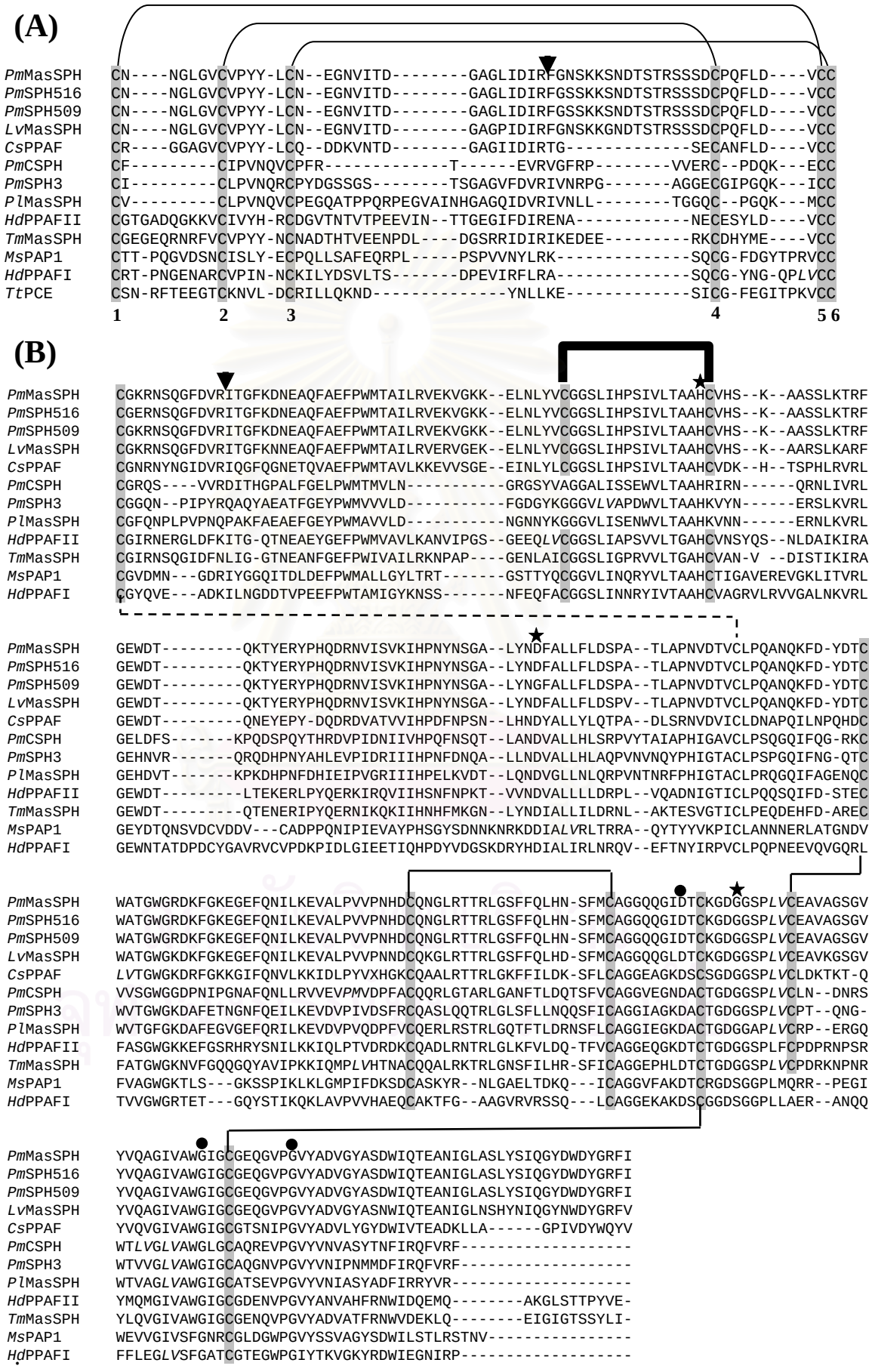



**Figure 3.8 (continued)**

**Figure 3.8** Multiple alignments of amino acid sequence of clip domains (A) and SP-like domains (B) of clip-SPHs and SPs. The amino acid sequence of *P. monodon* MasSPH (*Pm*MasSPH, ABE03741) was aligned with those of *P. monodon* SPH516 (*Pm*SPH516, ABD6288), *P. monodon* SPH509 (*Pm*SPH516, ABI78947), *L. vannamei* MasSPH (*Lv*MasSPH), *C. sapidus* PPAF (CsPPAF, AAS60227), *P. monodon* clip-SPH (*Pm*CSPH, AAT42131), *P. monodon* SPH3 (*Pm*SPH3, ABO33174), *P. leniusculus* MasSPH (PlMasSPH, AAX55746), *H. diomphalia* PPAFII (HdPPAFII, CAC12665), *T. molitor* MasSPH (TmMasSPH, BAC15605), *M. sexta* PAP1 (MsPAP1, AAX18636) *H. diomphalia* PPAFI (HdPPAFI, BAA34642) and *T. tridentatus* proclotting enzyme (TtPCE, AAA30094) (A) The six conserved cysteines in the clip domain are numbered and the linkages were demonstrated as solid line. The putative proteolytic activation cleavage site is shown by an arrow head. Gaps (-) were introduced to maximize the alignment of the clip domain cysteines. (B) Arrows, stars and black dots indicate the putative proteolytic activation cleavage site, amino acid residues corresponding to the catalytic triad and the amino acid residues forming the substrate specificity pocket, respectively. The putative inter-domain disulfide bond between clip domain and SP-like domain is indicated by a dotted line; the disulfide linkages in the SP-like domain are shown by solid lines, and the typical disulfide bond in trypsin is represented by a bold line.

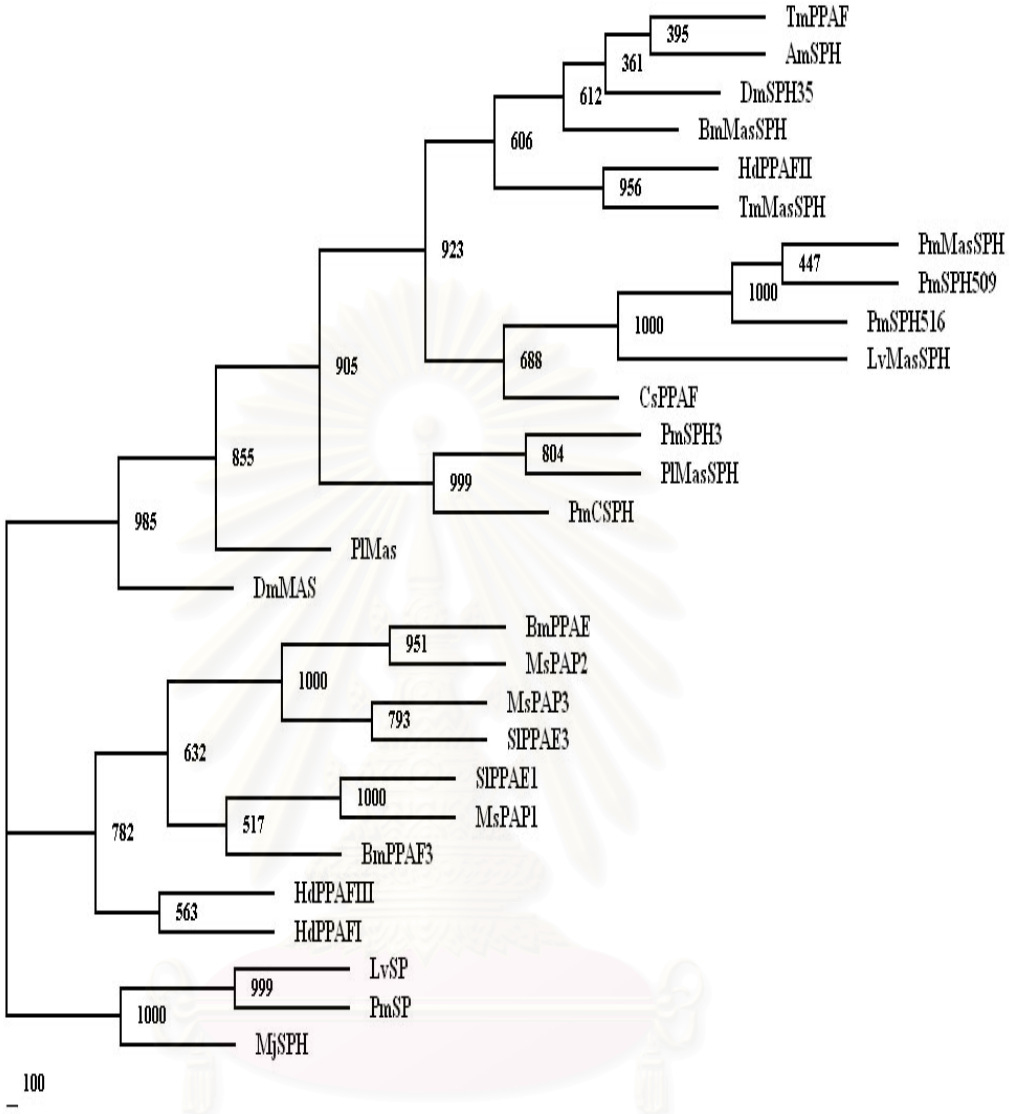



**Figure 3.9** A bootstrapped neighbour-joining tree illustrating relationships between different families of SPHs and SPs in arthropods including *Pm*MasSPH (ABE03741), *Pm*SPH516 (ABD6288), *Pm*SPH509 (ABI78947), *Lv*MasSPH, *Cs*PPAF (AAS60227), *Pl*MasSPH (AAX55746), *Pl*Mas (CAA72032), *Pm*CSPH (AAT42131), *Pm*SPH3 (ABO33174), *Dm*Mas (AAC46512), *Tm*PPAF (CAC12696), *Tm*MasSPH (BAC15605), *Dm*SPH35 (AAF52904), *Am*PPAF (XP_623150), *Bm*MasSPH (AAN77090), *Bm*PPAF3 (AAL31707), *Bm*PPAE (BAA76308), *Hd*PPAFI (BAA34642), *Hd*PPAFII (CAC12665), *Hd*PPAFIII (BAC15604), *Sl*PPAE1 (AAW24480), *Sl*PPAE3 (AAW24481), *Ms*PAP2 (AAL76085), *Ms*PAP3 (AAO74570), *Ms*PAP1 (AAX18636), *Mj*SPH (BAD34945), *Pm*SP (AAQ93679) and *Lv*SP (AAQ92356). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

## 3.6 Expression analysis of the *Pm*MasSPH gene in hemocytes of *V. harveyi*-challenged *P. monodon* using *in situ* hybridization

From the RT-PCR analysis, the expression of *Pm*MasSPH was observed in the hemocytes and *Pm*MasSPH mRNA levels was increased between 24 to 48 h after *V. harveyi* challenged ($P < 0.05$) (Pulsook, 2005). The highest expression was observed at 24 h post injection (4.13 times compared with relative expression at 0 h.). To confirm the transcript expression and determine the localization of *Pm*MasSPH gene *in vivo*, *in situ* hybridization was performed.

The localization and expression level of *Pm*MasSPH transcript in saline-injected (control) and *Vibrio*-injected shrimp were determined by *in situ* hybridization. After DIG-labeled *Pm*MasSPH antisense or sense riboprobes were incubated with hemocytes, the hemocytes were hybridized with alkaline phosphatase-conjugated sheep anti-DIG antibody and detected by color development using NBT/BCIP until the purple color appeared. The sense probe and the RNase A pretreatment were used as a control for signal specificity and to verify that the signal was indeed from the hybridized probe. Positive cells were counted under microscope and the percentage of positive cells was calculated from the number of purple stained cells divided by total cells. The positive cells were detected in hemocytes at 6, 24, 48 and 72 h after injection (Figure 3.10A-b to A-e), whereas the positive cells were not found in hemocytes of normal shrimp (Figure 3.10A-a), saline-injected shrimp, and RNase-treated hemocytes (Figure 3.10A-f). No positive cells observed in the sense probe treated hemocytes (Figure 3.10A-g). The time course analysis of % positive hemocyte of shrimps after *V. harveyi* injection at the different times (0, 6, 24, 48, and 72 h) was evaluated and shown in the Figure 3.10B. The hemocytes expressing *Pm*MasSPH mRNA were significantly increased at 6 h ($p < 0.05$) and continues until 72 h after

injection. These implied that *Pm*MasSPH mRNAs were synthesized in circulating hemocytes and were up regulated upon microbial injection. The statistical values were determined using ANOVA and DUNCAN and demonstrated the significance of the different tests (Appendix B).

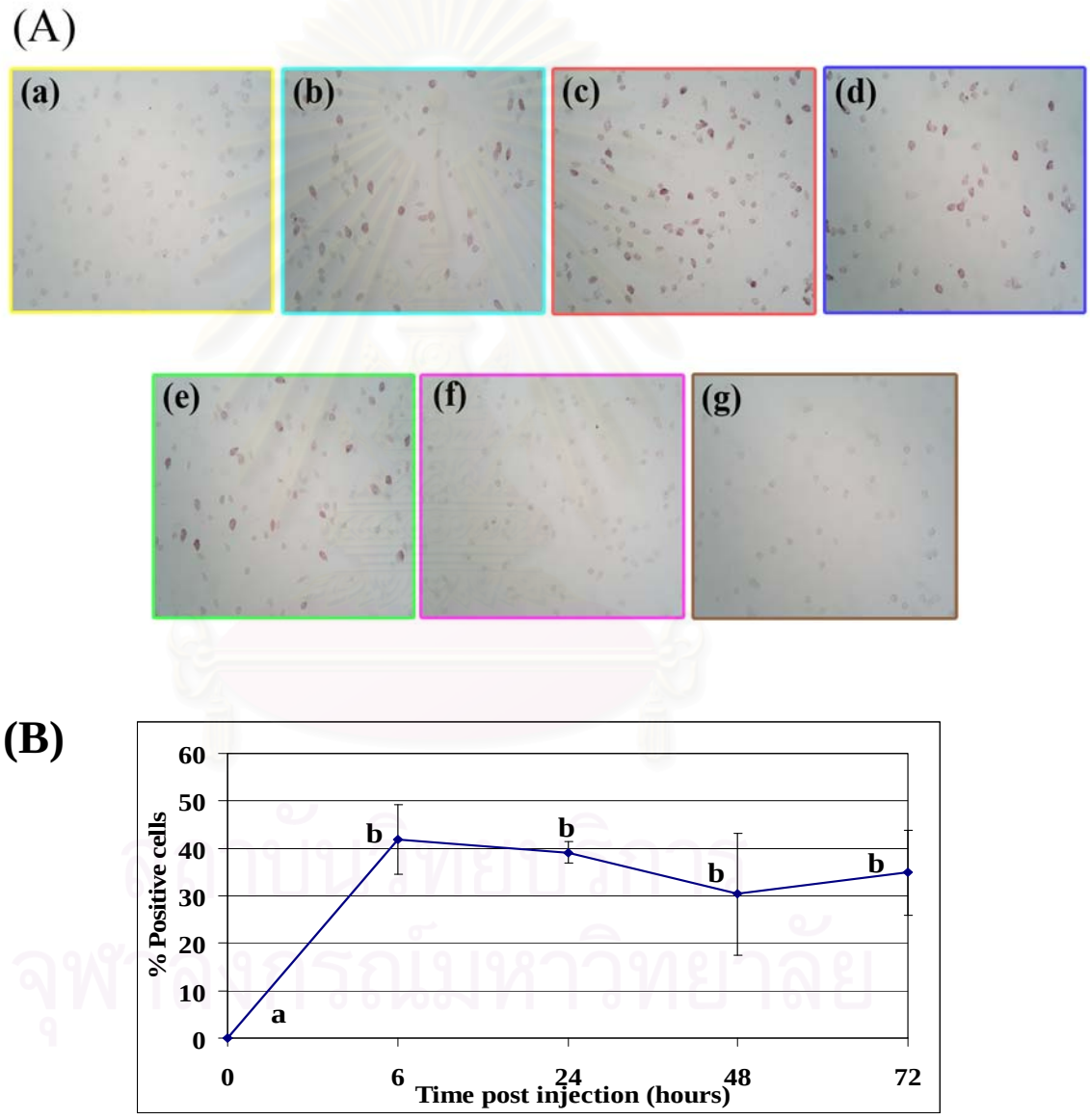



**Figure 3.10** *In situ* hybridization of *P. monodon* hemocytes probed with DIG-labelled *Pm*MasSPH-antisense riboprobe. (A-a) normal shrimp; (A-b to A-e) *V. harveyi* injected shrimp at 6, 24, 48 and 72 h post injection, respectively; (A-f) control: RNase-treated shrimp hemocytes; (A-g) control: shrimp hemocytes probed with sense riboprobes (B) Time-course analysis of percentage of *Pm*MasSPH expressing haemocyte after *V. harveyi* challenge at 0, 6, 24, 48, and 72 h. The assays were performed in triplicate from each individual shrimp. The statistical calculations were analyzed by ANOVA and DUNCAN. Different letters indicate significant difference ($p>0.05$) in mean expression level of immune-related gene.

