การแสดงออกและลักษณะสมบัติของตัวยับยั้งซีรีนโปรตีเนส *Pm*SERPIN8 จากกุ้งกุลาดำ *Penaeus monodon*

นางสาวสุภาพร สมนึก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

EXPRESSION AND CHARACTERIZATION OF A SERINE PROTEINASE INHIBITOR *Pm*SERPIN8 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2011

Thesis Title	Expression and Characterization of a Serine Proteinase Inhibitor *Pm*SERPIN8 from the Black Tiger Shrimp *Penaeus monodon*
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สุภาพร สมนึก: การแสดงออกและลักษณะสมบัติของตัวยับยั้งซีรีนโปรตีเนส *Pm*SERPIN8 จากกุ้งกุลาดำ *Penaeus monodon* (EXPRESSION AND CHARACTERIZATION OF A SERINE PROTEINASE INHIBITOR *Pm*SERPIN8 FROM THE BLACK TIGER SHRIMP. *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.วิเชียร ริมพณิชยกิจ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.ดร.อัญชลี ทัศนาขจร, 78 หน้า

SERPIN คือตัวยับยั้งซีรีนโปรตีเนสที่พบในสิ่งมีชีวิตชนิดต่างๆ รวมถึงกุ้งกุลาดำ (*Penaeus monodon*) การศึกษาวิจัยในแมลงหวี่ (*Drosophila melanogaster*) และ หนอนใบยาสูบ (*Manduca sexta*) พบว่า SERPIN ยับยั้งการทำงานของโปรตีเนสในเชื้อจุลชีพและยังควบคุมการทำงานอย่างเป็นลำดับขั้นของโปรตีเนสในระบบโพรฟีนอลออกซิเดส (prophenoloxidase system)และระบบการแข็งตัวของเลือด (blood coagulation) อีกด้วย ในการศึกษานี้จะทำการศึกษายีน *Pm*SERPIN8 จากกุ้งกุลาดำ โดยศึกษาโครงสร้างของยีน *Pm*SERPIN8 ในจีโนม พบว่า มี 5 exon และ 4 intron โดยยีน *Pm*SERPIN8 มีการแสดงออกในกุ้งทุกระยะแต่ในระยะ nauplii พบการแสดงออกของยีนมากที่สุด ซึ่งการแสดงออกของยีน *Pm*SERPIN8 ส่วนใหญ่อยู่ที่เม็ดเลือดและพบน้อยลงที่เหงือก ต่อมน้ำเหลือง กระเพาะอาหาร และเอพิโพไดท์ หลังจากกุ้งติดเชื้อแบคทีเรีย *V. harveyi* และไวรัสหัวเหลือง(YHV) พบการแสดงออกของยีนสูงสุดอย่างมีนัยสำคัญที่ 24 และ 48 ชั่วโมงตามลำดับ การใช้เทคนิค immunocytochemistry พบโปรตีน *Pm*SERPIN8 แสดงออกในเซลล์เม็ดเลือดชนิด hyaline, semi-granular และ granular และพบว่ากุ้งที่ติดเชื้อแบคทีเรีย *V. harveyi* มีการแสดงออกของโปรตีน *Pm*SERPIN8 มากที่สุดที่ 48 ชั่วโมง ยีน *PmSERPIN8* มีขนาด 1,254 เบส แปลรหัสให้เป็นโปรตีนตัวสมบูรณ์ (mature protein) ได้ 417 กรดอะมิโน จากนั้นได้โคลนเข้าสู่เวกเตอร์ pVR600 และทำการผลิตโปรตีนรีคอมบิแนนท์ *Pm*SERPIN8 ในเชื้อแบคทีเรีย *Escherichia coli* สายพันธุ์ BL21(DE3) พบว่าโปรตีนรีคอมบิแนนท์ *Pm*SERPIN8 ที่ผลิตได้มีขนาด 43 กิโลดาลตัน (kDa) ซึ่งอยู่ในรูปของ inclusion bodies เมื่อนำโปรตีนรีคอมบิแนนท์ *Pm*SERPIN8 ที่ผ่านการทำให้บริสุทธิ์มาทดสอบแอคติวิตี้ในการยับยั้งเอนไซม์พบว่ามีแอคติวิตี้ในการยับยั้งการทำงานของเอนไซม์ subtilisin และ α-chymotrypsin และพบว่าแอคติวิตี้ในการยับยั้งการทำงานของเอนไซม์ subtilisin ไม่เพิ่มขึ้นเมื่อมี heparin นอกจากนั้นพบว่าโปรตีนรีคอมบิแนนท์ *Pm*SERPIN8 สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย *Bacillus sublilis* แต่ไม่สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย *Vibrio harveyi* สายพันธ์ 639 จากการทดสอบการแข็งตัวของเลือดพบว่า โปรตีนรีคอมบิแนนท์ *Pm*SERPIN8 ไม่สามารถป้องกันการแข็งตัวของเลือดได้ แต่สามารถยับยั้งการทำงานของระบบโพรฟีนอลออกซิเดส(prophenoloxidase system) ซึ่งแสดงให้เห็นว่า *Pm*SERPIN8 ทำหน้าที่ในการควบคุมการทำงานในระบบโพรฟีนอลออกซิเดส(prophenoloxidase system)

ภาควิชา..... ชีวเคมี ..... ลายมือชื่อนิสิต .....

สาขาวิชา..... ชีวเคมี ..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา..... 2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5272598423: MAJOR BIOCHEMISTRY
KEYWORDS: PROPHENOLOXIDASE/ SERPIN/ PROTEINASE INHIBITOR/ MICROBIAL CHALLENGE / YALLOW HEAD VIRUS/ *Penaeus monodon*

SUPHAPHON SOMNUK: EXPRESSION AND CHARACTERIZATION OF A SERINE PROTEINASE INHIBITOR *Pm*SERPIN8 FROM THE BLACK TIGER SHRIMP. *Penaeus monodon*. ADVISOR: ASSOC. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D., CO-ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., 78 pp.