## 3.7 Analysis of the *Lv*MasSPH expression in hemocytes of *V. harveyi* –injected shrimp by quantitative Real-Time RT-PCR

The expression level of *Lv*MasSPH transcript in the saline (control) and *V. harveyi* injected shrimp at various time points (0, 6, 24, 48, and 72 h) was examined by real-time RT-PCR. The cDNA at each time point was prepared from the pooled total RNA of three individuals of the control shrimp and the challenged shrimp. The β-actin, the housekeeping gene, was used as a reference gene. For an accurate assessment of gene expression by real-time PCR, the PCR efficiency and the PCR specificity of gene must be taken into consideration. real-time PCR efficiency was calculated from the slope, obtained from the curve plotted in log scale between the five serial dilutions (1:5 to $1:5^5$ dilutions) of cDNA and the threshold cycle (Ct) (Figure 3.11), following the equation $E = 10^{[-1/slope]}$. The correlation coefficients, melting temperatures and real-time PCR efficiencies were represented in Table 3.3.

The specificity of the product amplified by SYBR Green PCR was monitored by analysing the dissociation curve of each amplicon. The dissociation curve showed a single peak at expected melting temperature, indicating that *Lv*MasSPH gene was specifically amplified and there was no non-specific amplification or primer-dimer (Figure 3.12). The mRNA expression level of *Lv*MasSPH gene was examined by normalizing the Ct values of the *V. harveyi*-injected shrimp with saline-injected shrimp. The expression ratios of *Lv*MasSPH gene at each time point after injection were calculated relative to β-actin.

The temporal expression of the *Lv*MasSPH transcript in *V. harveyi*-challenged shrimps is shown in Figure 3.13. The transcription level of *Lv*MasSPH mRNA slightly increased at 6 h until 24 h after *V. harveyi* injection, dramatically increased at 48 h post injection ($P < 0.05$), and then at 72 h decreased to the same level as those of

6 h and 24 h. The highest expression was observed at 48 h (5.7 times compared with relative expression at 0 h). The result revealed that *Lv*MasSPH gene is an inducible gene upon *V. harveyi* challenge. The statistical calculations were analyzed by ANOVA and DUNCAN (Appendix B).

**Table 3.3** The correlation coefficients, PCR efficiency for amplified *Lv*MasSPH gene, and the melting temperature ($T_m$) of ampicon.

| Gene name | Tm (°C) | Correlation coefficient | Slope | PCR efficiency |
|---|---|---|---|---|
| *Lv*MasSPH | 87.5 | 0.999 | -3.436 | 1.954 |
| β-actin | 88 | 1.000 | -3.704 | 1.862 |

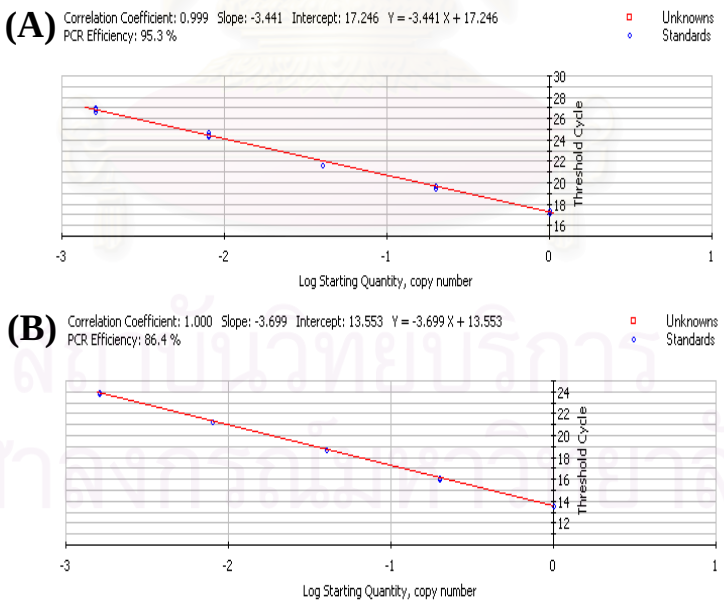



**Figure 3.11** Standard curves generated from $C_T$ values for 5-fold dilution series. The data were plotted on a semilog graph. A, amplification of *Lv*MasSPH gene ($R^2$ = 0.999; slope = -3.441); B, Amplification of the β-actin gene ($R^2$ = 1.000; slope = -3.699). The amplification reactions were performed in triplicate.

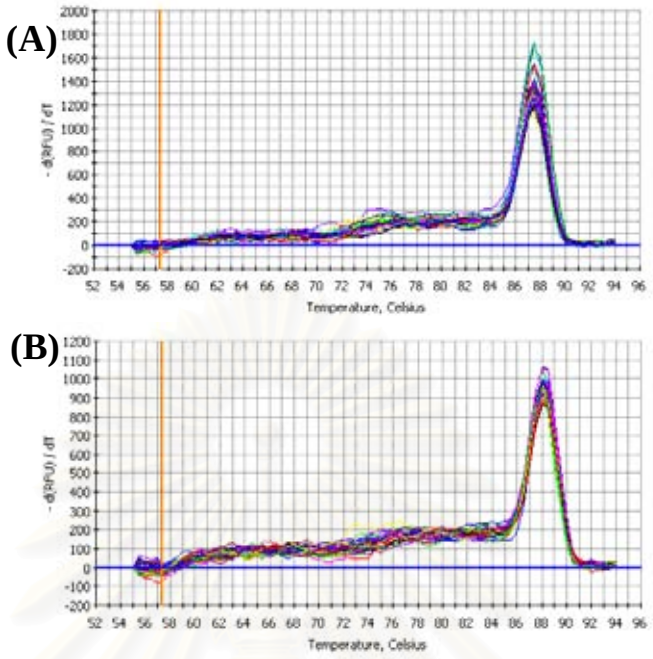



**Figure 3.12** Dissociation curve analysis of the *Lv*MasSPH (A) and β-actin (B) has a different melting temperature of specific product. Dissociation curve analysis is performed after a completed PCR. Data is obtained by slowly decreasing the temperature of reaction solutions from 55 to 94 °C while continuously collecting fluorescence data. The increase in temperature causes PCR products to undergo denaturation. dRFU/dt indicate the Derivative Melting Curve.

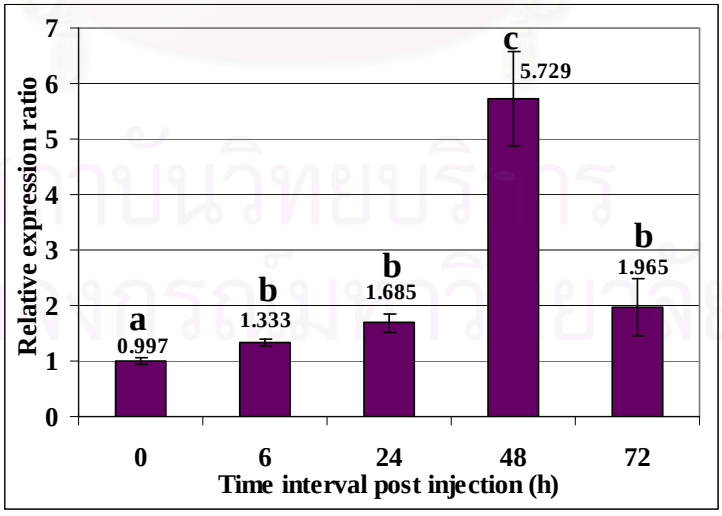



**Figure 3.13** Time course analysis of the change in *Lv*MasSPH mRNA expression of *V. harveyi*-challenged shrimp after 0, 6, 24, 48, and 72. The β-actin was used as a reference gene. All assays were performed in triplicate. Statistical analysis of data was analyzed with a one-way ANOVA followed by Duncan.

## 3.8 Recombinant expression of a serine proteinase-like (SP-like) domain of *Pm*MasSPH in the *E. coli* system

### 3.8.1 Construction of the recombinant plasmid pET28b(+)-SPL

To begin understanding the functions of *Pm*MasSPH, the C-terminal SP-like domain of *Pm*MasSPH was recombinantly expressed in *E.coli* system and characterized (Figure 3.14).

At first, the SP-like domain of *Pm*MasSPH with the size of 849 bp was amplified by PCR. The obtained PCR product was assessed by 1.2% agarose gel electrophoresis (Figure 3.15). The SP-like domain fragment was cloned into pGem-T-easy and then sub-cloned into an expression plasmid pET-28b(+) at the *Nco*1-*Hin*dIII sites fused with a His Tag at the N terminus. The recombinant clones were screened by colony PCR (data not shown) and the recombinant pET-28b(+)-SPL was extracted and verified by restriction enzyme (*Nco*1-*Hin*dIII) digestion. The recombinant plasmid screening was demonstrated in Figure 3.16. The construct was checked by nucleotide sequencing.

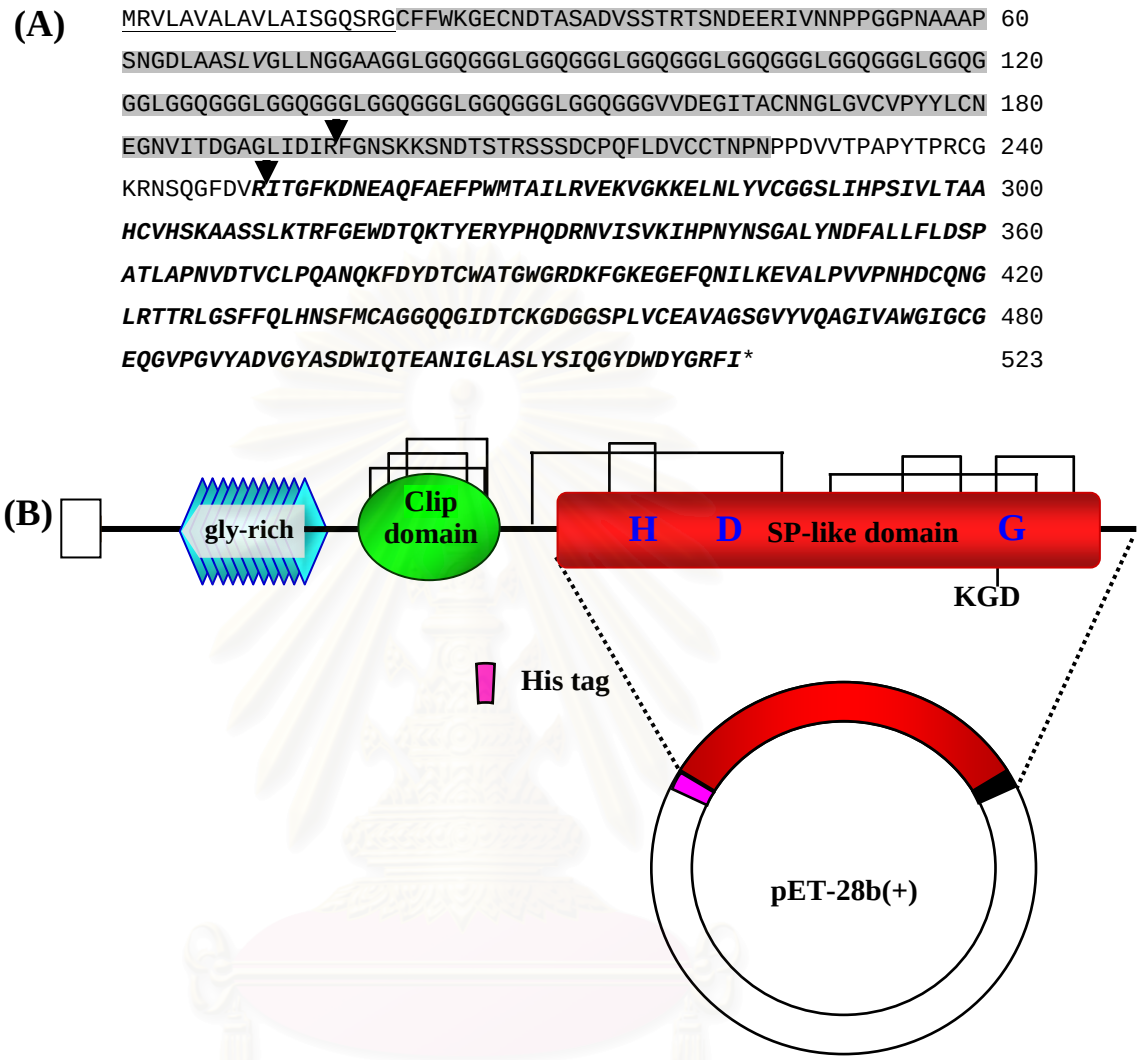



**Figure 3.14** The cloning of a recombinant protein encoding the C-terminal SP-like domain of *Pm*MasSPH from *P. monodon*. (A) A deduced amino acid sequence of a *Pm*MasSPH. The putative signal cleavage site is underlined. The two putative activation sites are indicated by arrowhead. The shaded and bold italicized amino acid sequences are the N-terminal domain comprising the repeated glycine-rich, the clip domain and the C-terminal SP-like domain, respectively. (B) Schematic drawing of structural domains of *Pm*MasSPH. The open box represents a putative signal peptide. The repeated glycine-rich motif, clip domain and SP-like domain are in stacked diamond, elliptical shape and bold box, respectively. The C-terminal SP-like domain was cloned into pET-28b(+) vector with His tag fusion. Solid lines indicate the putative disulfide bridges. The replacement catalytic triad is marked as the H, D, G letters and KGD indicates the integrin binding motif.

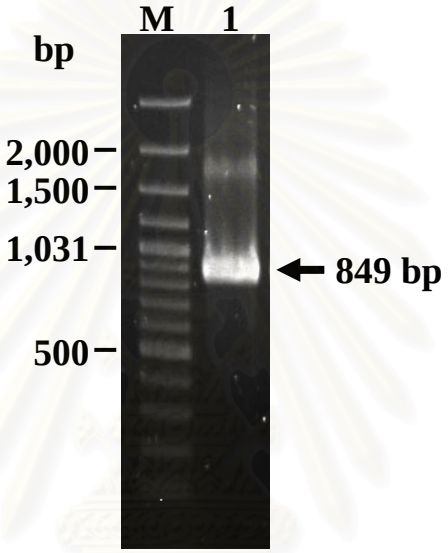



**Figure 3.15** The amplified SP-like domain fragment was analyzed on 1.2% agarose gel electrophoresis. Arrow indicates the expected SP-like domain DNA fragment.
Lane M: GeneRuler™ 100 bp DNA ladder (Fermentas)
Lane 1: The PCR product of SP-like domain

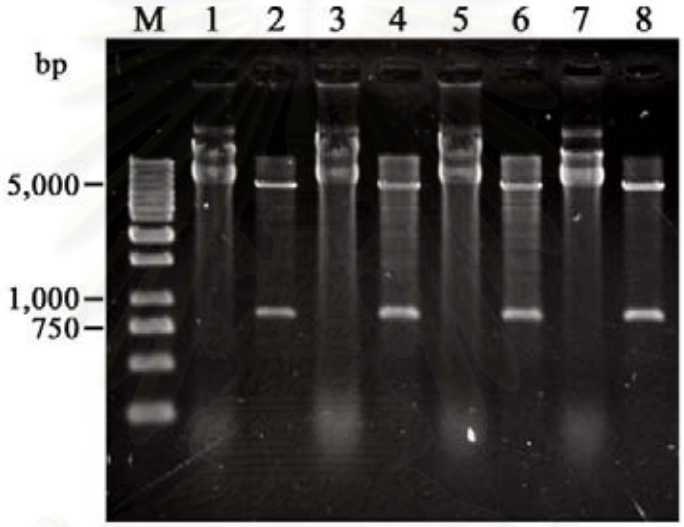



**Figure 3.16** Screening and detection of the recombinant plasmid digesting with *Nco*1-*Hin*dIII on 1.2% agarose gel electrophoresis. Lane M is GeneRuler™ 1kb DNA ladder (Fermentas). The odd numbers represent the uncut recombinant plasmid and even number indicate the pET28b(+)-SPL/ *Nco*1/*Hin*dIII.

### 3.8.2 Over-expression of the C-terminal SP-like domain in the *E. coli* Rosetta(DE3) strain

The constructed plasmid was directionally transformed into an *E. coli* Rosetta(DE3) strain and the selected clone was grown. The recombinant protein was overproduced in LB medium containing kanamycin and chloramphenicol. Protein expression was induced by addition of 1 μM IPTG. The cells were harvested at 0, 1, 2, 3, 4, 5 and 6 h after IPTG induction, then analyzed by coomassie stained 12%. SDS-PAGE. The result showed that the protein expression was continuously increased after 1 h until 6 h of IPTG induction; however, the optimum of IPTG induction time was 4 h (Figure 3.17). This condition therefore was employed for a large-scale expression of the recombinant C-terminal SP-like domain.

After 4 h of IPTG induction, the recombinant protein, expressed as inclusion bodies, were solubilized in denaturing solution, but no recombinant protein band was detected in the cell lysates of cells containing the parental plasmid pET28b(+) after 4 h IPTG induction and uninduced cells carrying the recombinant pET28b(+)-SPL using as the control (Figure 3.18).

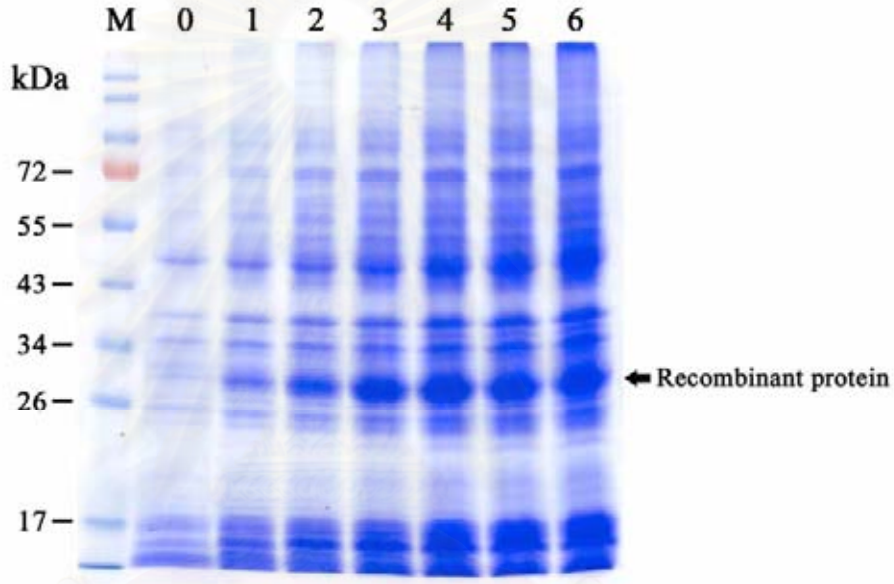



**Figure 3.17** Expression of the recombinant SP-like domain after IPTG induction at 0, 1, 2, 3, 4, 5, and 6 hrs, respectively (lanes 0 to 6). Lane M indicates the pre-stained protein molecular weight marker (Fermentas).

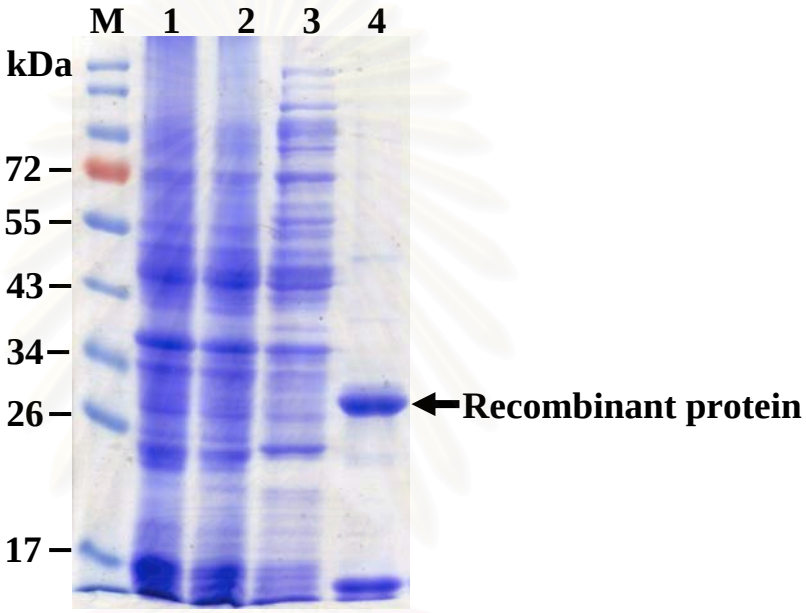



**Figure 3.18** SDS-PAGE analysis of the recombinant SP-like domain in the different cell lysates. Lane M indicates the pre-stained protein molecular weight marker (Fermentas). Lane1 is the lysate of cells carrying the pET28b(+) after 4h IPTG induction, Lane 2 shows the lysate obtained from uninduced cells containing the recombinant pET28b(+)-SPL plasmid, Lanes 3 and 4 represent the soluble protein and inclusion bodies of cells carrying the recombinant pET28b(+)-SPL plasmid after 4h of IPTG induction**.**

### 3.8.3 Detection of recombinant protein in crude total protein by Western blot analysis

To confirm that the major band of cell lysate is the rSP-like domain, immunoblotting with anti-His antibody was performed. The crude protein from induced cell carrying the recombinant pET28b(+)-SPL plasmid was electrophoresed on SDS-PAGE, subsequently blotted to nitrocellulose membrane, then hybridized with mouse anti-His antibody as well as second antibody conjugated with alkaline phosphatase. Finally, the rSP-like domain was detected with colorimetric method (Figure 3.19). The result showed that the specific band of overproduced protein was the rSP-like domain.

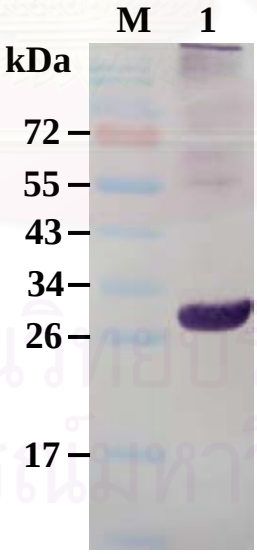



**Figure 3.19** Western blot analysis of rSP-like domain expressed by pET28b(+)-SPL clone. Lane M is the prestained protein molecular weight marker and lane 1 represents the crude protein of cell carrying the recombinant pET28b(+)-SPL plasmid hybridized with anti-His Ab.

### 3.8.4 A single-step purification of the recombinant SP-like domain

The recombinant protein was efficiently purified with a single-step purification using a Ni–NTA affinity column under denaturing conditions in 8 M urea. After removing the urea in three dialysis steps, the recombinant protein was refolded and analyzed for the identity and purity on 12% (w/v) SDS-PAGE and confirmed by immunoblotting using anti-His antibody. A single band of the recombinant C-terminal SP-like domain (rSP-like domain), approximately 31 kDa, was observed (Figure 3.20A) which is in good agreement with the calculated molecular mass of the purified rSP-like domain (31,060.39 Da) of *Pm*MasSPH based on its respective deduced amino acid sequence including the extra glycine and six histidine residues. Moreover, MALDI-TOF mass fingerprint analysis revealed a single mass spectrum for the rSP-like domain preparation with no contaminating other proteins and molecular masses of 30,964.263 Da corresponding to the expected theoretical mass (Figure 3.20C). The native PAGE of the refolded rSP-like domain showed a single band indicating only one form presumably the correctly folded protein was obtained (Figure 3.20B). The purified rSP-like domain was used for preparation of the specific polyclonal antibody in rabbit.

### 3.8.5 The specificity of polyclonal antibody against the rSP-like domain

The specificity and optimal quantity of polyclonal antibody against the rSP-like domain were examined using the immunoblotting analysis. The purified rSP-like domain was run on SDS-PAGE, transferred to membrane, probed with various dilutions of rabbit anti-rSP-like domain antibody in (1 to 2,500; 1 to 5,000; 1 to 10,000 and 1 to 20,000 dilutions, respectively), followed by incubation with goat anti-

rabbit IgG antibody linked alkaline phosphatase, and detected using NBT/BCIP substrates. The result revealed that the obtained polyclonal antibody against the rSP-like domain was greatly specific to rSP-like domain and the 1:5,000 dilution of anti-rSP-like domain antibody was a proper concentration for further study (Figure 3.21).

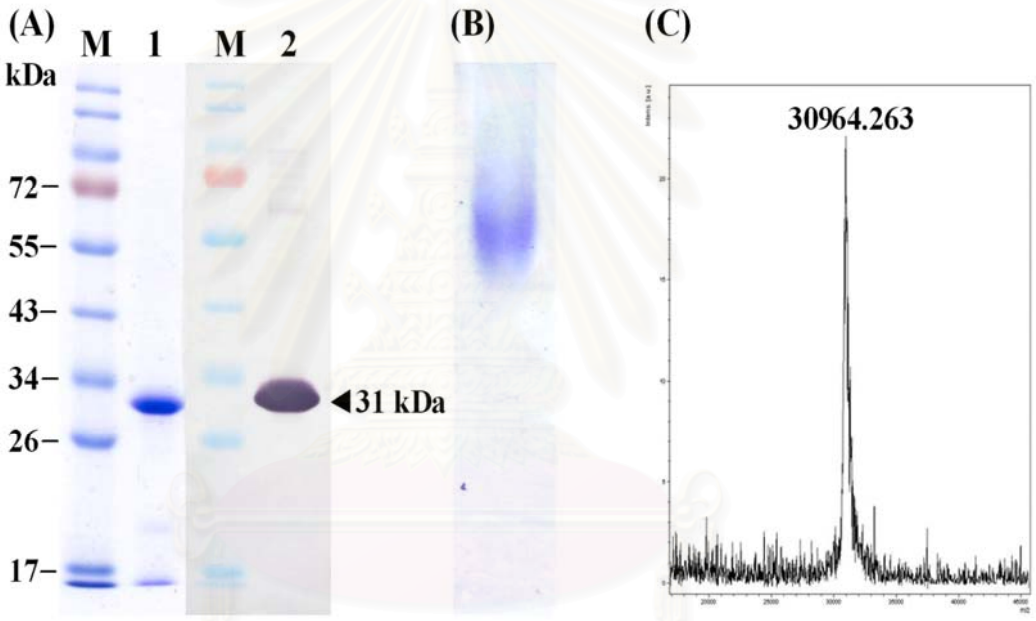



**Figure 3.20** SDS–PAGE, native PAGE and Western blot analysis of refolded recombinant SP-like domain. Lane M, prestained protein standard marker (Fermentas); (A) lane 1: the purified recombinant protein through Ni affinity chromatography on 12% SDS-PAGE and lane 2: immunoblot detection of recombinant protein using anti-His tag antibody, (B) the purified recombinant protein on 6% non-denaturing PAGE, and (C) mass spectrum of the purified rSP-like domain with a molecular mass of 30,964.263 Da.

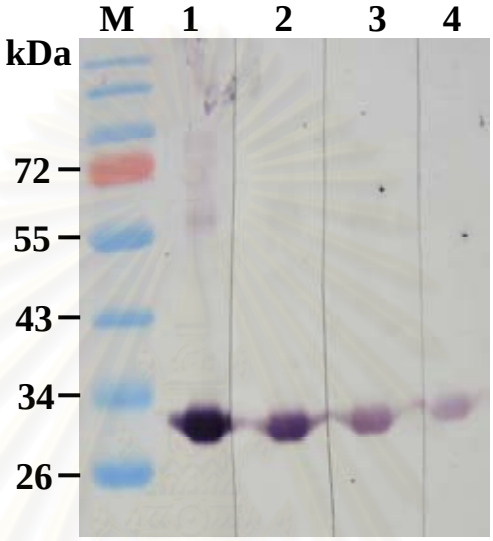



**Figure 3.21** Specificity of the polyclonal anti-rSP-like domain antibody to rSP-like domain. The western blot analysis using the rabbit anti-rSP-like domain antibody was performed. Lane M: pre-stained protein standard marker. Lanes 1 to 4 were the purified rSP-like domain probed with anti-rSP-like domain antibody with different dilutions of 1:2,500; 1:5,000; 1:10,000 and 1: 20,000, respectively, followed by incubation with anti-rabbit conjugated with alkaline phosphatase. The signal detection was observed using the colorimetric method.

## 3.9 Determination of the proteolytic cleavage site of *Pm*MasSPH

To investigate the non-processed and processed forms of *Pm*MasSPH, the hemocyte lysate supernatant prepared in the presence and absence of EDTA, HLS1 and HLS2, respectively were assessed by 12% SDS-PAGE under reducing condition and immunoblotting analysis using the antibody against the C-terminal SP-like domain (Figure 3.22). Due to a lot of proteins obtained from hemocyte lysates from both HLSs, non-processed and processed forms of *Pm*MasSPH could not be identified by SDS-PAGE (Figure 3.22A). However, the immunoblotting of a HLS1 showed a major band of a non-processed form of *Pm*MasSPH with the size of 54 kDa (Figure 3.22B). This mass is larger than 51.58 kDa predicted from the open reading frame, suggesting the possibility of post-translational modifications such as glycosylation and phosphorylation of native protein.

From the deduced amino acid sequence of *Pm*MasSPH, the putative activation cleavage sites were predicted after arginine within the clip domain (Arg195) and at the beginning of the SP-like domain (Arg250) as shown in Figure 3.14, probably producing the processed forms of *Pm*MasSPH with size of 35.87 and 29.89 kDa based on amino acid sequence, respectively (Appendix A). The immunoblotting of HLS2-prepared in condition without EDTA and activated with laminarin-showed a single band of approximately 36 kDa, probably the processed form of the *Pm*MasSPH. The result implied that the authentic activation site is the position between Arg195 and Phe196.

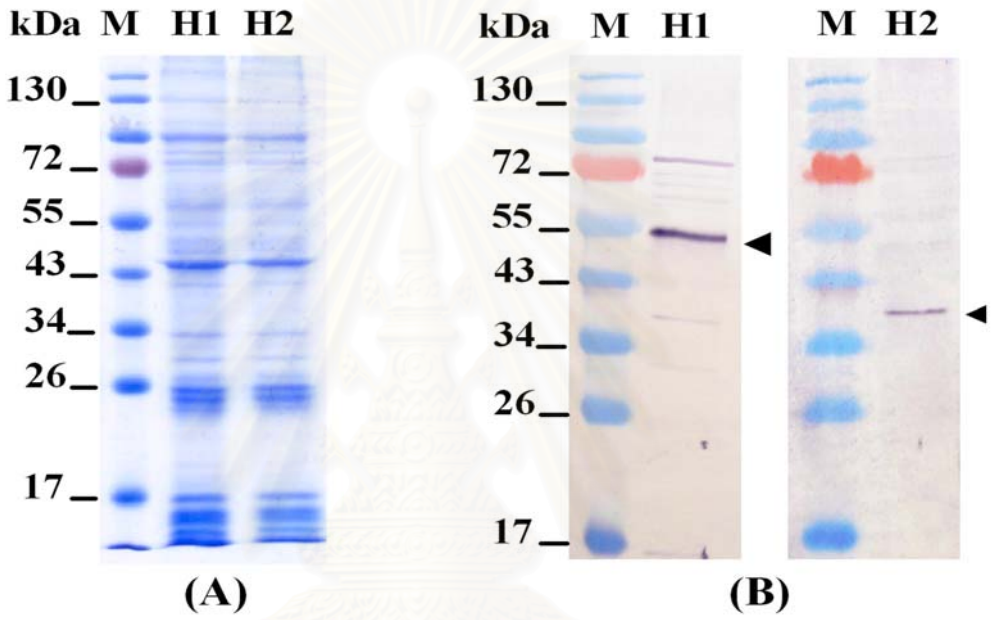



**Figure 3.22** The intact and processed forms of *Pm*MasSPH in hemocyte lysates. (A) 12% SDS-PAGE under reducing condition and (B) immunoblotting of shrimp hemocyte lysates with anti-rSP-like domain antibody, Lane H1: cells were homogenized in buffer containing the EDTA; Lane H2: cells were activated by laminarin; and M indicates Molecular mass markers.

## 3.10 Functional characterization of the recombinant SP-like domain

The purified recombinant protein was assayed for various biological functions: *in vitro* proteinase activity, hemocyte adhesion, bacterial binding, and phenoloxidase (PO) activity.

### 3.10.1 Proteinase activity assay

The active site serine in the catalytic triad of rSP-like domain of *Pm*MasSPH is replaced with glycine, suggesting that it might lose serine protease enzymatic activity. To confirm this notion, various chromogenic substrates for trypsin, chymotrypsin and elastase were tested. The enzymatic activity was monitored as the release of p-nitroaniline at $A_{405}$ nm. These experiments were performed in duplicate. No protease activity was detected in all assays using the rSP-like domain of *Pm*MasSPH, in contrast to the positive controls (Table 3.4).