SERPIN is a serine proteinase inhibitor found ubiquitously in various organisms including the black tiger shrimp *Penaeus monodon*. Research studies in *Drosophila melanogaster* and *Manduca sexta* revealed that the SERPINs inhibit microbial proteinases and control the proteinase cascades in prophenol oxidase system and blood coagulation. In this study, the *Pm*SERPIN8 from the black tiger shrimp was investigated. The structure of *PmSERPIN8* genomic gene was found to have 5 exons and 4 introns. The *PmSERPIN8* gene was expressed in shrimp at all developmental stages but highest in nauplii. The *PmSERPIN8* gene was expressed mainly in the hemocyte and lesser in gill, lymphoid organ, stomach and epipodite. The *PmSERPIN8* expression is up-regulated at 24 and 48 h after *V. harveyi* and YHV injection, respectively. Using technique in immunocytochemistry, the *Pm*SERPIN8 protein was found in hyaline, semi-granular and granular cells. The expression of *Pm*SERPIN8 protein in the hemocytes was up regulated at 48 h after *V. harveyi* infection. An open reading frame of *PmSERPIN8* gene of 1,254 bp encoding a mature protein of 417 amino acid residues was cloned into the pVR600 expression vector and expressed the r*PmSERPIN8* in *Escherichia coli* BL21(DE3). The recombinant protein (r*Pm*SERPIN8) whose size approximately 43 kDa was expressed as inclusion bodies. The purified r*Pm*SERPIN8 exhibited inhibitory activity against subtilisin and α-chymotrypsin, and the inhibitory activity against subtilisin is not enhanced by heparin. The r*Pm*SERPIN8 was able to inhibit the growth of *Bacillus sublilis* but not *Vibrio harveyi* 639. The r*Pm*SERPIN8 was unable to prevent blood coagulation but could impede the proPO activating system. It was most likely that the *Pm*SERPIN8 functioned to control the proPO activating system.

Department: ……Biochemistry..... Student's Signature: …….

Field of Study: …Biochemistry….. Advisor's Signature: …….

Academic Year: ……2011……..… Co-advisor's Signature: ……

ACKNOWLEDGEMENTS

This thesis would not have been completed without good suggestions from many people. I would like to express my deepest gratitude to my advisor Associate Professor Dr. Vichien Rimphanichayakit and my co-advisor Professor Dr. Anchalee Tassanakajon for their guidance, supervision, and supports throughout this thesis.

I thank Professor Dr. Aran Incharoensakdi, Associate Professor Dr. Apinunt Udomkit and Dr. Tanakarn Monshupanee for serving as thesis committees, for their valuable comments and also useful suggestions.

My appreciation is also to Dr. Kunlaya Somboonwiwat, Dr. Siriporn Pongsomboon, Dr. Premruethai Supungul, Dr. Piti Amparyup, and Miss Sureerat Tang for instruction and helps. Thanks are also expressed to all members at CEMs laboratory for their helps throughout my study.

Thanks are expressed to all students and researchers in the Department of Biochemistry for their friendships.

Finally, my greatest indebtedness is express to my parents and family members for their guidance, understanding, encouragement and support throughout my study.

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LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DEPC	diethylpyrocarbonate
EtBr	ethidium bromide
M	molar
mM	milimolar
µM	micromolar
h	hour
kb	kilobase
mg	milligram
ml	mililitre
ng	nanogram
nm	nanometer
°C	degree Celcius
O.D.	optical density
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
sec	second
µg	microgram
µl	microlitre

CHAPTER I

INTRODUCTION

1.1. Shrimp farming in Thailand

The black tiger shrimp is an economically important shellfish and it is cultured most intensively in China, Taiwan, Bangladesh, India and Thailand. Shrimp farming in Thailand started in 1970 and really began to expand in the mid 1980s. Before 1984, more than 90% of the shrimp in Thailand are harvested from natural resources that are mainly in the gulf of Thailand. By 1987, shrimp culture mainly the black tiger prawn took off in Thailand spreading quickly along the coast. The east and south coastal areas were major area used in shrimp farming. In early 1990, Thailand emerged as the world's leading farmed shrimp producer and exporter of mainly the black tiger shrimp (*Penaeus monodon*) (Fig. 1.1)

In 1992, the economically devastating pandemic caused by white spot syndrome virus after its first appearance in China (Flegel and Alday-Sanz, 1998) had spread rapidly around Asia. In 1993, another virus, yellow head virus, was first reported in Thailand. The name of virus was called from the clinical signs of infected shrimp which include a yellowish cephalothorax and very pale overall coloration of moribund shrimp. Other viruses followed (Wongteerasupaya et al. 1995b). As the situation deteriorated, diseases caused also by bacteria amounted. The shrimp production was reduced for several years (Fig. 1.1). Attempt had been made to prevent the diseases and improve the culturing methods which helped increase the production for some years later. But with the introduction of white shrimp,

Litopenaeus vannamei, which was resistance to diseases and easy to rear, the culture of tiger shrimp had come to the end.

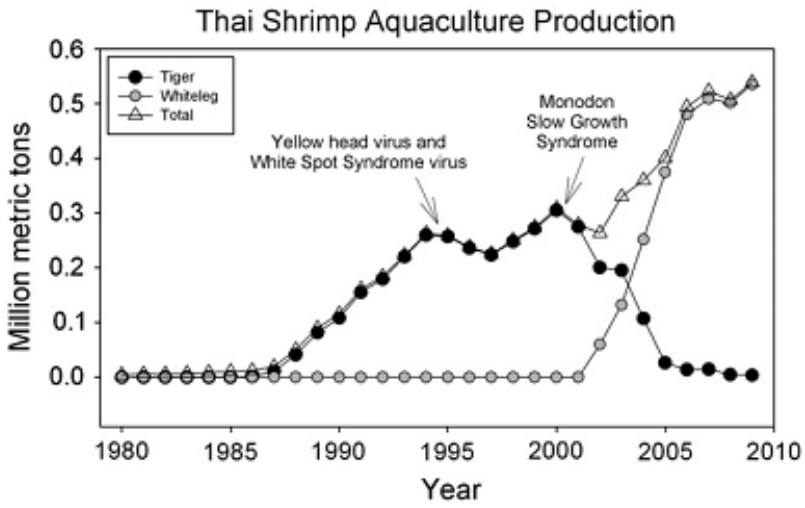


Fig. 1.1. The production of cultured penaeid shrimp in Thailand. Despite periodic disease setbacks, the tiger shrimp production was steadily increased from 1987-2000. The *L. vannamei* has gradually replaced the tiger shrimp from 2002 onward.

The viral and bacterial diseases are primary concerns in the farming of black tiger shrimp. Others are the environmental factors that are related to the system of culture such as water quality, temperature, organic sediments, etc. Shrimp are easily stressed when exposed to the poor conditions such as high organic matter concentration, pH, and soft sediment. Thus, the good quality environment in ponds is the primary objective to prevent stress and reduce the risk of shrimp diseases.

In Thailand, there are five different vial infectious diseases in penaeid shrimp and crustacean species which are currently studied for their impact on commercial farming. The five viruses are white-spot syndrome virus (WSSV), yellow-head virus (YHV) (Sittidilokratna et al., 2006; Walker et al., 2010), hepatopancreatic parvo-like

virus (HPV), infectious hypodermal and hematopoeitic necrosis virus (IHHNV) and monodon baculovirus (MBV). The other non-viral pathogens are, for example, *Vibrio* species and rickettsia (Defoirdt et al., 2004).

1.2. Shrimp diseases

1.2.1. Viral diseases of shrimp

Viral disease causes mainly dead of the black tiger shrimp. White spot syndrome virus (WSSV) is the most serious pathogen in terms of overall production losses because it is lethal for all cultivated species and because mortality can be very high and rapid (Flegel, 2006). The next most severe pathogen is probably yellow head virus (YHV) (Walker et al., 2010). The WSSV and YHV cause white spot syndrome disease (WSS) and yellow head disease (YH) (Boonyatatpalin et al., 1993; Wongteerasupaya et al., 1995b)

1.2.1.1. White spot syndrome virus (WSSV)

White spot syndrome virus is a viral disease affecting most of the commercially culture marine shrimp, not just in Asia but globally because the mortality can be very high and rapid. The white spot syndrome virus has a wide host range which infects all species of penaeid shrimp and invertebrate aquatic organisms such as crab and crayfish (Wang et al., 1998; Chen et al., 2000). Most of the cultured penaeid shrimp are natural hosts for the virus such as *P. monodon*, *Marsupenaeus japonicus*, *P. penicillatus*, *P. semisulcatus*, *P. indicus*, *P. setiferus*, *Litopenaeus vannamei*, *P. stylirostris* and *Metapenaeus ensis*. Other natural hosts are crabs *Scylla serrata*, *Charybdis feriatus*, *Portunus pelagicus*, *P. sanguino-lentus*, *Sesarma* spp., *Uca pugilator*, and *Helice tridens* (Lo et al., 1996a; Supamattaya et al., 1998;

Kanchanaphum et al., 1998; Chen et al., 2000; Maeda et al., 2000; Jiravanichpaisal et al., 2001; Pantoja and Lightner, 2000).

WSSV has been the cause of mass mortality often reaching 100% within 2 to 5 days from the onset of clinical signs. It is a tailed, rod shaped, double stranded DNA virus with a very large circular genome in the order of 300 kb. A new viral family (Nimaviridae) and genus (Whispovirus) are erected to accommodate this virus (Mayo, 2002). Transmission of the virus is mainly through oral ingestion and waterborne routes in the farms. Vertical transmission is found in shrimp hatcheries. The tissue targets of viral infection are gills, lymphoid organ and cuticular epithelium. The WSSV can be detected in early stages of shrimp but significant mortality is observed in post-larvae and juvenile shrimp (Yoganandhan et al., 2003).

Diagnostic methods used to detect WSSV infection are polymerase chain reaction (PCR), in situ DNA hybridization (Wongteerasupaya et al., 1996) or histopathology that can detect WSSV from the organs of shrimp by microscopic examination of rapidly fixed and stained organ fragments.

1.2.1.2. Yellow head virus (YHV)

Yellow head virus (YHV) is one of the important pathogen of shrimp farming operations throughout South East Asia countries. In Thailand, it was first report in 1990 in shrimp farms near Bangkok, such as Samutsakorn and Samutprakarn. It was, subsequently, reported from more southerly farming areas on both sides of the Gulf of Thailand such as Rayong on the east coast and prachuapkhirikhan on the west coast (Limsuwan, 1991).

YHV is an enveloped rod-shaped ssRNA virus (Chantanachookin et al., 1993; Flegel, 1997; Wongteerasupaya et al., 1995b) and is classified as the type

species of genus *Okavirus,* family *Roniviridae,* in the order *Nidovirales* (Walker et al., 2004). When a shrimp is infected with YHV, it has typical signs of yellow head disease (YHD) including a yellowish discoloration of the cephalothorax and yellow hepatopancreas compared with the brown hepatopancreas of normal shrimp (Chantanachookin et al., 1993). The mortality rate may reach as high as 100% of affected population within 3-5 days from the onset of disease. At present, the best diagnosis of YHV is RT-PCR assays (Wongteerasupaya et al., 1997) rather than in situ hybridization because of the instability of viral RNA. The samples of RT-PCR assay must be processed quickly, since storage at −80 °C does not prevent deterioration of the RNA genome

1.2.2. Bacterial diseases

Bacteria are the major causative agents for some of the most serious diseases in fish, shellfish and penaeid. Bacterial diseases are mainly due to *Vibrio,* species which are part of the natural microflora of wild and cultured shrimp and become opportunistic pathogens when natural defense mechanisms are suppressed. There have been reports in penaeid shrimp culture systems implicating at least 14 species of pathogenic bacteria such as *V. harveyi, V. vulnificus and V. parahaemolyticus* causing massive epidemics in Thailand (Nash et al., 1992) and Philippines (Lavilla-Pitogo et al., 1990). Luminescent *V. harveyi* appears to release exotoxins and may cause 80-100% mortality in *P. monodon* hatcheries (Harris and Owens, 1999).

V. harveyi is a Gram-negative bacterium. It has a rod shape and is able to emit a blue-green color light. The affected shrimp have milky white body with weakness and disoriented swimming. The organs affected are haemolymph and

hepatopancreas. Diagnosis is made on basis of clinical signs and bacterial culture of hepatopancreas suspension or blood on culture plate containing 2% NaCl and grow overnight at 30 °C. The colonies show strong luminescence and yellow color on a selective TCBS (thiosulfate-citrate-bile salts-sucrose) plate.

Vibiosis and bacterial diseases can be treated with antibiotics such as chloramphenicol, erythromycin, furazolidone, neomycin, prefuran, streptomycin, and sulphadiazine. However, frequent and improper uses of antibiotics may cause antibiotic resistance strain of bacteria which may also affect human in a long run. Alternatively, attempts have been made to use the probiotic bacteria, such as *Lactobacillus* sp., *Bacillus subtilis*, in the shrimp culture instead of antibiotics. Such probiotic bacteria may provide resistance to infection by other pathogenic bacteria. Currently, the effectiveness of probiotic bacteria is under vigor investigation.

1.3. Innate immune system in crustacean

The major defense system in crustacean is innate immune response which is based on humoral immune response, such as antimicrobial peptides (AMP), prophenoloxidase cascade, lectin, and cellular immune response, such as phagocytosis, nodule formation and encapsulation. Like those of invertebrates, the innate immune system of crustacean is activated by pattern-recognition proteins that recognize glycans, glycolipids, glycoproteins, peptidoglycans, or lipopolysaccharides on the surfaces of Gram-negative and Gram-positive bacteria, viruses, and fungi.

The hemocytes are important effectors destroying the foreign matter that invaded into the crustacean body cavity. Phagocytosis is the action of hemocyte to remove the foreign particles that are too large to be internalized by encapsulation. In

crustacean, there are three morphologically different hemocytes, namely hyaline, semi-granular and granular cells. The classification of hemocytes is also based on different functions in immunity (Table 1.1).

Table 1.1. Three hemocyte types and their known biological functions

Hemocyte type	**Function in immunity**
Hyaline cell	Phagocytosis
Semi-granular cell	Encapsulation, phagocytosis, storage and release of the proPO system and cytotoxicity
Granular cell	Storage and release of the proPO system and cytotoxicity

Each cell type performs its different function in defense reactions. The cell-cell communication is necessary for the defense reactions. When the microorganism infected host cells, the immune response was activated by the microbial cell wall components. Then, the semi-granular and granular cells release the proPO activation system, the cell adhesion factors and peroxinectin by degranulation from the storage granules. The peroxinectin stimulates the phagocytosis by hyaline cells. The affected hemocytes also synthesize and release antimicrobial effectors to participate in the defense mechanism.