**Table 3.4** The proteinase activity assay of recombinant SP-like domain. Trypsin, chymotrypsin and elastase were adopted as the positive control.

| **Proteinase** | **Average $A_{405}$ - blank** | | |
|---|---|---|---|
| | **Trypsin substrate** | **Chymotrypsin substrate** | **Elastase substrate** |
| Trypsin (0.005 µM) | 0.771 | - | - |
| Chymotrypsin (0.003 µM) | - | 0.364 | - |
| Elastase (0.039 µM) | - | - | 0.520 |
| rSP-like domain (0.404 µM) | 0.026 | 0.020 | 0.032 |
| rSP-like domain (4.04 µM) | 0.037 | 0.029 | 0.033 |

**Chromogenic substrate**: N-benzoyl–Phe–Val–Arg–p-nitroanilide, N-succinyl–Ala–Ala–Pro–Phe–p-nitroanilide and N-succinyl–Ala–Ala–Ala–p-nitroanilide as substrate for trypsin, chymotrypsin and elastase, respectively.

### 3.10.2 Cell adhesion assay

Many cell adhesion molecules have been discovered to be involved in invertebrate immunity (Johansson, 1999). The crayfish masquerade-like protein as well as the clip-serine proteinase homologues (c-SPH) of the black tiger shrimp have been shown to exhibit cell adhesion activity and that this is derived from the SP-like domain and not the clip domain (Huang et al., 2000; Lin et al., 2006). To investigate whether *Pm*MasSPH could function as a cell adhesion molecule, the rSP-like domain protein was tested for the ability to promote cell adhesion *in vitro*. The number of attached cells was counted under an inverted microscope, and cell adhesion activity (%) was calculated in comparison with the number of initial hemocytes (3,600 cells). The assays were performed in triplicate from each individual shrimp. The cell adhesion assay revealed that an average of 51% of shrimp hemocytes adhered to the rSP-like domain-coated coverslips, whereas only 25% of hemocytes adhered to the bovine serum albumin-coated coverslips (Figure 3.23). These results suggested that *Pm*MasSPH acts as a cell adhesive molecule through the adhesion activity of the C-terminal SP-like domain.

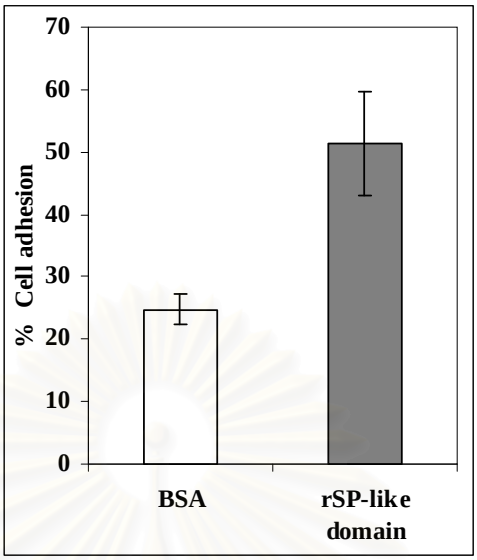



**Figure 3.23** Cell adhesion activity of the recombinant C-terminal SP-like domain to shrimp hemocytes. Coverslips coated with BSA or the C-terminal SP-like domain was used to determine the cell adhesion activity. The results were shown as means with standard error bars. The assays were performed in triplicate with three different hemocyte suspensions from individual shrimp.

### 3.10.3 Assay of binding activity to bacteria, LPS, laminarin and peptidoglycan

The binding ability of the rSP-like domain protein to bacterial cells (Gram-negative bacteria *E. coli* 363 and *V. harveyi* 1526; Gram-positive bacteria *S. aureus*) and certain cell wall components (LPS, laminarin and peptidoglycan) was investigated. The CFU value of each bacterium was shown in Table 3.5 (Appendix c).

These biding assays revealed that the rSP-like domain was found in the eluted fractions whereas no band was detected in the supernatant or the washed fractions (Figure 3.24), suggesting that the C-terminal SP-like domain strongly binds to the Gram-negative and Gram-positive bacteria as well as each of the microbial cell wall components including laminarin from brown algae. The binding activities of the

recombinant proteins to bacteria and microbial cell wall components suggest that *Pm*MasSPH is likely to play a role as a pattern recognition protein to stimulate the innate immune system in shrimp.

**Table 3.5** The colony forming units of bacteria using in binding activity assay

| **Bacteria type** | **A600** | **CFU (cells/ml)** |
|---|---|---|
| *E. coli* 363 | 0.510 | $1.4x10^8$ |
| *V. harveyi* 1526 | 0.518 | $1.6x10^8$ |
| *S. aureus* | 0.635 | $2x10^{10}$ |

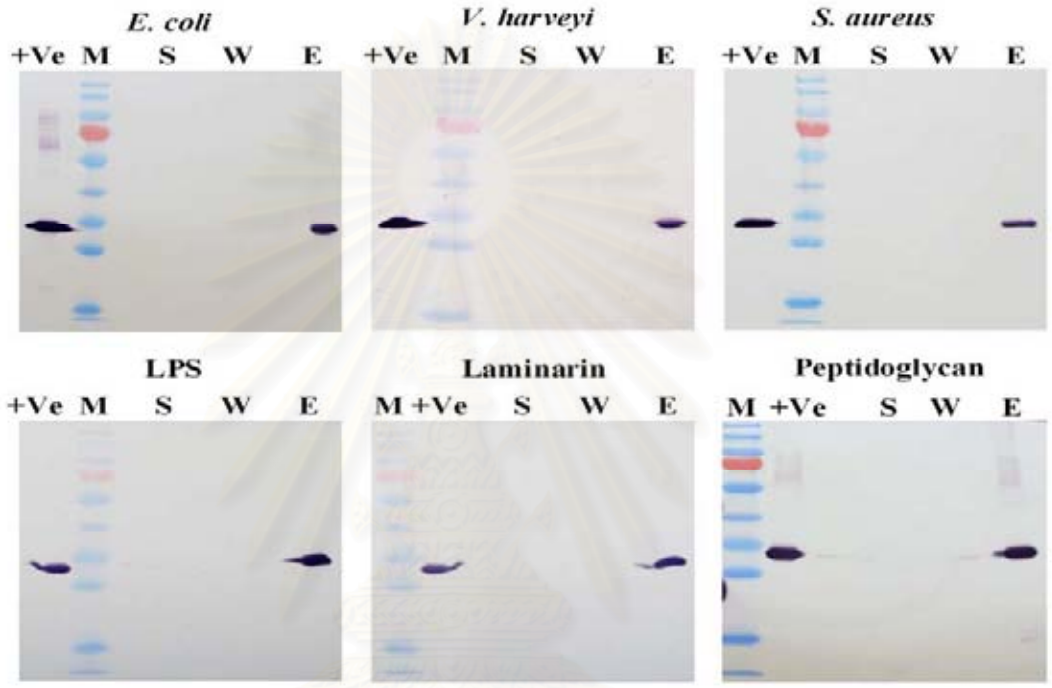



**Figure 3.24** Binding activity of the recombinant C-terminal SP-like domain to Gram-negative bacteria (*E. coli and V. harveyi*), Gram-positive bacteria (*S. aureus*) and microbial cell wall components (LPS, laminarin and peptidoglycan). Initially, the recombinant protein was incubated with formaldehyde-fixed bacteria, LPS, laminarin or peptidoglycan. After incubation, the supernatants were separated by centrifugation. The pellets were washed with PSS buffer and the bound proteins were eluted with SDS-PAGE sample loading buffer. The supernatants (S), washed (W) and eluted (E) fractions were examined by Western blot analysis under reducing condition using the anti-His antibody. Lane +Ve, positive control (rSP-like domain); lane M, pre-stained protein standard marker (Fermentas)

### 3.10.4 Assay for phenoloxidase activity

The proPO activation system in arthropods begins with stimulation triggered by microbial cell wall components, such as β-glucan or laminarin from fungi or lipopolysaccharide and peptidoglycans from bacteria (Brookman et al., 1989; Söderhäll and Cerenius, 1998). Similarly, in the crustaceans examined to date, the proteolytic activation of proPO is mediated by an endogenous serine proteinase enzyme, or *in vitro* by commercial trypsin, resulting in the production of active phenoloxidase (Asokan et al., 1997; Aspan and Söderhäll, 1991). In insects, SPHs have been demonstrated to be essential for proPO activation. Here, C-terminal region of *Pm*MasSPH was tested for the involvement in the prophenoloxidase activation system. Fresh hemocyte lysate (HLS) in carcodylate (CAC) buffer containing $Ca^{2+}$ were incubated with laminarin, rSP-like domain or BSA (the control) prior to assaying for PO activity. The PO activity was determined by measurement of the absorbance at 490 nm using L-DOPA as a substrate and specific PO activity was defined as an increase in OD490/min/mg protein of HLS. Statistical analysis of data was analyzed with a one-way ANOVA followed by a post hoc test (Duncan's new multiple range test). Differences were considered significant at $P < 0.05$ (Appendix B). The incubation of HLS with laminarin significantly enhanced PO activity, whilst the addition of the rSP-like domain showed a slight increase in the PO activity compared to the non-specific protein, BSA (Figure 3.25). Our results suggested that *Pm*MasSPH probably involved in the proPO system.

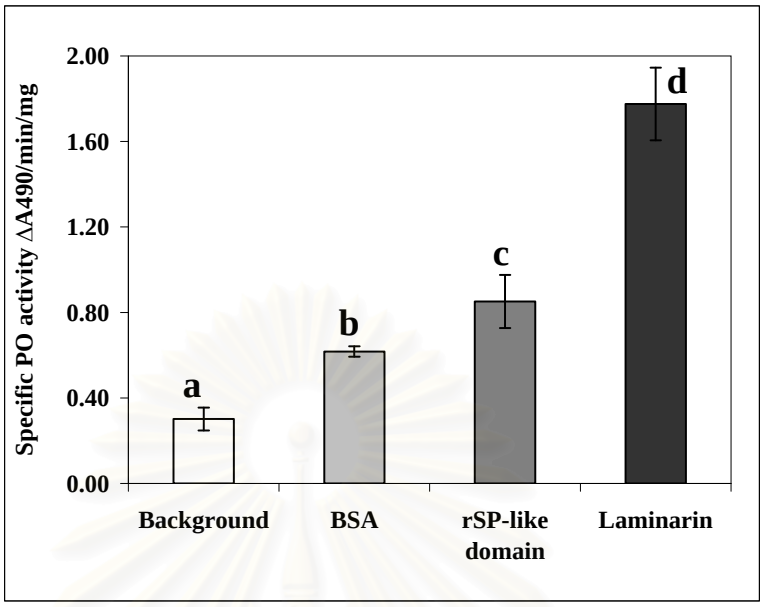



**Figure 3.25** The effect of the C-terminal SP-like domain of *Pm*MasSPH on PO activity. Hemocyte lysates of black tiger shrimp were pre-incubated laminarin, the rSP-like domain, BSA or CAC buffer as the control before PO activity assay. The PO activity was determined by measurement of the absorbance at 490 nm using L-DOPA as a substrate and specific PO activity was defined as an increase in OD490/min/mg protein of HLS. The reaction mixtures of each column were represented on the bottom of figure. All assays were performed in triplicate. Statistical analysis of data was analyzed with a one-way ANOVA followed by a post hoc test (Duncan's new multiple range test). Differences were considered significant at $P < 0.05$.

# CHAPTER IV

# DISCUSSIONS

Shrimp farming has been a commercially successful global industry during the past two decades, but now, tremendous losses of the shrimp production are seriously affected by infectious outbreaks (Lightner and Redman, 1998). Those problems have not been solved because the basic knowledge of shrimp immunity is still lacking. In order to effectively solve the disease problems and increase sustainability of shrimp farming, the study of immune-related genes and their products that would lead to better understanding the immune system is necessary (Bachere, 2000). There are several techniques to identify the genes involved in immune response of shrimp such as subtractive hybridization, differential display PCR as well as the Expressed Sequenced Tags (ESTs) analysis (Gross et al., 2001; He et al., 2004; Rojtinnakorn et al., 2002; Somboonwiwat et al., 2006; Supungul et al., 2002).

cDNAs encoding immune related proteins such as antimicrobial peptides (ALFs, crustin and penaeidin), lectins, protease inhibitors (Kazal and Kunitz-type inhibitors), heat shock proteins, genes involved in prophenoloxidase system (prophenoloxidase activating factor, prophenoloxidase, serine proteinase and their homologue), and some other immune molecules (transglutaminase, profilin, survivin, techylectin-5B, thymosin beta-4) have been identified from the black tiger shrimp by the EST approach (Supungul et al., 2002).

Serine proteinases (SPs) and serine proteinase homologues (SPHs) appear to be abundant in both vertebrates and invertebrates and are involved in a diverse variety

of biological functions (Campanelli et al., 1990; Jiang and Kanost, 2000; Lee and Söderhäll, 2001). It is thus not surprising that several partial sequences of cDNAs encoding serine proteinases and serine proteinase homologues have been identified from hemocyte cDNA libraries of the black tiger shrimp *P. monodon*. A putative serine proteinase homologue from *P. monodon* (*Pm*MasSPH) is one of the interested gene because the transcript of *Pm*MasSPH significantly increased upon *Vibrio harveyi* infection (Pulsook, 2005) indicating its potential role in shrimp immune responses.

To investigate the involvement of *Pm*MasSPH on shrimp immunity, a full-length cDNA encoding the *Pm*MasSPH from the black tiger shrimp was identified by the RACE-PCR technique. The powerful application of this technique was achievable isolation of the unknown 3’ or 5’ sequence of target gene (Frohman et al., 1988). Only a DNA fragment (800 bp) was obtained from the first round of 5’RACE-PCR representing still the partial cDNA sequence. In the second round of RACE-PCR, DMSO was included in PCR reaction because of the high GC content in the 5' region of this gene (Wang et al., 2000), the resulted RACE-PCR product contained the complete 5’ end of the *Pm*MasSPH gene. This result revealed that some difficulties of RACE technology were encountered and these may probably have resulted from the strong secondary structure of the *Pm*MasSPH mRNA.

Analysis of the nucleotide sequence at the 5’ region of *Pm*MasSPH sequence of 175 bp exhibited the very interesting characteristics. The 5’ region had a high GC content (73%) and the corresponding mRNA can form a peculiar structure *in vivo*. The secondary structure in 5’ region of *Pm*MasSPH mRNA was predicted by the GeneBee software package (http://www.genebee.msu.su/services/rna2_reduced.html). The prediction showed the formation of a very stable stem-loop structure ($\Delta G$ = -57.3 kcal/mol), suggesting that the secondary structure existed in 5’ region of *Pm*MasSPH

mRNA of *P. monodon* (data not shown). The presence of such a structure could explain the difficulties that we encountered in isolation the 5' end of this cDNA. It is very likely that the stable secondary structure of the *Pm*MasSPH mRNA made it difficult for the reverse transcriptase and polymerase to copy the 5' end of this molecule (Pan and Halper, 2003). In light of these results, it promised to be quite interesting to verify whether the peculiar sequence that found in the 5' region of *Pm*MasSPH mRNA might play an important role in the regulation of gene expression by means of post-transcriptional or translational control.

Moreover, a putative cDNA of *Lv*MasSPH from *Litopenaeus vannamei* was also identified using designed primers based on *Pm*MasSPH sequence. The amplicon could not be amplified by PCR using the designed primers based on 5' and 3' UTR of *Pm*MasSPH (5UTR-PmMas and 5PmRACE primers). When using the primers designed from the coding region of *Pm*MasSPH (PmMas-F and PmMas-R), a resulted PCR product was achieved. This result implied that the untranslated region is less conserved than the coding sequences. This finding was in concordance with the previous evidence in eukaryote that the rate of polymorphism was found to be greater in the UTR sequence of eukaryotic gene compared to that in the coding sequences (Fay and Benavides, 2005; Hellmann et al., 2003) .

To date, the full-length of several clip-domain SPH cDNAs have already been identified and cloned from different crustaceans such as blue crab *Callinectes sapidus* (Buda and Shafer, 2005), freshwater crayfish *Pacifastacus leniusculus* (Huang et al., 2000), kuruma shrimp *Marsupenaeus japonicus* (Rattanachai et al., 2005), black tiger shrimp *P. monodon* (Amparyup et al., 2007; Lin et al., 2006; Sriphaijit et al., 2007) and white shrimp *L. vannamei*. Most of SPH had a predicted amino acid sequence ranged from 350 to 500 residues with a single clip domain whilst the Mas-like protein

of *P. leniusculus* had 978 amino acid residues with seven repeats of a putative clip domain.