1.4. The prophenoloxidase activating system (proPO-AS)

In response to infection, melanization reaction is an immediate response that produces chemical intermediates and melanin that are deleterious to the foreign entities. It is said that the foreign entities are melanized. An enzyme phenoloxidase (PO) is responsible for melanization reaction. It exists as proprotein and is required to be activated to form active PO by the prophenoloxidase activating system (proPO-AS) (Söderhäll and Cerenius 1998).

The proPO is a polypeptide of approximately 80 kDa and contains 0.14–0.22% copper, indicating that there are two atoms of copper per protein molecule (Ashida and Brey, 1997; Aspán et al., 1995). Amino acid sequences for proPOs were first reported in 1995 from four arthropod species, the crayfish *Pacifastacus leniusculus*, and three insect species, *Manduca sexta, B. mori*, and *Drosophila melanogaster* (Aspán et al., 1995; Fujimoto et al., 1995; Hall et al.,1995; Kawabata et al., 1995). Since then, the gene sequences for proPOs have been determined for at least 26 insects and 7 crustacean species. In *P. monodon*, the proPO gene has an open reading frame of 2,121 bp encoding a protein of 688 amino acids. It has a calculated molecular mass of 78.7 kDa (Sritunyalucksana et al., 1999). The sequence analysis reveals that the amino acid sequence of *P. monodon* proPO is 74% similar to that of crayfish.

The proPO is synthesized as zymogen in the hemocytes and is activated by proteolytic cleavage at a specific site in response to infection to active PO. The proPO activation requires a fast and efficient cascade of proteinase activation in the proPO activating system. The system is activated by the pattern-recognition proteins that bind to β-1,3–glucan, lipopolysacchride (LPS) and peptidoglycan from cell wall components of bacteria and fungi (Fig. 1.2). The active PO catalyzes the oxidation of phenols into quinones, which then polymerize into melanin (Söderhäll and Cerenius 2004). Since the proPO activation system uses proteinases to activate the system, it is, thus, controlled by serine proteinase inhibitors.

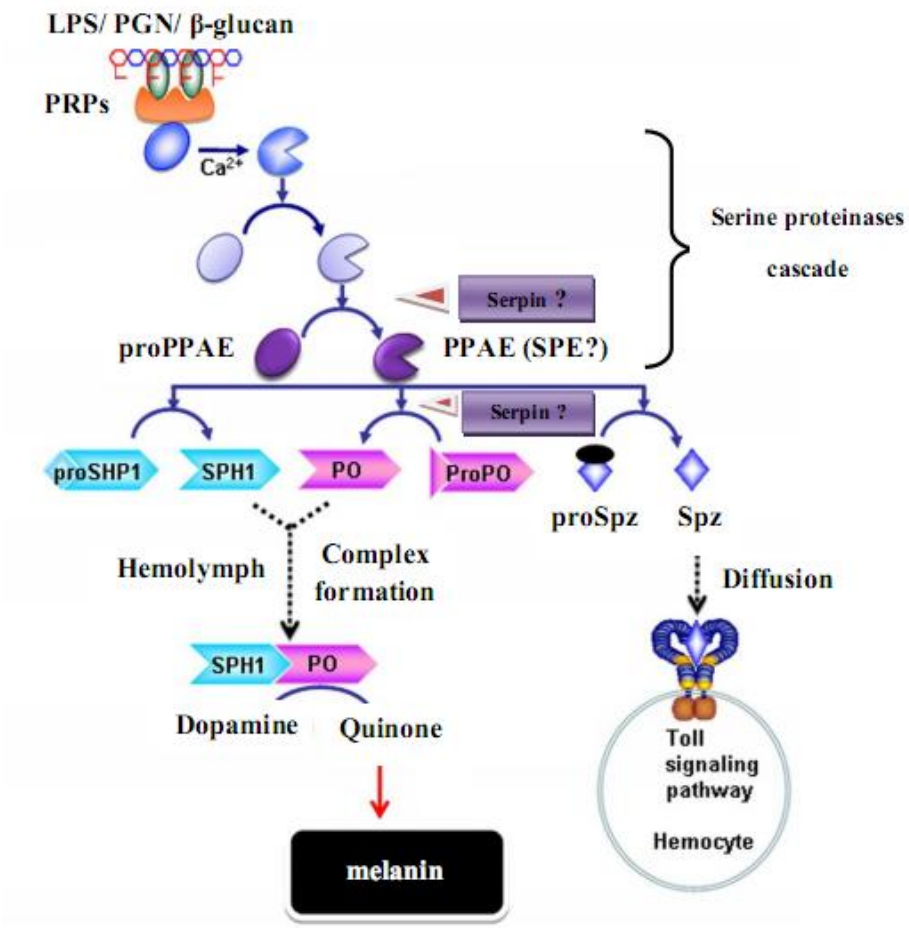


Fig. 1.2. An overview of the insect prophenoloxidase (proPO)-activating system.

1.5. Coagulation system

Hemolymph coagulation is a defense response of crustacean to prevent both loss of hemolymph through break in the exoskeleton and dissemination of bacteria throughout the body. The microbial cell wall activates the coagulation system and proPO-activating system through proteolytic cascades. The systems have been reported in horseshoe crab and freshwater crayfish but are rarely defined in penaeid shrimp. The blood clotting system is the polymerization process of clotting protein (CP) cross-linked by transglutaminase (TGase) (Kopacek et al., 1993).

Blood clot in insects and crustaceans is formed by polymerization of the clotting protein. The CP was first cloned and characterized from *P. leniusculus* (Hall et al., 1999) followed by CP from *P. monodon*. CP crayfish, a dimeric protein consisting of 210 kDa subunits, is a very-high-density lipoprotein (VHDL) (Hall et al., 1995) and each of the 210-kDa subunits has both free lysine and glutamine, which are recognized and become covalently linked to each other by calcium ion dependent transglutaminase (TGase) released from hemocyte (Fig. 1.3).

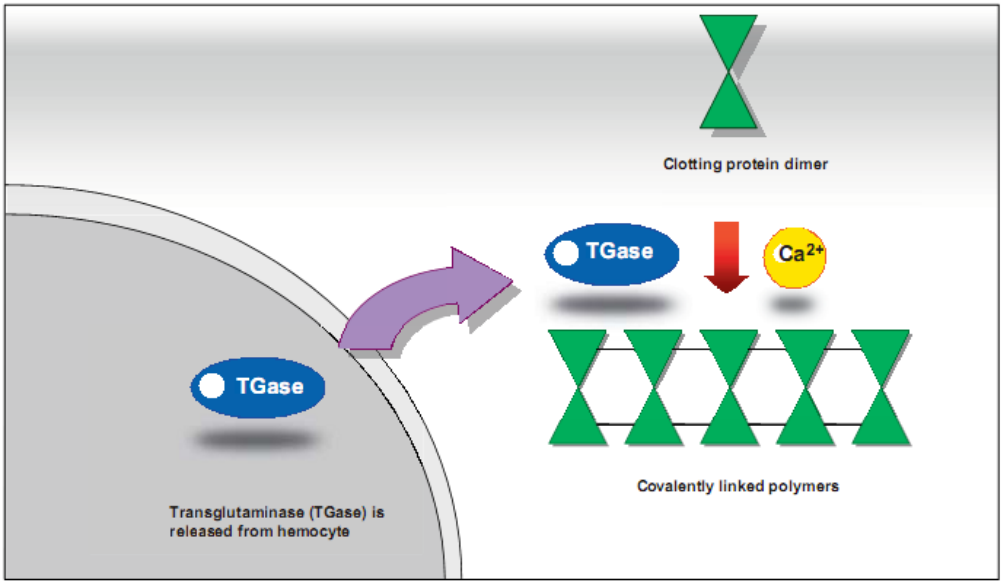


Fig. 1.3. An overview of the blood coagulation system. The clotting occurs through polymerization of CP in the plasma by calcium ion dependent transglutaminase (TGase) released from the hemocytes

1.6. Serine proteinase inhibitors

Serine proteinase inhibitors (SPIs) are widely found in many organisms. They are involved in various biological processes including prophenoloxidase activation,

blood coagulation, complement system, metamorphosis and development, among others (Kanost, 1999). Recent report has categorized SPIs into at least 59 families such as Kazal, Kunitz, α-macroglobulin and pacifastin. Serine protease inhibitors from several gene families have been identified in hemolymph as regulators of proPO activation (Kanost, 1999) such as pacifastin from crayfish that can blocks proPO activation by inhibiting the proPO activating protease (Hergenhahn et al., 1987; Liang et al., 1997)

Serpins belong to a SPI family that had been identified in organisms including viruses, plants, insects, animals and prokaryotes (Irving et al., 2000). All serpins have the same tertiary structure, a single core domain consisting of three β-sheets (A-C) and eight to nine α-helices (Gettins, 2002). Serpins are 40-50 kDa single chain protein with approximately 400 amino acid residues long and a reactive center loop (RCL) composed of amino acid residues P_{16} to P_{10}'. The RCL of active serpin protrudes out from the body of fold, providing a bait peptide bond (P_1-P_1') that mimics a substrate of protease (Fig. 1.4 a). The serpins target specific proteinase though recognition of the P_1 amino acid in the RCL. (Huntington et al., 2000). The inhibition was initiated by the interaction of RCL with the proteinase. Once the bait is cleaved, the proteolysis is arrested at the acyl enzyme stage, resulting in a covalent serpin-proteinase complex. The newly cleaved and unconstrained RCL, then, inserts itself into the β-sheet A of the serpin (Fig. 1.4 b) (Huber and Carrell, 1989) causing drastic conformational change that traps the proteinase with the inhibitor (Fig. 1.4 c). The serpin is, therefore, known as the suicide-type inhibitor.

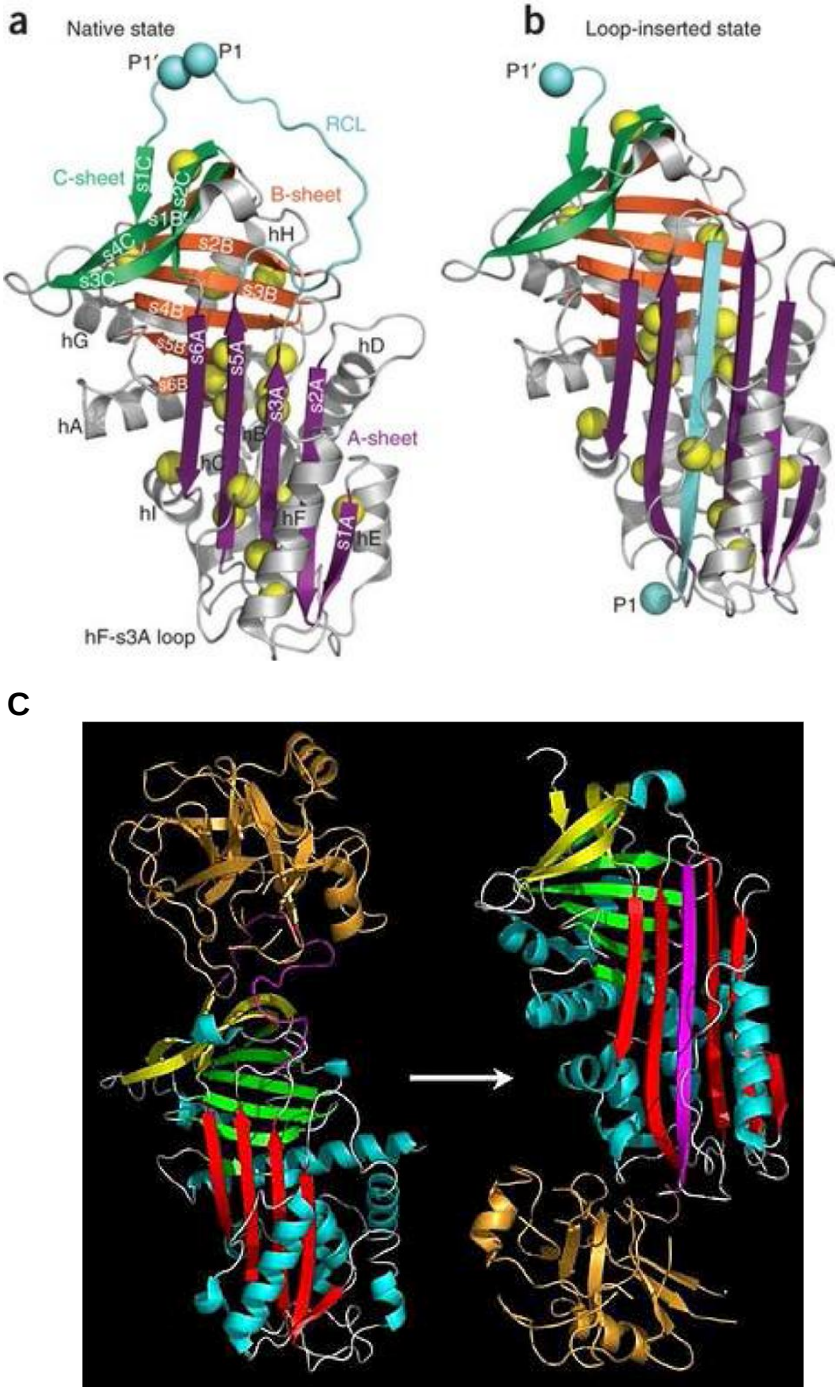


Fig. 1.4. Structures of serpin. (a and b) A human serpin (α_1-proteinase inhibitor) in native state and loop-inserted state, respectively. The reactive center loop (RCL), β-sheets A (purple), B (orange), C (green) and the eight α-helices (A through H) are indicated. (c) The serpin-protease complex showing structural change after the RCL is cleaved causing the covalent linkage between RCL and proteinase and the insertion of RCL into the β-sheet A.