According to the previous report, more than one c-SPH isoforms were identified from the *Drosophila melanogaster* genome sequence. Two mas-like and low molecular mass mas-like molecules were found in crayfish hemocytes and three c-SPHs also existed in *Manduca sexta*. Remarkably, not only SPH isoforms were found in insect and crayfish but in shrimp also. At least four SPH sequences (C-SPH, *Pm*MasSPH, SPH509 and SPH516) have been identified from *P. monodon* and the different properties have been reported. A comparison of the deduced amino acid sequences of the shrimp SPHs (*Pm*MasSPH, *LvMasSPH*, SPH509 and SPH516) revealed that their sequences were highly similar but the most noticeable difference between these SPHs was the variation of the number of glycine repeats (*Pm*MasSPH: 11 repeats, SPH516: 10 repeats, SPH509: 9 repeats and *Lv*MasSPH: 9 repeats, data not show).

The N-terminal amino acid sequence of *Pm*MasSPH and *Lv*MasSPH were highly hydrophobic and represented a putative signal sequence (Nielsen et al., 1997), which was similar to the other arthropods SPHs. All known arthropod SPHs were secreted proteins for instance secreted SP-like molecule in the Drosophila embryo (Murugasu-Oei et al., 1995) and serine proteinase homologue Vn50 from the parasitoid, *Cotesia rubecula* (Asgari et al., 2003). The transcription expression of the SPH in *P. monodon* was determined by Western blotting and immunohistochemistry suggesting that SPH was transcribed in hemocytes and then secreted into the hemolymph. This was consistent with the presence of a putative signal peptide in the deduced protein sequence (Sriphaijit et al., 2007). Two potential of the putative N-glycosylation site were found in the both shrimp MasSPHs (Hansen et al., 1998),

suggesting that *Pm*MasSPH and *Lv*MasSPH were a glycoprotein. Asgari et al., (2003) reported that Vn50 containing the putative glycosylation site was indeed glycosylated protein, which was experimentally confirmed.

The *Pm*MasSPH and *Lv*MasSPH contained a clip domain at the N terminus and conserved inactive serine proteinase (SP)-like domain at the C terminus similar to most of the known arthropod SPHs. These molecules show homology to serine proteinases, except for a substitution within the catalytic triad that will render them without proteinase activity (Jiang and Kanost, 2000). The *Pm*MasSPH and *Lv*MasSPH, however, contain a somewhat different N terminus consisting of the repeated glycine-rich region.

Eleven and nine unique glycine-rich repeats presented at the N-terminal part of *Pm*MasSPH and *Lv*MasSPH proteins, respectively. No sequence homology with any other known proteins was found within the glycine-rich repeat domain. However, the pattern of glycine residues in this domain was similar to novel glycine-rich anti-microbial peptide (acanthoscurrin) constitutively expressed in the hemocytes of the spider, *Acanthoscurris gomesiana*. Acanthoscurrin was linear cationic peptides with a high glycine (72-73%) content and presence of the 3 repeats of GGGLGGGGLGGGG LGGGKGLGGGGLG (Lorenzini et al., 2005). It was therefore possible that the glycine-rich repeat domain might exhibit the antibacterial activity by reference to the function of glycine-rich antimicrobial peptides from the spider. Besides, the crayfish mase-like protein also contained a seven-repeated glycine-rich sequence at the N-terminal domain (Huang et al., 2000). This suggested that the glycine-rich repeat region in the N-terminal region of SPHs might have an important function for these proteins. Therefore, the role of the repeated glycine-rich region of a *Pm*MasSPH protein requires further investigation.

Clip domains are features only found in N-terminus of arthropod SPs and SPHs. The clip-domain of those proteins had six cysteine residues involving conserved disulfide bonds. They are cleaved from the proteinase domain upon the activation of the pro-protein but remain attached by disulfide bridges (Jiang and Kanost, 2000). By comparing to the amino acid sequence between different clip domains of *Pm*MasSPH with other arthropod SPHs, six Cys residues were strictly conserved in all clip domains formed the same disulfide bond patterns as in proclotting enzyme from horseshoe crab (Muta et al., 1990). Moreover, the arrangement of Cys in clip domain was identical to the β-defensins suggested that it had the folding patterns similar to these antibacterial proteins (Iwanaga et al., 1998). On the other hand, Piao et al. (2005) demonstrated that the crystal structure of clip domain of HdPPAF-II was different from that of the defensins. Although, shrimp SPs (Jimnez-Vega et al., 2005) and SPH of *M. japonicus* SPH (Rattanachai et al., 2005), there are only four and five instead of the six conserved Cys. These modified domains were called pseudo-clip domain.

The functions of clip domain in arthropod SP family were not yet well established. It seems likely that they were implicated in regulating the function of the enzymes through their interaction with other proteins (Jiang and Kanost, 2000). The recombinant clip domain in the crayfish ppA exhibited antibacterial activity towards Gram-positive bacteria (Wang et al., 2001). Clip domains linked to serine proteinase domains might serve as an inhibitor of the proteinase domain (Boudreault et al., 1998; Smith et al., 1995). Examination of clip structures of *M. sexta* PAP2 revealed that a highly positive charged on clip domain surface might interact with polyanionic bacterial surfaces through the electrostatic attraction (Huang et al., 2007). In the insect, *H. diomphalia*, crystal structure analysis of PPAF-II revealed the potential role of the

clip domain as the protein part that interacts with proPO through its central cleft (Piao et al., 2005). From these evident, the clip domain might have the multiple functions in arthropod immune responses.

The amino acid sequence of the C-terminal SP-like domain of *Pm*MasSPH also shared significant homology with those of typical arthropod PPAFs, such as *C. sapidus* PPAF, *A. mellifera* PPAF, *M. sexta* SPH4, mas-like SPH of *B. mori* and *T. molitor* PPAF. They had a glycine residue instead of the critical serine at the C-terminul SP-like domain. Analysis of amino acid sequence alignment, SP-like domain of *Pm*MasSPH and insect SPHs had four putative disulfide bonds but there were only three disulfide linkages in the SP-like domain of *Pm*cSPH (AAT42131), *Pm*SPH3 (ABO33174) and crayfish mas-like SPH (AAX55746). The insertion included two Cys residues which formed a disulfide bond resulting in a loop structure. This structure was potentially involved in the regulation of the prophenoloxidase system (Jiang and Kanost, 2000). A seventh Cys (at position 239 of *Pm*MasSPH) in the linker sequence presumably formed the inter-domain link with a Cys inside the SP-like domain. The inter-domain disulfide bridge probably maintained the clip domain and the SP-like domain together after proteolytic activation (Piao et al., 2005).

The phylogenetic tree clearly indicates that *Pm*MasSPH and *Lv*MasSPH were more closely related to non-catalytic SPHs rather than to the active SP. Moreover, *Pm*MasSPH and *C. sapidus* PPAF showed in the same branch pattern indicated that *Pm*MasSPH was the closest to *C. sapidus* PPAF among the examined species. Not only the unique feature of the Gly substitution for the Ser in SP-like domain was able to discriminate the SPHs from SPs but the presence of a calcium-binding site and four signature sequences were also used to classify these proteins (Piao et al., 2005).

In the present study, we examined the expression and localization of *Pm*MasSPH transcript in saline-injected (control) and *Vibrio*-injected shrimp using *In situ* hybridization. *In situ* hybridization technique was initially introduced in 1969 (John et al., 1969; Pardue and Gall, 1969) for the localization of DNA sequences. After that non-radioactive protocol to detect mRNA transcripts in different tissues was described (Macville et al., 1995). The immune reaction of shrimp and crustaceans is mainly achieved through circulating hemocytes and many immune factors located in hemocytes or released into plasma from hemocytes (Bachere, 2000). In addition, a masquerade-like SPH has been isolated and cloned from granular hemocytes from a crayfish (Sricharoen et al., 2005).

The reducing in total circulating hemocyte counts (THC) at early post-infection was observed in crustaceans. (Lorenzon et al., 1999). These phenomena were also reported in shrimp. THC of white shrimp significantly decreased at 6 h after bacterial, fungal infections (Muñoz et al., 2002). In white shrimp, THC also decreased after Taura syndrome virus infection compared with control shrimp (Song et al., 2003). WSSV-infected shrimp, *P. indicus* showed the reduction of THC compared to control shrimp (Yoganandhan et al., 2003).

The attribution of hemocytes to different defense responses caused of a reduced cell concentration in the hemolymph. The decrease in THC caused from many phenomena; such as hemocyte migration to the injection site (Muñoz et al., 2002; Van De Braak et al., 2002), hemocyte aggregation, phagocytosis (Martin et al., 1996) and hemocyte lysis (Aono and Mori, 1996; Johansson and Söderhäll, 1985).

*In situ* hybridisation analysis, purple stained hemocytes indicated that expression of *Pm*MasSPH transcript localized in hemocytes. The positive cells were detected in hemocytes at 6, 24, 48 and 72 h after *V. harveyi* injection. The result

suggested that the hemocytes expressing *Pm*MasSPH mRNA were significantly increased at 6 h ($p$ <0.05) and continues until 72 h after injection. These implied that *Pm*MasSPH mRNA was synthesized in circulating hemocytes and was up regulated upon microbial injection. This result was in agreement with that obtained from the real-time RT- PCR, which showed the highest expression of *Pm*MasSPH at 24 h after injection with *V. harveyi* (4.13-fold difference compared with relative expression at 0 h). However, the positive cells were not found in hemocytes of normal shrimp and at 0 hour post *V. harveyi* injection, the *Pm*MasSPH mRNA level of control shrimp could detect with low expression by real-time RT-PCR analysis. This result showed that *in situ* hybridization technique had a comparatively low sensitivity (Melton et al., 1984), whilst, quantitative RT-PCR was the most sensitive and accurate of the quantification methods (Wang and Brown, 1999)

Besides, expression profile of *Lv*MasSPH also was determined by the quantitative real-time RT-PCR. β-actin gene was constitutively expressed in most cell types and one of the most widely used as reference genes (Kusakabe, 1997; Romans et al., 1985), whereas, rRNAs were housekeeping genes and useful for the internal controls (Haeusler and Engelke, 2006). However, rRNA transcription might vary by biological factors and drugs (Spanakis, 1993). Therefore, β–actin was used as an internal control in this experiment. The transcription level of *Lv*MasSPH mRNA revealed the highest expression at 48 h post injection (P < 0.05) (5.7 times compared with relative expression at 0 h). The result suggested that .*Lv*MasSPH expression was also induced in response to infection stress corresponding to the expression profile of *Pm*MasSPH.

The obtained *in situ* hybridization and real-time RT-PCR results showed that both of shrimp *Pm*MasSPH and *Lv*MasSPH genes were an inducible gene upon

bacterial challenge. The shrimp SPH expression were principally localized in hemocytes and are significantly up-regulated in response to bacterial and fungal infections (Lin et al., 2006; Rattanachai et al., 2005). In insect, the expression of *H. diomphalia* masquerade-like SPH mRNA at various times after *E. coli* injection was examined by Northern blot analysis. The results showed that small amounts of mRNA were detected but mRNA levels were increased at 8 h after *E. coli* injection and the expression continued for 48 h (Kwon et al., 2000). Recently, SPH516 and SPH509 transcripts from *P. monodon* were highly expressed in hemocytes and gills. They exhibited the down-regulated after YHV infection that might be the result of a direct or indirect virus shut-off mechanism (Sriphaijit et al., 2007).

Like other proteinases, *Pm*MasSPH is produced as a precursor (inactive) and is activated by proteolytic cleavage. The non-processed and processed forms of *Pm*MasSPH in hemocyte lysate supernatant (HLS) were investigated by immunoblotting analysis using anti-SP-like domain antibody. HLS1-prepared in the presence of EDTA showed a major band of a non-processed form of the *Pm*MasSPH, whilst, HLS2-prepared in condition without EDTA and activated with laminarin-showed a band of processed form of the *Pm*MasSPH. There was evidence indicating that some serine proteinases in the HLS were involved in the activation of precursor form of SPH in crayfish (Huang et al., 2000). The result implied that the inhibitory effect of EDTA on proteolytic activity of proteinase in HLS1 was observed, therefore, the non-processed *Pm*MasSPH was successfully prepared. There were some reports supporting that the proteolytic activity of proteinase was inhibited by metal chelators such as EDTA (Moczon, 1995; Wu et al., 1998). The HLS1 represented a minor band (approximately 72 kDa) that probably corresponded to a contamination by

hemocyanin. Since hemocyanins were abundant proteins in the crustacean hemolymph (Chen et al., 2007), they might cross react with this antibody.

Analysis the size of processed form demonstrated that the activation site was represented at the position between Arg195 and Phe196 within clip domain. According to previous reports, *H. diomphala* PPAFII showed the cleavage site at clip domain that was cleaved by the serine proteinase PPAFIII (Kim et al., 2002). The *M. sexta* SPHs were processed at the site within the clip domain shown by unknown proteinases in the hemolymph (Yu et al., 2003). In contrast, the arthropod prophenoloxidase activating enzymes (ppA), *B. mori* (Satoh et al., 1999), *P. leniusculus* (Aspan and Söderhäll, 1991; Lee and Söderhäll, 2001), *M. sexta* (Jiang et al., 1998; Jiang et al., 2003) and *H. diomphalia* (Lee et al., 1998) represented the cleavage site at the beginning of SP domain. Recently, Piao et al. (2005) described that the proteolytic activation site in clip-domain was found in the PPAFII not in PAP2. These could explain by the different structure between Cys3 and Cys4 in clip domain. PAP2 adopted a helix-turn-helix fold whereas in PPAFII formed a long loop containing a small piece of $\beta$-strand. Notably, Clip domain is tightly associated with SPL domain after proteolytic cleavage in arthropods SPH (Piao et al., 2005).

In arthropods, serine proteinase homologues are produced as a precursor and need a specific proteinase to produce the active protein (Kim et al., 2002; Lee et al., 2002; and Söderhäll, 2001). Recently, *M. sexta* SPH precursors were expressed in insect cells and searched for their activating proteinases. The proteolytic cleavage of proSPH-1 was incomplete (Lu and Jiang, 2008). The information of activation was still limit. Although, it would be better to express the whole *Pm*MasSPH protein, its activation by the proteolytic cleavage can be problematic. So, our study begins with over-expression of SP-like domain in the *E. coli* system.

The *E. coli* expression system was one of the most widely used hosts for the production proteins from eukaryotic organisms such as insect, shrimp, and human (De-La-Re-Vega et al., 2004; Savopoulos et al., 2000; Yu and Kanost, 2003). Moreover, advantages of this expression system were simple, time saving, large quantities of protein and cost effective. Therefore, the SP-like domain of *Pm*MasSPH was expressed in the *E. coli* strain Rosetta (DE3). The Rosetta strains supplied rare codons, provide enhanced the expression of eukaryotic proteins that contain codons rarely. Moreover, it had the compatible chloramphenicol resistant (Brinkmann et al., 1989; Kane, 1995; Seidel et al., 1992)

After 4 h IPTG induction, the recombinant SP-like domain was expressed in a large production in *E. coli* expression system but it was formed as the inclusion bodies. Unfortunately, the recombinant protein expression in prokaryotes is often to obtain the precipitated protein as inclusion bodies distributed in the cytoplasm (Hockney, 1994; Krueger et al., 1989; Kurland and Gallant, 1996).

From the SDS-PAGE result (Figure 3.18), we obtained the inclusion bodies that were quite pure. The inclusion bodies, rSP-like domain, were solubilized in the denaturing buffer containing urea before purification. The recombinant protein was efficiently purified a Ni affinity column. Subsequently, the purified recombinant protein was slowly refolded in three dialysis steps. To slightly reduced urea concentration in dialysis buffer, the precipitated protein was found less than dialysis in buffer without urea. The identity and purity of rSP-like domain were analyzed on 12% (w/v) SDS-PAGE and confirmed by immunoblotting using anti-His antibody. A single band of the rSP-like domain, approximately 31 kDa, was observed corresponding to the molecular mass of rSP-like domain based on MALDI-TOF mass fingerprint analysis and its deduced amino acid sequence including the extra glycine

and six histidine residues. Moreover, MALDI-TOF mass fingerprint analysis confirmed that the rSP-like domain preparation was pure without other proteins contamination. Since the obtained recombinant protein contained the histidine residues at the C terminus, the immunoblotting analysis could be employ to confirm the identity of recombinant protein using the anti-His antibody. The result indicated that the 31 kDa protein was actually the rSP-like domain and successfully over-expressed in the *E. coli* expression system. Only one band of the C-terminal SP-like domain was detected on the native PAGE indicating that this protein adopted one conformation and presumably the correctly folded protein.

The SP-like domain of *Pm*MasSPH has a serine replaced with glycine in the catalytic triad suggested that it losses enzymatic activity. To confirm this notion, various chromogenic substrates for trypsin, chymotrypsin and elastase were tested. In fact, the recombinant C-terminal SP-like domain of *Pm*MasSPH had no detectable proteinase activity. In former research, *M. sexta* SPH in the hemolymph, that did not has the proteolytic activity, enhanced activation of proPO in connection with other components in hemolymph (Yu et al., 2003). SPHs including Vn50 was experimentally determined the proteolytic activity. The result showed that no proteolytic activity was detected (Asgari et al., 2003).