The first serpin ever studied is the human α_1-proteinase inhibitor. It is involved in inflammation and blood clotting. Other mammalian serpins has been shown to have similar structure but have difference functions. In insects, serpins from *Manduca sexta* and *Bombyx mori* have been reported to regulate the proPO system. Three serpins from *M. sexta* hemolymph (serpin-1J, serpin-3, and serpin-6) which disrupt proPO activation when added as recombinant proteins to the plasma, directly inhibit proPO activating proteases (Jiang et al., 2003; Wang and Jiang, 2004; Zhu et al., 2003). In *Drosophila melanogaster*, serpin-27A regulates melanization, and it can inhibit the proPO activating protease from *Holotrichia diomphalia* (De Gregorio et al., 2002; Ligoxygakis et al., 2002; Nappi et al., 2005). In crustaceans, serpin has been found in *Pacifastacus leniusculus* (crayfish) (Liang and Soderhall, 1995), *Tachypleus tridentatus* (Japanese horseshoe crab) (Miura et al., 1994), *Scylla paramamosain* (Chen et al., 2010) and *Eriocheir sinensis*. In shrimp, serpin has been found in *Fenneropenaeus chinensis* (Liu et al., 2009) and *Marsupenaeus japonicus*. The expression of these serpin genes are changed upon bacterial infections and the serpins are believed to be involved in immune response.

In *P. monodon*, there are nine different serpins, eight serpins *Pm*SERPIN1-8 are identified from the *P. monodon* database and one serpin *Pm*SERPINB3 from the differential display technique (Somboonwiwat et al., 2006; Homvises et al., 2010). To date, there are 5 serpins, *Pm*SERPIN3, 6, 7, 8 and B3 with complete open reading frames. Amino acid sequence comparison among the related serpins reveals that the *Pm*SERPIN8 has 61%, 56%, 56% and 34% sequence identity to *Pm*SERPIN6, 7, *Fc*-serpin and *M. sexta* serpin-6, respectively. It is interesting that the serpin-6 of *M. sexta* has been found to regulate the prophenoloxidase system (Zou and Jiang, 2005).

1.7. Objectives of the thesis

To study the function of *Pm*SERPIN8 in the proPO activating system by constructing an expression plasmid containing the *PmSERPIN8* gene from an EST clone GL_H_SO1_0891_LF. The protein is expressed in an *E. coli* system. The r*Pm*SERPIN8 was assayed for its inhibitory activity against serine proteinases, the blood clotting system, the proPO activating system and the growth of certain bacteria. Tissue expression of *PmSERPIN8* in normal shrimp is studied. The expression levels of *PmSERPIN8* gene after infection with *V. harveyi* 639, YHV and developmental stages are detected by quantitative RT-PCR and RT-PCR. Protein expression of r*Pm*SERPIN8 in *V. harveyi* 639 infected shrimp is studied by immunocytochemistry using rabbit anti-*Pm*SERPIN8 antiserum. Gemomic structure of *PmSERPIN8* gene is elucidated.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Equipments

Amicon Ultra-4 Concentrator (Millipore)

Autoclave model # MLS-3750 (SANYO E&E Europe (UK Branch) UK Co.)

Automatic micropipette P10, P20, P100, P200, P1000 (Gilson Medical Electrical)

Biological safety cabinets (Nuaire)

Centrifuge 5804R (Eppendorf)

Centrifuge Avanti™ J-301 (Beckman Coulter)

96-well cell culture cluster, flat bottom with lid (Costar)

-20 °C freezer (Whirlpool)

-80 °C freezer (Thermo Electron Corporation)

Gel document (Syngene)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Microcentrifuge tube 0.6 ml and 1.5 ml (Bio-Rad)

Microplate reader: (SpectraMAX®M5)

PD-10 column (GE Healthcare)

pH meter model #SA720 (Orion)

Pipette tip 10, 20, 200 and 1000 µl (Axygen)

Refrigerated incubator shaker (New Brunswick Scientific)

Trans-Blot® SD (Bio-Rad)

Thermal cycler mastercycler gradient (Eppendorf)

Touch mixer model # 232 (Fisher Scientific)

2.1.2. Chemicals and reagents

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

100 bp plus DNA ladder (Fermentas)

1 kb DNA ladder (Fermentas)

2-Mercaptoethanol, C_2H_6OS (Fluka)

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Fermentas)

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

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH. (BDH)

Agarose (Sekem)

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

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

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bovine serum albumin (Bio-Rad)

Bromophenol blue (Merck)

Calcium chloride, ($CaCl_2$) (Merck)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

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

Ethidium bromide (Sigma)

Ethylenediaminetetraacetic acid (EDTA), disodium salt dehydrate (Fluka)

Formaldehyde (BDH)

Glycerol, $C_3H_8O_3$ (BDH)

Hydrochloric acid, HCl (Merck)

Isopropanol (Merck)

Isopropyl-β-D-thiogalactoside (IPTG) (Sigma)

Nitrocellulose membrane (Bio-Rad)

Potassium chloride, KCl (Ajex)

Potassium dihydrogen phosphate, KH_2PO_4 (Ajex)

Prestained protein molecular weight marker (Fermentas)

RNase A (Sigma)

Sodium acetate, CH_3COONa (Merck)

Sodium bicarbonate, Na_2CO_3 (Ajex)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma)

Sodium dihydrogen orthophosphate, $NaH_2PO_4 \cdot H_2O$ (Carlo Erbo)

Disodium dihydrogen orthophosphate, $Na_2HPO_4 \cdot H_2O$ (Carlo Erbo)

Sodium hydroxide, NaOH (Eka Nobel)

TRI Reagent (Molecular Research Center)

TEMED $(CH_3)_2NCH_2CH_2N(CH_3)_2$ (Amresco)

2.1.3. Kits

Geneaid High-speed Plasmid Mini Kit

NucleoSpin® Extract II Kit (Macherey–Nagel)

RevertAid™ First strand cDNA synthesis Kit (Fermentas)

2.1.4. Enzymes

*Bam*HI, *Eco*RI, *Eco*RV, *Msc*I, *Nco*I, *Pst*I and *Xho*I (Biolabs)

α-chymotrypsin, bovine pancreas (Sigma)

Elastase, porcine pancreas (Pacific Science)

Phusion ® Hot Start High-Fidelity DNA polymerase (Finnzymes)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

Subtilisin Carlsberg, *Bacillus licheniformis* (Sigma)

Taq DNA polymerase (Fermentas)

T4 DNA ligase (Fermentas)

T7 DNA polymerase (Sigma)

Trypsin, bovine pancreas (Sigma)

2.1.5. Substrates

N-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Sigma)

N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma)

N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma)

2.1.6. Antibiotics

Ampicilin

Chloramphenicol

Kanamycin

Tetracycline

2.1.7. Bacterial and virus strains

Bacillus subtilis

Escherichia coli XL-1 Blue

Escherichia coli BL21(DE3)

Vibio harveyi 639

Yellow head virus (YHV)

2.1.8. Vectors

pET-28b(+)

pVR600, a pET-28b(+) derivative

2.1.9. Softwares

ClustalX (Thompson et al., 1997)

GENETYX (Software Development)

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

2.2. Samples

Black tiger shrimp *Penaeus monodon* 10 to 15 g body weight were purchased from Suratthani Province.