So far, several SPHs found in vertebrates and invertebrates have been demonstrated to possess variety biological functions. Human azurocidin, which is a serine proteinase homologue, exhibits antimicrobial activity against Gram-positive and Gram-negative bacteria and the yeast *Candida albicans* (Campanelli et al., 1990) A masquerade-like serine proteinase homologue in *Drosophila* has been shown to be involved in embryonic muscle development (Murugasu-Oei et al., 1995). In arthropods, SPHs participate in an antimicrobial action in the horseshoe crab

*Tachypleus tridentatus* (Kawabata et al., 1996), as an immune molecule in the mosquito *Anopheles gambiae* (Dimopoulos et al., 1997), in the regulation of the proPO system in some coleopteran and lepidopteran insects (Kwon et al., 2000; Lee et al., 2002; Yu et al., 2003), in pattern recognition, opsonization and cell adhesion activity (Lee and Soderhall, 2001). Moreover, A SPH venom protein inhibited melanization was that having structural and the protein might function as an antagonist molecule competing with host SPHs for binding sites of immunolectins and proPO (Asgari et al., 2003). However, the molecular mechanism by which crustacean SPHs were involved in the regulation of the prophenoloxidase system was yet to be clarified.

In the present study, the potential functions of *Pm*MasSPH were investigated by screen for various activities of the C-terminal SP-like domain such as cell adhesion, binding activity to bacteria as well as phenoloxidase (PO) activity. In cell adhesion assay, a high number of hemocytes adhered to SP-like domain-coated coverslips, whereas less hemocyte adhered to BSA-coated coverslips. The result suggested that the recombinant C-terminal SP-like domain of *Pm*MasSPH revealed a likely cell adhesion activity.

In cell adhesion assay, all populations of hemocytes containing granular, semigranular and hyaline cells were used. In crayfish, *in vivo* the cell adhesion factors were store in the semigranular and the granular cells in a putative inactive pro-form, which can be released during exocytosis (Johansson and Söderhäll, 1988; Kobayashi et al., 1990). Examination of cell attached to the coverslips for an hour after incubation, the semigranular cells was lysed and thereby released other cell adhesion molecules (Johansson and Söderhäll, 1989; Smith and Söderhäll, 1983). In control experiment, we found that some hemocytes (25%) could attach to the BSA-coated

coverslips. It may be result of the lyses of semigranular hemocytes and it released other cell adhesion factors. These may affect on the assay. However, the cell adhesion activity was significantly higher in the coverslips coated with the C-terminal-domain (51%) than those coated with the BSA (25%) and all assays were performed in triplicate. The crayfish and shrimp myeloperoxidase homologue and peroxinectin contained the integrin binding (KGD/RGD) motif that able them to directly bind the integrin receptor on blood cells (Johansson and Söderhäll, 1989; Sritunyalucksana et al., 2001). Indeed, the *Pm*MasSPH also contained a KGD motif at the C-terminal SP-like domain that may participate in cell adhesion activity. Sequence alignment also showed that the KGD motif at the position within the C-terminal SP-like was conserved in several insect and crustacean SPs and SPHs (data not shown). Nevertheless, a shrimp clip domain serine proteinase homologue (c-SPH) (Lin et al., 2006) and a crayfish mas-like protein (Huang et al., 2000) which also exhibited cell adhesion activity did not contain the KGD motif and the mechanism(s) by which they bind to blood cells remains unknown. A shrimp c-SPH has also been shown to have cell adhesion activity but in this case no antimicrobial and opsonic activities (Lin et al., 2006).

The recombinant C-terminal SP-like domain of *Pm*MasSPH also showed ability to bind strongly to both Gram-positive and Gram-negative bacteria as well as specifically bind to the microbial cell wall components; LPS, peptidoglycan and laminarin. The crystal structure of the SP-like domain of PPAF-II demonstrated that this domain contained two clefts that might serve as the site for binding a protein (Piao et al., 2005). The specific binding of the crayfish mas-like protein to Gram-negative bacteria and yeast suggested it roles as a pattern recognition molecule. After

proteolytic activation, the crayfish mas-like protein reveals cell adhesion and opsonic activity but does not have antimicrobial activity (Lee and Söderhäll, 2001). Recently, the C-terminal domain of a serine proteinase homologue, SPH516, from *P. monodon* was shown to interact with a metal ion-binding domain (MIB) of yellow head virus (Sriphaijit et al., 2007). SPH516 is most likely an allelic variant of *Pm*MasSPH since it contains an identical predicted amino acid sequence except for one less repeat of the septapeptide in the N-terminal glycine-rich region. Therefore, *Pm*MasSPH potentially involved in both antiviral and bacterial responses.

Previous reports in insects have shown that SPHs are necessary for activating the proPO cascade and function as cofactors (Kwon et al., 2000; Lee et al., 2002; Yu et al., 2003). However, the essential role of SPHs in the ProPO activation has not been demonstrated neither in the silkworm nor crustaceans. In silkworm and crayfish, the prophenoloxidase activating enzyme can convert proPO to active PO without the need of an SPH cofactor (Satoh et al., 1999; Wang et al., 2001). In this study, the addition of laminarin using as a positive control significantly enhanced PO activity representing that the proPO system was activated by larminarin. In the earlier report on the invertebrate proPO system indicated that the activation of proPO system was specifically activated by microbial cell wall component such as larminarin, β-1,3-glucan, lipopolysaccharides, peptidoglycans (Cerenius and Söderhäll, 2004; Sooderhall and Cerenius, 1998). The C-terminal rSP-like domain of *Pm*MasSPH did not strongly enhance the PO activity. In fact, the recombinant C-terminal SP-like domain of *Pm*MasSPH had no detectable proteinase activity, which presumably prevented it from direct activation of the proteinase cascade in the proPO system. However, this result unclearly demonstrates the involvement of this protein in proPO

system, further investigation is required. The results reported here, thus, suggest an important role for *Pm*MasSPH in shrimp immunity.

# CHAPTER V

# CONCLUSIONS

1. The full-length cDNA of a serine proteinase homologue of *Penaeus monodon* (*Pm*MasSPH) and *Litopenaeus vannamei* (*Lv*MasSPH) were successfully identified by RACE method.
2. A full-length cDNA of *Pm*MasSPH (1,958 bp) contained an open reading frame (ORF) of 1,572 bp encoding a 523 amino acid protein. The calculated molecular mass of the mature protein is 51.58 kDa with an estimated pI of 4.86.
3. A complete sequence of *Lv*MasSPH (1,955 bp) consisted of an open reading frame of 1,536 bp encoding for a polypeptide of 511 amino acids. The calculated molecular mass of the mature protein 50.93 kDa and its estimated pI is 5.20.
4. Both of *Pm*MasSPH and *Lv*MasSPH contained a similar domain structures: a glycine-rich repeated region, disulfide knotted clip domain at the N-terminus and a SP-like domain at the C terminus. The replacement catalytic triad of SP-like domain has a Gly substitution for critical serine suggested that both of them were the non-catalytic serine proteinase homologues (SPHs).
5. Sequence comparison with data in the GenBank database showed that the deduced amino acid of *Pm*MasSPH had an identity of 58%, 55%, 53%, 52% and 50% to those of *Callinectes sapidus* PPAF, *Apis mellifera* PPAF, *Bombyx*

*mori* masquerade-like SPH, *Lonomia obliqua* PPAF1 and *Tenebrio molitor* PPAF, respectively

6. Homology search of *Lv*MasSPH against the GenBank database showed identity of 93%, 92%, 92%, 57% and 57% to those of *P. monodon* *Pm*MasSPH, *P. monodon* SPH509, *P. monodon* SPH516, *C. sapidus* PPAF and *N. vitripennis* PPAF , respectively.
7. A phylogenetic tree clearly revealed that *Pm*MasSPH and *Lv*MasSPH were more closely related to the non-catalytic SPHs than to the active SP.
8. *In situ* hybridization showed tha*t Pm*MasSPH mRNA was transcribed in hemocytes of *P. monodon* and upregulated within 6 h after *Vibrio harveyi* injection. Moreover, the expression level of *Lv*MasSPH transcript was investigated by real-time RT-PCR analysis. *Lv*MasSPH transcript was also induced in response to *V. harveyi* infection. The results suggested that *Pm*MasSPH and *Lv*MasSPH gene were the immune-inducible genes associated with bacterial invasion.
9. *Pm*MasSPH was produced as a precursor and need a specific proteinase to produce the active protein in hemocyte lysate.
10. A recombinant C-terminal SP-like domain from *Pm*MasSPH of *P. monodon* was successfully over-expressed in *E. coli* and purified by a Ni column.
11. The recombinant SP-like domain lacked the proteolytic activity confirming that this protein was the non-catalytic SPH.
12. The SP-like domain mediates hemocyte adhesion and displays the binding ability to Gram-negative and Gram-positive bacteria and microbial cell wall. The binding ability of SP-like domain to bacteria and cell wall components implied that it may act as a pattern recognition molecule.

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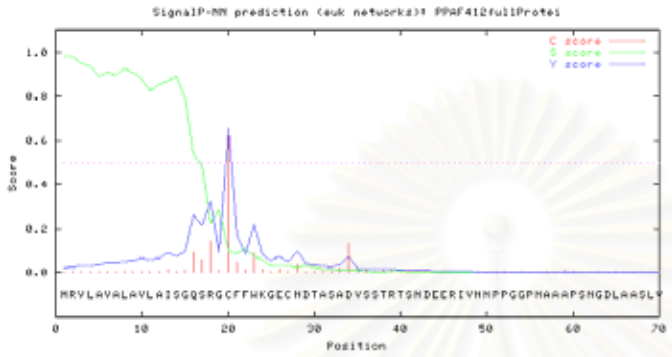
# APPENDICES

# APPENDIX A

# The signal peptide prediction by SignalP

## 1. *Pm*MasSPH

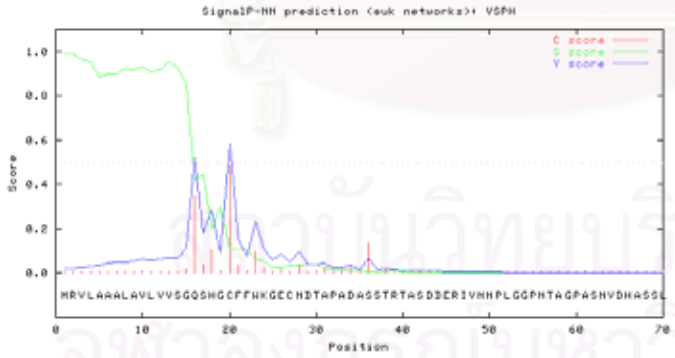



> *Pm*MasSPH length = 70

| # Measure | Position | Value | Cutoff | signal peptide |
|---|---|---|---|---|
| max. C | 20 | 0.623 | 0.32 | YES |
| max. Y | 20 | 0.654 | 0.33 | YES |
| max. S | 2 | 0.980 | 0.87 | YES |
| mean S | 1-19 | 0.790 | 0.48 | YES |
| D | 1-19 | 0.722 | 0.43 | YES |

# Most likely cleavage site between position 19 and 20: SRG-CF

## 2. *Lv*MasSPH




> *Lv*MasSPH length = 70

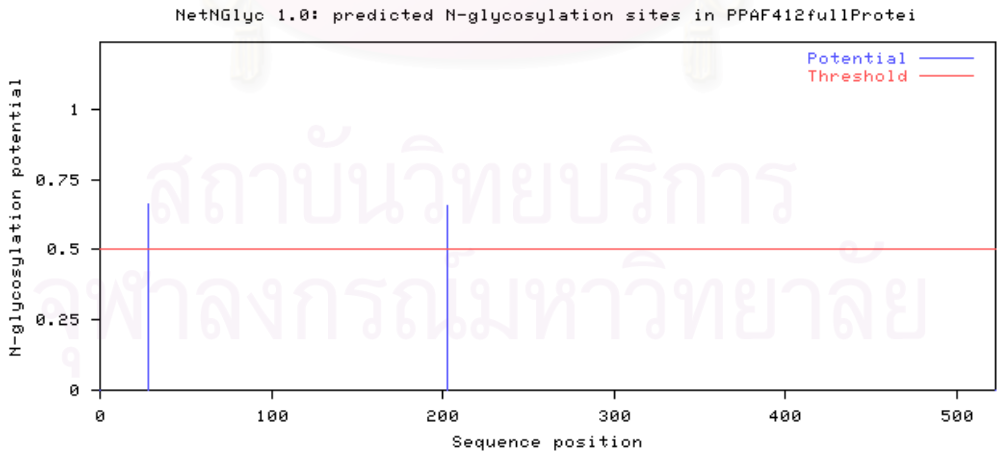
| # Measure | Position | Value | Cutoff | signal peptide |
|---|---|---|---|---|
| max. C | 20 | 0.488 | 0.32 | YES |
| max. Y | 20 | 0.584 | 0.33 | YES |
| max. S | 1 | 0.987 | 0.87 | YES |
| mean S | 1-19 | 0.804 | 0.48 | YES |
| D | 1-19 | 0.694 | 0.43 | YES |

# Most likely cleavage site between position 19 and 20: SHG-CF4

# The N-Glycosylation sites prediction with NetNglyc server

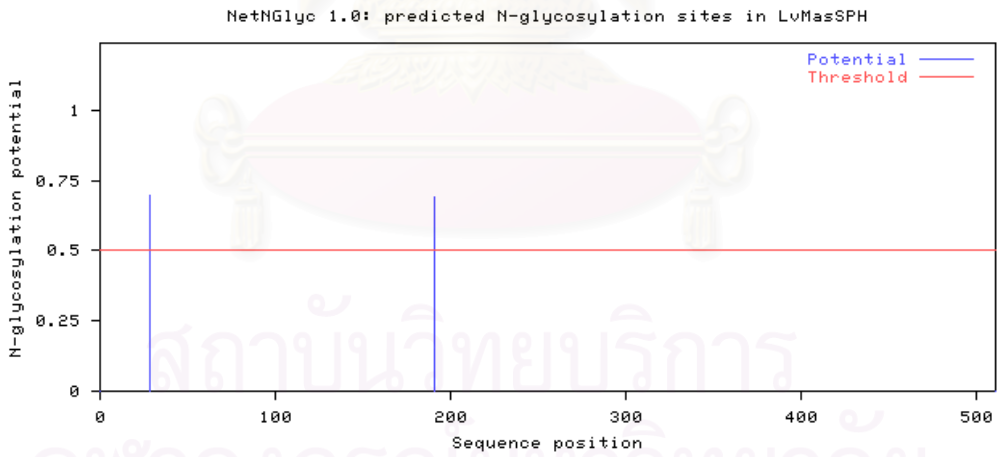
## 1. *Pm*MasSPH

```
Name:  PmMasSPH          Length:  523
MRVLAVALAVLAISGQSRGCFFWKGECNDTASADVSSTRTSNDEERIVNNPPGGPNAAAPSNGDLAASLVGLLNGGAAGG      80
LGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGVVD      160
EGITACNNGLGVCVPYYLCNEGNVITDGAGLIDIRFGNSKKSNDTSTRSSSDCPQFLDVCCTNPNPPDVVTPAPYTPRCG      240
KRNSQGFDVRITGFKDNEAQFAEFPWMTAILRVEKVGKKELNLYVCGGSLIHPSIVLTAAHCVHSKAASSLKTRFGEWDT      320
QKTYERYPHQDRNVISVKIHPNYNSGALYNDFALLFLDSPATLAPNVDTVCLPQANQKFDYDTCWATGWGRDKFGKEGEF      400
QNILKEVALPVVPNHDCQNGLRTTRLGSFFQLHNSFMCAGGQQGIDTCKGDGGSPLVCEAVAGSGVYVQAGIVAWGIGCG      480
EQGVPGVYADVGYASDWIQTEANIGLASLYSIQGYDWDYGRFI
.........................N.................................................      80
.....................................................................................      160
.........................................N.....................................      240
.....................................................................................      320
.....................................................................................      400
.....................................................................................      480
.........................................                                            560
(Threshold=0.5)
---------------------------------------------------------------------------
SeqName       Position    Potential      Jury          N-Glyc
                                                     agreement result
---------------------------------------------------------------------------
PmMasSPH         28         NDTA         0.6616          (9/9)   ++
PmMasSPH        203         NDTS         0.6586          (9/9)   ++
---------------------------------------------------------------------------
```



## 2. *Lv*MasSPH

```
Name:  LvMasSPH     Length:  511
MRVLAAALAVLVVSGQSHGCFFWKGECNDTAPADASSTRTASDDERIVNNPLGGPNTAGPASNVDHASSLVGLLNGAVAG      80
GLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGQGGGLGGLGGGLGGGVSDQDITPCNNGLGV     160
CVPYYLCNEGNVITDGAGPIDIRFGNSKKGNDTSTRSSSDCPQFLDVCCTNPNPPDVVTPAPYTPRCGKRNSQGFDVRIT     240
GFKNNEAQFAEFPWMTAILRVERVGEKELNLYVCGGSLIHPSIVLTAAHCVHSKAARSLKARFGEWDTQKTYERYPHQDR     320
NVISVKIHPNYNSGALYNDFALLFLDSPVTLAPNVDTVCLPQANQKFDYDTCWATGWGKDKFGKEGEFQNILKEVALPVV     400
PNNDCQKGLRTTRLGSFFQLHDSFMCAGGQQGLDTCKGDGGSPLVCEAVKGSGVYVQAGIVAWGIGCGEQGVPGVYADVG     480
YASNWIQTEANIGLNSHYNIQGYNWDYGRFV
.........................N.....................................................      80
.....................................................................................     160
.............................N.....................................................     240
.....................................................................................     320
.....................................................................................     400
.....................................................................................     480
.............................                                                         560
(Threshold=0.5)
---------------------------------------------------------------------
SeqName       Position       Potential      Jury          N-Glyc
                                                        agreement result
---------------------------------------------------------------------
LvMasSPH         28            NDTA         0.6962       (9/9)   ++
LvMasSPH        191            NDTS         0.6908       (9/9)   ++
---------------------------------------------------------------------
```



# The molecular mass prediction by Genetyx program

**1. The mature protein of *Pm*MasSPH**

Sequence Size : 504
Sequence Position: 1 - 504
hydrophobic: 279( 55.36)
neutral : 126( 25.00)
hydrophilic: 99( 19.64)
other : 0( 0.00)
[ hydrophobic residues ]
Gly(G) 103( 20.44%) Ala(A) 35( 6.94%) Val(V) 32( 6.35%) Leu(L) 39( 7.74%)
Ile(I) 19( 3.77%) Met(M) 2( 0.40%) Phe(F) 18( 3.57%) Trp(W) 8( 1.59%)
Pro(P) 23( 4.56%)
[ neutral residues ]
Ser(S) 28( 5.56%) Thr(T) 24( 4.76%) Asn(N) 29( 5.75%) Gln(Q) 27( 5.36%)
Cys(C) 18( 3.57%)
[ hydrophilic residues ]
Asp(D) 28( 5.56%) Glu(E) 17( 3.37%) Lys(K) 17( 3.37%) His(H) 7( 1.39%)
Arg(R) 15( 2.98%) Tyr(Y) 15( 2.98%)
[ other residues ]
Asx(B) 0( 0.00%) Glx(Z) 0( 0.00%) Xaa(X) 0( 0.00%) ???(?) 0( 0.00%)

Average Molecular Weight = 51588.23 Da

Monoisotopic Molecular Weight = 51556.7404 Da

**2. The recombinant SP-like domain of *Pm*MasSPH**

Sequence Size : 282
Sequence Position: 1 - 282
hydrophobic: 142( 50.35)
neutral : 65( 23.05)
hydrophilic: 75( 26.60)
other : 0( 0.00)
[ hydrophobic residues ]
Gly(G) 31( 10.99%) Ala(A) 23( 8.16%) Val(V) 20( 7.09%) Leu(L) 20( 7.09%)
Ile(I) 14( 4.96%) Met(M) 3( 1.06%) Phe(F) 13( 4.61%) Trp(W) 7( 2.48%)
Pro(P) 11( 3.90%)
[ neutral residues ]
Ser(S) 15( 5.32%) Thr(T) 14( 4.96%) Asn(N) 13( 4.61%) Gln(Q) 14( 4.96%)
Cys(C) 9( 3.19%)
[ hydrophilic residues ]
Asp(D) 16( 5.67%) Glu(E) 12( 4.26%) Lys(K) 13( 4.61%) His(H) 13( 4.61%)
Arg(R) 9( 3.19%) Tyr(Y) 12( 4.26%)
[ other residues ]
Asx(B) 0( 0.00%) Glx(Z) 0( 0.00%) Xaa(X) 0( 0.00%) ???(?) 0( 0.00%)

Average Molecular Weight = 31060.39
Monoisotopic Molecular Weight = 31041.1198

**3. The processed form of *Pm*MasSPH was obtained from the activation cleavage sites after arginine within the clip domain (Arg195).**

Sequence Size : 328
Sequence Position: 1 - 328
hydrophobic: 159( 48.48)
neutral : 88( 26.83)
hydrophilic: 81( 24.70)
other : 0( 0.00)
[ hydrophobic residues ]
Gly(G) 33( 10.06%) Ala(A) 24( 7.32%) Val(V) 24( 7.32%) Leu(L) 21( 6.40%)
Ile(I) 14( 4.27%) Met(M) 2( 0.61%) Phe(F) 16( 4.88%) Trp(W) 7( 2.13%)
Pro(P) 18( 5.49%)
[ neutral residues ]
Ser(S) 22( 6.71%) Thr(T) 19( 5.79%) Asn(N) 18( 5.49%) Gln(Q) 16( 4.88%)
Cys(C) 13( 3.96%)
[ hydrophilic residues ]
Asp(D) 21( 6.40%) Glu(E) 12( 3.66%) Lys(K) 16( 4.88%) His(H) 7( 2.13%)
Arg(R) 12( 3.66%) Tyr(Y) 13( 3.96%)
[ other residues ]
Asx(B) 0( 0.00%) Glx(Z) 0( 0.00%) Xaa(X) 0( 0.00%) ???(?) 0( 0.00%)

Average Molecular Weight = 35868.57 Da
Monoisotopic Molecular Weight = 35846.3553 Da

**4. The processed forms of PmMasSPH was obtained from the activation cleavage sites at the beginning of the SP-like domain (Arg250).**

Sequence Size : 273
Sequence Position: 1 - 273
hydrophobic: 140( 51.28)
neutral : 65( 23.81)
hydrophilic: 68( 24.91)
other : 0( 0.00)
[ hydrophobic residues ]
Gly(G) 30( 10.99%) Ala(A) 23( 8.42%) Val(V) 20( 7.33%) Leu(L) 20( 7.33%)
Ile(I) 14( 5.13%) Met(M) 2( 0.73%) Phe(F) 13( 4.76%) Trp(W) 7( 2.56%)
Pro(P) 11( 4.03%)
[ neutral residues ]
Ser(S) 15( 5.49%) Thr(T) 14( 5.13%) Asn(N) 13( 4.76%) Gln(Q) 14( 5.13%)
Cys(C) 9( 3.30%)
[ hydrophilic residues ]
Asp(D) 16( 5.86%) Glu(E) 12( 4.40%) Lys(K) 13( 4.76%) His(H) 7( 2.56%)
Arg(R) 8( 2.93%) Tyr(Y) 12( 4.40%)
[ other residues ]
Asx(B) 0( 0.00%) Glx(Z) 0( 0.00%) Xaa(X) 0( 0.00%) ???(?) 0( 0.00%)

Average Molecular Weight = 29893.11 Da
Monoisotopic Molecular Weight = 29874.6034 Da

**5. The mature protein of *Lv*MasSPH**

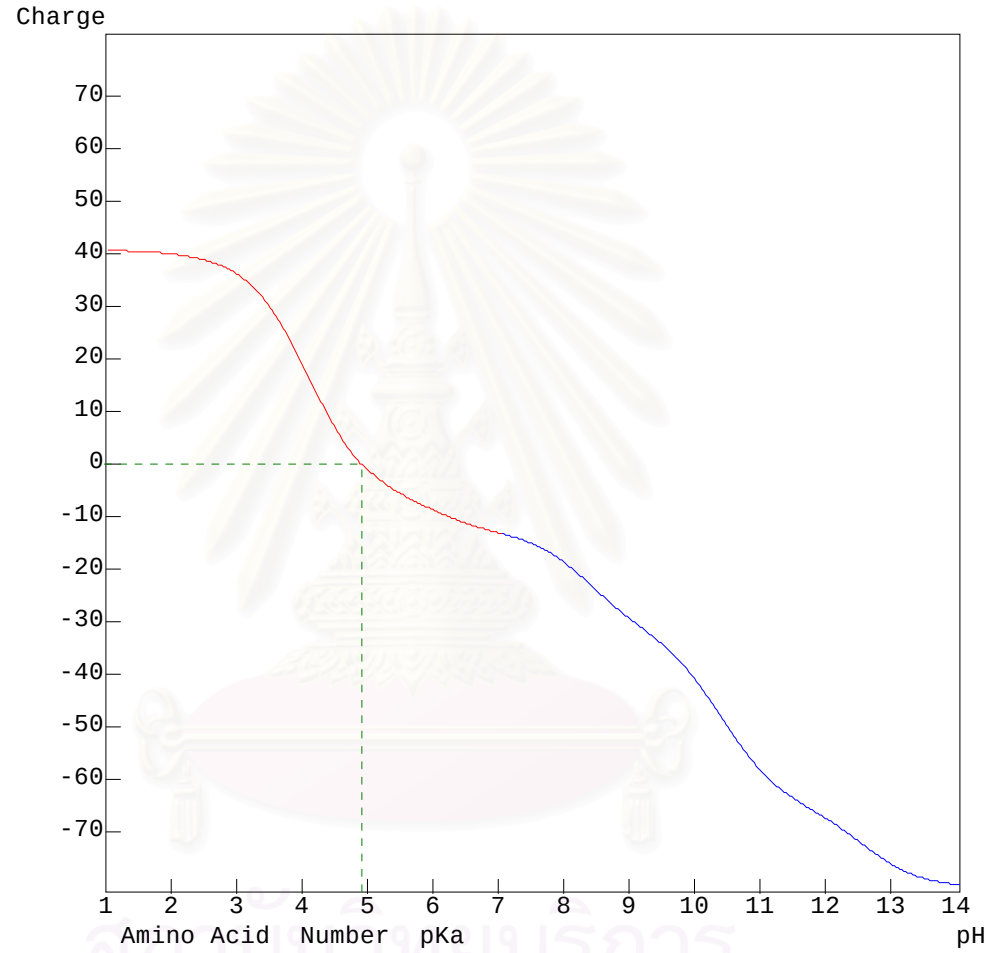
Sequence Size : 492
Sequence Position: 1 - 492
hydrophobic: 267( 54.27)
neutral : 124( 25.20)
hydrophilic: 101( 20.53)
other : 0( 0.00)
[ hydrophobic residues ]
Gly(G) 93( 18.90%) Ala(A) 32( 6.50%) Val(V) 34( 6.91%) Leu(L) 38( 7.72%)
Ile(I) 17( 3.46%) Met(M) 2( 0.41%) Phe(F) 18( 3.66%) Trp(W) 8( 1.63%)
Pro(P) 25( 5.08%)
[ neutral residues ]
Ser(S) 26( 5.28%) Thr(T) 24( 4.88%) Asn(N) 32( 6.50%) Gln(Q) 24( 4.88%)
Cys(C) 18( 3.66%)
[ hydrophilic residues ]
Asp(D) 28( 5.69%) Glu(E) 16( 3.25%) Lys(K) 18( 3.66%) His(H) 8( 1.63%)
Arg(R) 16( 3.25%) Tyr(Y) 15( 3.05%)
[ other residues ]
Asx(B) 0( 0.00%) Glx(Z) 0( 0.00%) Xaa(X) 0( 0.00%) ???(?) 0( 0.00%)

Average Molecular Weight = 50933.63 Da
Monoisotopic Molecular Weight = 50902.5066 Da

# The prediction of putative pI by Genetyx program

## 1. The mature protein of PmMasSPH

Sequence position : 1 - 504



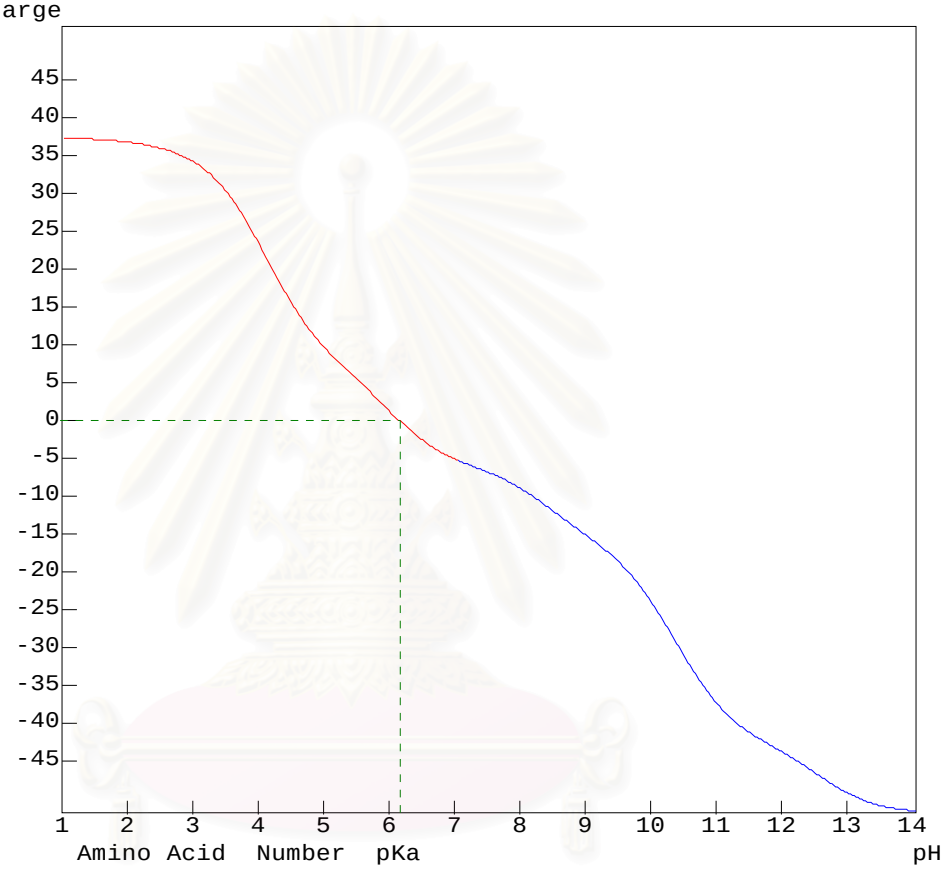

| Amino Acid | Number | pKa |
|---|---|---|
| Arg (R) | 15 | 12.5 |
| His (H) | 7 | 6.0 |
| Lys (K) | 17 | 10.5 |
| Asp (D) | 28 | 3.9 |
| Cys (C) | 18 | 8.3 |
| Glu (E) | 17 | 4.3 |
| Tyr (Y) | 15 | 10.1 |
| N-terminal Cys (C) | | 10.8 |
| C-terminal Ile (I) | | 2.3 |

Isoelectric point: 4.86

## 2. The recombinant SP-like domain of PmMasSPH

```
[GENETYX : Calculate isoelectric point]
Date              : 2007.07.29
Filename          : AA_SPH domain in pET28b+
Sequence size     : 282
Sequence position : 1 - 282
```

Charge




| Amino Acid | Number | pKa |
|---|---|---|
| Arg (R) | 9 | 12.5 |
| His (H) | 13 | 6.0 |
| Lys (K) | 13 | 10.5 |
| Asp (D) | 16 | 3.9 |
| Cys (C) | 9 | 8.3 |
| Glu (E) | 12 | 4.3 |
| Tyr (Y) | 12 | 10.1 |