2.3. Primer design

The Primers were designed from the nucleotide sequences of DNA templates. The T_m values were calculated using GENETYX program (Software Development).

2.4. General techniques for genetic engineering

2.4.1. DNA determination

The DNA was diluted with water and the concentration was determined by measuring the A_{260} using a spectrophotometer (SpectraMAX M5). The concentration of DNA was calculated from the A_{260} value in µg/ml using an equation: [DNA] (µg/ml) = A_{260} × dilution factor × 50 for one A_{260} corresponds to 50 µg/ml of DNA (Sambrook et al., 1989)

2.4.2. Determination of RNA sample

The RNA was diluted in DEPC-treated water, detected by spectrophotometer at 260 nm and the total RNA concentration was calculated in µg/ml using the following equation: [RNA] = A_{260} × dilution factor × 40. The purity of RNA could be assessed by determining the ratio of A_{260}/A_{280}. The purified RNA should not have an A_{260}/A_{280} ratio below 1.6 (Sambrook et al., 1989).

2.4.3. Plasmid preparation

The plasmid was prepared from overnight bacterial cells. The bacteria were cultured in 1.5 ml of LB medium (1% (w/v) peptone, 1% (w/v) NaCl and 0.5% (w/v) yeast extract) containing 100 µg/ml ampicillin for T&A vector (Bio-Rad) or 30 µg/ml kanamycin for pVR600 at 37 °C overnight. The bacterial cells were collected by centrifugation at 10,000 rpm for 5 min. The plasmid was extracted from the cells using a High-speed Plasmid Mini Kit (Geneaid). The cells were suspended in 200 µl

PD1 containing RNase A, added 200 µl PD2 and mixed gently. Then, the mixture was added 400 µl PD3, mixed and centrifuged at 12,000 rpm for 10 min. The supernatant containing the plasmid was transferred into a PD column, centrifuged at 12,000 rpm for 1 min, washed twice with 400 µl W1 buffer and 600 µl wash buffer and centrifuged to remove the remaining wash buffer. The PD column was removed to a 1.5-ml microcentrifuge tube. The plasmid was eluted by adding 50 µl of elution buffer into the PD column, incubated 1-2 min at room temperature and centrifuged to collect the plasmid solution.

2.4.4. Preparation of competent cells

The competent cells were prepared from *Escherichia coli* strain XL1-Blue, BL21(DE3) using a calcium chloride method. A single colony was cultured in 1 ml LB medium containing 12.5 µg/ml tetracycline for *E. coli* XL1-Blue or 30 µg/ml kanamycin for *E.coli* BL21(DE3) as a starter, grown with shaking at 37 °C overnight. The starter was diluted 1:100 in 100 ml LB medium, grown at 37 °C with 250 rpm shaking until the OD_{600} was about 0.2 – 0.4 after which the culture was chilled on ice for 10 min. The cells were collected by centrifugation at 5,000 rpm for 5 min at 4 °C. The cells were resuspended with 0.5 volume of 0.1 M calcium chloride ($CaCl_2$) and incubated on ice for 30 min. The cells were collected again by centrifugation at 8,000 rpm for 5 min at 4 °C, suspended with 0.1 volume of 0.1 M $CaCl_2$ and incubated on ice for 30 min. The cells were said to be competent and ready for transformation. For storage, the glycerol was added to a final concentration of 15% glycerol and frozen at -80 °C.

2.4.5. Transformation

About 10-15 µl of ligation mixture were mixed with 100 µl of competent cells, incubated on ice for 30 min, heat-shocked for 1 min at 42 °C, added with 1 ml of LB medium, incubated at 37 °C for 1 h. The cell pellet was separated by centrifugation, resuspended and spread on LB agar plate containing antibiotic, incubated 37 °C overnight.

2.4.6. Agarose gel electrophoresis

The PCR products, plasmid DNA and DNA fragments were analyzed using 0.8-1% agarose gel. The agarose gel was melted in 1× TBE buffer (89 mM Tris-HCl, 2.5 mM EDTA pH 8, 8.9 mM boric acid). After cooling down, the gel solution was poured onto a casting tray equipped with a well-former. The 6-10× loading dye was added into the samples, mixed and loaded into the sample wells. The electrophoresis was run in 1× TBE buffer at 100 volts for 40 min. The gel was stained in ethidium bromide (EtBr) for 1 min and washed with water for 15 min. The DNA bands were visualized under UV transilluminator. The size of DNA band was estimated by comparing with those of standard DNA markers.

2.4.7. Extraction DNA from agarose gel

The DNA fragment was extracted from agarose gel using NucleoSpin® Extract II Kit (Macherey–Nagel). The gel slice containing the DNA fragment of interest was cut from the agarose gel. It was added 200 µl NT buffer per 100 g gel slice and melted at 50 °C for 10 min. The melted sample was added into a NucleoSpin® column. The column was centrifuged at 11,000 rpm for 1 min, washed with 600 µl of NT3 buffer and centrifuged at 11,000 for 1 min. The filtrate was poured off and the column was centrifuged at 11,000 rpm for 2 min to dry the

membrane. Elution buffer usually 50 µl was added into the column, incubated at room temperature for 1 min and centrifuged at 11,000 rpm for 2 min to collect the DNA filtrate.

2.5. Genomic structure of *PmSERPIN8* gene

2.5.1. Preparation of genomic DNA

The genomic DNA was isolated from pleopods of black tiger shrimp by homogenizing the tissue in 400 µl of extraction buffer (100 mM Tris-HCl pH 9, 100 mM NaCl, 200 mM sucrose, 50 mM Na_2EDTA pH 8). Then, the sample was added 40 µl of 10% SDS, incubated at 65 °C for 1 h, added 10 µl of 50 µg/ml proteinase K, incubated at 65 °C for 3 h, added 90 µl of 5 M potassium acetate and chilled on ice for 30 min. The supernatant was separated by centrifugation at 12,000 rpm for 10 min, added 2 volumes of absolute ethanol and frozen at -20 °C overnight. The genomic DNA fiber was removed, dissolved in 200 µl of water at 4 °C, extracted with 200 µl phenol:chloroform:isoamyl alcohol. Liquid phase was separated by centrifugation at 7,000 rpm for 15 min at 4 °C. The liquid phase was added 2 volumes of absolute ethanol and frozen at -20 °C. The genomic DNA was pelleted, washed with 1 ml 70% ethanol and dissolved with 200 µl of water.