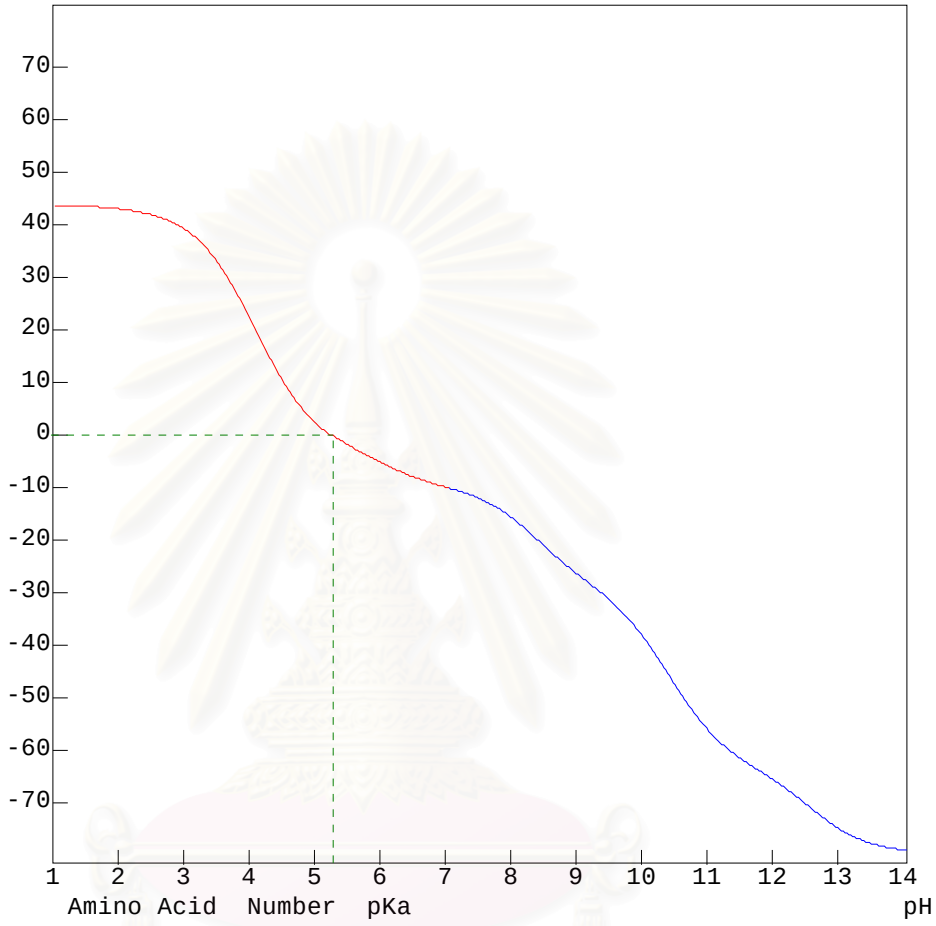
| | |
|---|---|
| N-terminal Met (M) | 9.3 |
| C-terminal Ile (I) | 2.3 |

Isoelectric point: 6.10

### 3. The mature protein of *Lv*MasSPH

Sequence position : 1 - 492

Charge




| Amino Acid | Number | pKa |
|---|---|---|
| Arg (R) | 16 | 12.5 |
| His (H) | 8 | 6.0 |
| Lys (K) | 18 | 10.5 |
| Asp (D) | 28 | 3.9 |
| Cys (C) | 18 | 8.3 |
| Glu (E) | 16 | 4.3 |
| Tyr (Y) | 15 | 10.1 |
| N-terminal Cys (C) | | 10.8 |
| C-terminal Val (V) | | 2.3 |

Isoelectric point: 5.20

# APPENDIX B

# Statistical analysis by ANOVA and DUNCAN test

## *in situ* hybridization experiment

1. ANOVA test of *Pm*MasSPH expression in hemocytes of *V. harveyi*-challenged *P. monodon*

**ANOVA**

PmMasSPH

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3438.681 | 4 | 859.670 | 14.037 | .000 |
| Within Groups | 612.439 | 10 | 61.244 | | |
| Total | 4051.120 | 14 | | | |

2. DUNCAN test of *Pm*MasSPH expression in hemocytes of *V. harveyi*-challenged *P. monodon*

**PmMasSPH**

Duncan

| Hours | N | Subset for alpha = .05 | |
|---|---|---|---|
| | 1 | 2 | 1 |
| .00 | 3 | .0000 | |
| 48.00 | 3 | | 30.3979 |
| 72.00 | 3 | | 34.8588 |
| 24.00 | 3 | | 39.1478 |
| 6.00 | 3 | | 41.8960 |
| Sig. | | 1.000 | .124 |

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

**Real-Time RT-PCR**

1. ANOVA test of *Lv*MasSPH expression in hemocytes of *V. harveyi*-challenged *L. vannamei*

**ANOVA**

LvMasSPH

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 44.639 | 4 | 11.160 | 55.419 | .000 |
| Within Groups | 2.014 | 10 | .201 | | |
| Total | 46.653 | 14 | | | |

2. DUNCAN test of *Lv*MasSPH expression in hemocytes of *V. harveyi*-challenged *L. vannamei*

**LvMasSPH**

Duncan

| Hours | N | Subset for alpha = .05 | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 1 |
| .00 | 3 | .9963 | | |
| 6.00 | 3 | 1.3327 | 1.3327 | |
| 24.00 | 3 | 1.6850 | 1.6850 | |
| 72.00 | 3 | | 1.9650 | |
| 48.00 | 3 | | | 5.7297 |
| Sig. | | .103 | .130 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

**Assay for phenoloxidase activity**

1. ANOVA test of the effect of recombinant protein and elicitor on phenoloxidase activity

**ANOVA**

PO activity

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 7.231 | 3 | 2.410 | 202.183 | .000 |
| Within Groups | .238 | 20 | .012 | | |
| Total | 7.469 | 23 | | | |

2. DUNCAN test of the effect of recombinant protein and elicitor on phenoloxidase activity

**PO activity**

Duncan

| | N | Subset for alpha = .05 | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 1 |
| Background | 6 | .3021 | | | |
| BSA | 6 | | .6173 | | |
| rSP-like | 6 | | | .8519 | |
| laminarin | 6 | | | | 1.7753 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

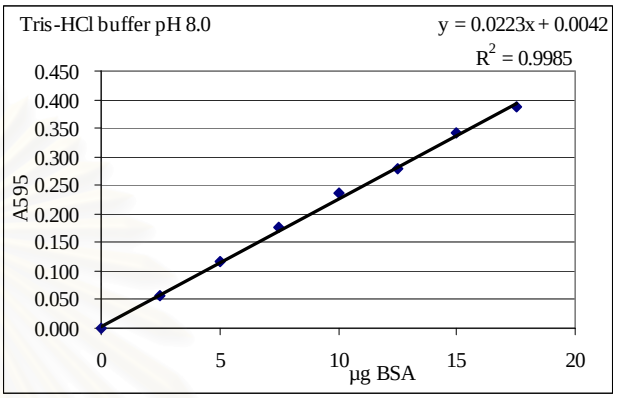
a Uses Harmonic Mean Sample Size = 6.000.

# APPENDIX C

# BSA standard curve using Bradford method

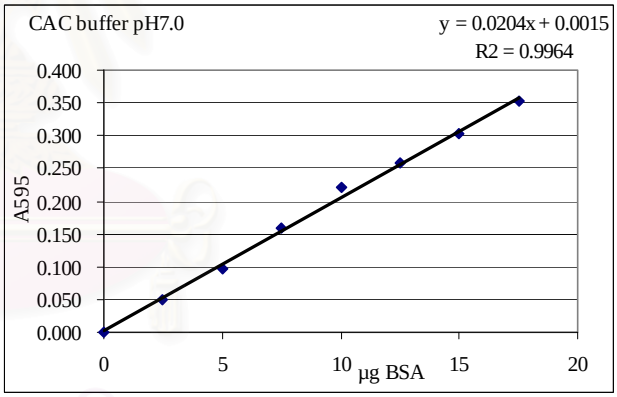
## 1. Tris-HCl buffer pH 8.0

| µg BSA | A595 | | Avr A595 |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 2.5 | 0.0547 | 0.0565 | 0.0556 |
| 5 | 0.1211 | 0.115 | 0.1181 |
| 7.5 | 0.1781 | 0.1763 | 0.1772 |
| 10 | 0.2385 | 0.2318 | 0.2352 |
| 12.5 | 0.2786 | 0.2809 | 0.2798 |
| 15 | 0.3448 | 0.3359 | 0.3404 |
| 17.5 | 0.3767 | 0.3989 | 0.3878 |
| 20 | 0.4398 | 0.4304 | 0.4351 |



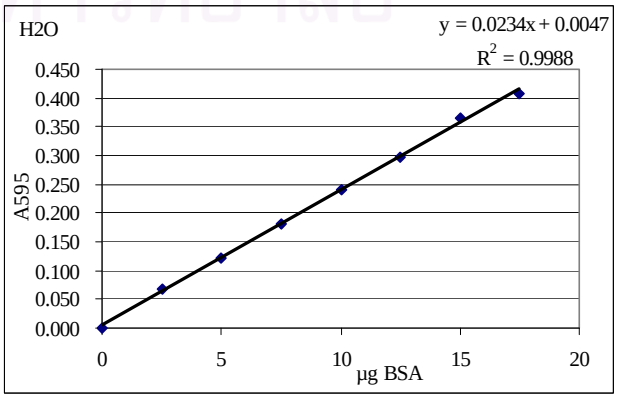

## 2. CAC buffer pH 7.0

| ug BSA | A595 | | Avr A595 |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 2.5 | 0.0454 | 0.0521 | 0.0488 |
| 5 | 0.0942 | 0.0989 | 0.0966 |
| 7.5 | 0.1607 | 0.159 | 0.1599 |
| 10 | 0.2365 | 0.2057 | 0.2211 |
| 12.5 | 0.2601 | 0.255 | 0.2576 |
| 15 | 0.3029 | 0.3028 | 0.3029 |
| 17.5 | 0.352 | 0.353 | 0.3525 |
| 20 | 0.3937 | 0.3784 | 0.3861 |

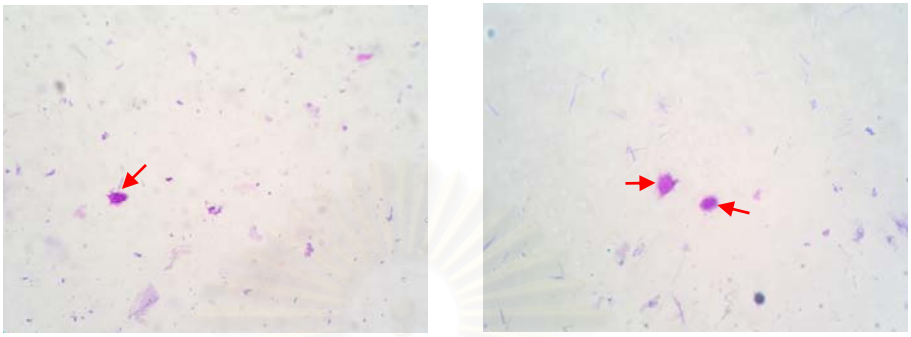



## 3. $H_2O$

| ug BSA | A595 | | Avr A595 |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 2.5 | 0.0717 | 0.0641 | 0.0679 |
| 5 | 0.1194 | 0.1223 | 0.1209 |
| 7.5 | 0.1834 | 0.1774 | 0.1804 |
| 10 | 0.2448 | 0.2351 | 0.2400 |
| 12.5 | 0.2918 | 0.3016 | 0.2967 |
| 15 | 0.3718 | 0.3569 | 0.3644 |
| 17.5 | 0.4128 | 0.4017 | 0.4073 |
| 20 | 0.4215 | 0.438 | 0.4298 |



## Cell adhesion experiment



**(A)** **(B)**

The attached cells on the protein coated cover slip from cell adhesion assay
A: Coverslip coated with BSA
B: Coverslip coated with recombinant C-terminal SP-like domain

## Bacterial binding assay

The colony forming units/mL (CFU/mL) in the original sample of E. coli 363, V. harveyi 1526 and S. aureus was calculated as follows:

| CFU/mL original sample = average colonies x volume plated x dilution factor |
|---|

A single colony of *E. coli* 363 and *S. aureus* 26 were inoculated in LB medium or TSB medum by shaking at 37 °C or 30 °C according to the strains whereas *V. harveyi* 1526 was grown in tryptic soy broth (TSB) medium at 30 °C overnight. The starters were cultured in new medium by shaking at 37 °C or 30 °C according to the strains until an optical density at 600 nm ($OD_{600}$) reached 0.5-0.6. The cultured bacteria in mid- logarithmic phase growth were then serially diluted from $10^{-4}$, $10^{-5}$, $10^{-6}$ to $10^{-7}$ with a sterile normal saline solution (0.85% NaCl, w/v) and 100 µl of each diluted cultured cells were plated on LB agar incubating at 37 °C or 30 °C overnight for colony counting (colony forming units; CFU).

**Calculation CFU of *E. coli* 363**

The *E. coli* 363 was cultured until $OD_{600}$ reached 0.510, and then 100 µl volume of $10^{-5}$ diluted *E. coli* 363 was spread on LB agar plate. We found that 120 and 154 colonies of $10^{-5}$ diluted *E. coli* 363 suspension were grown on the LB plate no. 1 and 2, respectively. The average number of colonies was 137 colonies.

CFU/ml = 137 colonies x 0.1ml (volume plated) x $10^5$ (dilution factor)

= $1.4 \times 10^8$ CFU/ ml

**Calculation CFU of *V. harveyi* 1526**

The *V. harveyi* 1526 was cultured until $OD_{600}$ reached 0.528, and then 100 µl volume of $10^{-5}$ diluted *V. harveyi* 1526 was spread on TSA agar plate. We found that 168 and 153 colonies of $10^{-5}$ diluted *V. harveyi* suspension were grown on the TSA plate no. 1 and 2, respectively. The average number of colonies was 160 colonies.

CFU/ml = 160 colonies x 0.1ml (volume plated) x $10^5$ (dilution factor)

= $1.6 \times 10^8$ CFU/ ml

**Calculation CFU of *S. aureus***

The *S. aureus* was cultured until $OD_{600}$ reached 0.635, and then 100 µl volumes of $10^{-7}$ diluted *S. aureus* was spread on LB agar plate. We found that 196 and 213 colonies of $10^{-7}$ diluted *S. aureus* suspension were grown on the LB plate no. 1 and 2, respectively. The average number of colonies was 204 colonies.

CFU/ml = 204 colonies x 0.1ml (volume plated) x $10^7$ (dilution factor)

= $2.0 \times 10^{10}$ CFU/ ml

# APPENDIX D

# Presentations

1. The 10th International Congress of International Society of Development and Comparative Immunology (ISDCI) congress. July 1-6, 2006. Charleston, South Carolina, USA. “Molecular cloning and characterization of a prophenoloxidase activating factor from black tiger shrimp, *Penaeus monodon*”. (Poster presentation)

2. The 32nd Congress on Science and Technology of Thailand for Sufficiency Economy to celebrate the 60th Anniversary of His Majestry the King's Accession to the Throne. October 10-12, 2006. Queen Sirikit National Convention Center, Bangkok, Thailand. “Recombinant expression and characterization of serine proteinase homologue (SPH) from the black tiger shrimp, *Penaeus monodon*”. (Poster presentation)

3. The 11th Biological Sciences Graduate Congress for Explorations Towards the Improved Quality of Life, Sustainable Development and Secured Future. December 15-17, 2006. Chulalongkorn University, Bangkok, Thailand. “Recombinant expression and characterization of serine proteinase homologue (SPH) from the black tiger shrimp, *Penaeus monodon*”. (Oral presentation)

4. The 33rd Congress on Science and Technology of Thailand Science and Technology for Global Sustainability. October 18-20, 2007. Walailak University, Nakhon Sri Thammarat, Thailand. “Overexpression and functional analysis of serine proteinase homologue from black tiger shrimp, *Penaeus monodon*”. (Oral presentation)

5. The 12th Biological Sciences Graduate Congress for Science Empowering life. December 17-19, 2007. University of Malaya, Kuala Lumpur, Malaysia. “Functional characterization of serine proteinase homologue from black tiger shrimp, *Penaeus monodon*”. (Oral presentation)

6. The RGJ-Ph.D. Congress IX Congress April 4-6, 2008. Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand. “Involvement of a Masquerade - Like Serine Proteinase Homologue from Black Tiger Shrimp in Innate Immunity”. (Oral presentation)

# Publications

1. Amparyup P, Jitvaropas R, Pulsook N, Tassanakajon A. Molecular cloning, characterization and expression of a masquerade-like serine proteinase homologue from black tiger shrimp *Penaeus monodon*. Fish Shellfish Immunol 2007; 22(5):535-46.

2. Jitvaropas R, Amparyup P, Gross PS, and Tassanakajon A. Functional characterization of a masquerade-like serine proteinase homologue from the black tiger shrimp *Penaeus monodon*. Fish Shellfish Immunol. (Submitted)

# BIOGRAPHY

Miss Rungrat Jitvaropas was born on March 25, 1981 in Bangkok. She graduated with the degree of Bachelor of Science (Hons) in 2002 and Doctor of philosophy (Ph.D) degree in 2007 from the Department of Biochemistry, Chulalongkorn University.

She had received a Certificate of Honor for Excellency of Examination-in Biochemistry from Prof. Dr. Thab Neelanithi Foundation (2003), a gold medal award for the first honor of the Department of Biochemistry, Faculty of Science, Chulalongkorn University (2003) and the Royal Golden Jubilee PhD program grants, Thailand Research Funds (TRF) (2003-2007).

She had published her works in the research journals on the topics of "Molecular cloning, characterization and expression of a masquerade-like serine proteinase homologue from black tiger shrimp *Penaeus monodon* " and "Functional characterization of a masquerade-like serine proteinase homologue from the black tiger shrimp *Penaeus monodon*".