2.5.2. PCR amplification of genomic *PmSERPIN8* gene

The genomic *PmSERPIN8* gene was PCR amplified fom 50 µg of genomic DNA. The reaction mixture consisted of 5 µl of genomic DNA template, 5 µl of 10× buffer, 1 µl of 10 mM dNTP, 2 µl of 5 µM forward primer *Pm*SERPIN8-F1, 2 µl of 5 µM reverse primer *Pm*SERPIN8-R2 (Table1) and 34 µl of water. The PCR reaction was started by adding 1 unit of Advantage *Taq* DNA polymerase (Clontech) and

conducted with an initial 5 cycles of denaturation at 94 °C for 25 sec, 62 °C for 45 sec and 72 °C for 3 min followed by 30 cycles of 95 °C for 25 sec, 58 °C for 45 sec and 67 °C for 3 min. The PCR products were analyzed by 1% agarose gel electrophoresis. The PCR product was eluted from the gel slice using NucleoSpin® Extract II Kit (Macherey–Nagel)

Table 2.1.
Nucleotide sequences and uses of primers

Primer	Sequence (5´ - 3´)	Use	Product size (bp)
*Pm*SERPIN8-F1	CAGTGTTCTATTTGCCAATGAA GTG	Forward primer for genome structure experiment	
*Pm*SERPIN8-F2	CGGATCCCCAGTGTCTTCCG	Forward primer with 5´ *Bam*HI site for RT-PCR and recombinant expression experiments	1,197
*Pm*SERPIN8-R2	CGAGCTCCTGAGCCCTTCC	Reverse primer with 5´ *Xho*I site for RT-PCR recombinant expression and genome structure experiments	
*Pm*SERPIN8-F3	GTACCAACAGCGCCAGGC	For qRT-PCR	161
*Pm*SERPIN8-R3	TGTGGGCGACCAGAGTCA		
EF-1α-F	GGTGCTGGACAAGCTGAAGGC	For amplification of internal control gene EF-1α	150
EF-1α-R	CGTTCCGGTGATCATGTTCTTGA TG		
*Pm*SERPIN8-F4	GTTCACCGTCGCCAATCGAG	Forward primer for sequencing the middle part of genome DNA sequence	
*Pm*SERPIN8-R4	TCAATCGCCAAGCACATCAA	Reverse primer for sequencing the middle part of genome DNA sequence	

2.5.3. Cloning of the PCR products into a T&A vector.

The eluted DNA was cloned into a T&A vector (RBC). The ligation mixture of 10 µl contained 1 µl of ligation buffer A, 1 µl of ligation buffer B, 2 µl of T&A vector, 5µl of elute DNA (100 ng) and 1 µl of T4 DNA ligase. The sample was

incubated at room temperature overnight and transformed into an *E. coli* strain XL1-Blue using calcium chloride method. The transformed cells were spread onto an LB agar plate containing 100 µg/ml ampicillin, 100 mM IPTG and 50 µg/ml X-gal. The recombinant plasmid was extracted by High-speed Plasmid Mini Kit (Geneaid) and might be digested with restriction enzymes to ensure the existence of DNA insert. The correct clone was subjected to DNA sequencing serviced by Macrogen Inc., Korea.

2.6. RT-PCR and quantitative RT-PCR analysis

2.6.1. Sample preparation

2.6.1.1. Samples for tissues distribution

The tissues analyzed in this experiment were hemocyte, epipodite, eyestalk, gill, heart, hepatopancreas, stomach, intestine, lymphoid organ and antennal gland from normal shrimp.

2.6.1.2. Samples for Vibrio harveyi and YHV challenge

This experiment used black tiger shrimp of 15 g body weight purchased from a farm in Suratthani Province. The shrimp were divided into 3 groups of 15 shrimp: first group was injected with 100 µl of *V. harveyi* in normal saline (10^5 CFU/ml), second group was injected with 100 µl YHV in normal saline and the last group was injected with 100 µl normal saline (0.85% NaCl) as a control group. The hemolymph was collected at 0, 6, 24, 48 and 72 h after infection using 200 µl 10% sodium citrate as an anticoagulant. The hemocytes were collected by centrifugation at 800 × g for 10 min.

2.6.1.3. Samples at different developmental stages of the black tiger shrimp

This experiment used 4 developmental stages of black tiger shrimp: 50 µg of nauplii, 50 µg of protozoae, 50 µg of mysis larvae and 15 postlarvae from Suratthani farm.

2.6.2. RNA extraction and first strand cDNA systhesis

2.6.2.1. RNA extraction

The samples were homogenized in 1 ml TRI Reagent®, added 200 µl of chloroform and mixed gently. The supernatant was separated by centrifugation at 12,000 rpm for 15 min at 4 °C. Total RNA was precipitated with equal volume of isopropanol (~500 µl) at room temperature for 10 min. The RNA was pelleted by centrifugation at 12,000 rpm for 15 min at 4 °C and washed with 1 ml 75% ethanol. The RNA pellet was air-dried for 5-10 min and dissolved in 10 µl of diethyl pyrocarbonate (DEPC)-treated water.

2.6.2.2. DNase treatment of total RNA

Total RNA was treated with RQ1 RNase-free DNase (Promega) (1 unit RNase per 5 µg total RNA) at 37 °C for 30 min to remove the contaminated chromosomal DNA, extracted with 250 µl TRI Reagent®, added 200 µl of chloroform, mixed gently and centrifuged at 12,000 rpm for 15 min at 4 °C. The total RNA in liquid phase was precipitated with an equal volume of isopropanol at room temperature. Total RNA was pelleted by centrifugation, washed with 1 ml 75% ethanol, centrifuged at 12,000 rpm for 15 min at 4 °C, air-dried for 5-10 min and dissolved in 10 µl of diethyl pyrocarbonate (DEPC)-treated water.

2.6.2.3. First-strand cDNA synthesis

Total RNA was used as a template for cDNA synthesis using the RevertAid™ First Stand cDNA Synthesis Kit (Fermentas). The 20-µl reaction contained 2.5 µg of total RNA, 1 µl of oligo$(dT)_{18}$, 4 µl of 5× reaction buffer, 1 µl of RiboLock™ RNase inhibitor (20 U/µl), 2 µl of dNTP mix and 1 µl of ReverstAid™ M-MuLV reverse transcriptase. The reaction was gently mixed, incubated at 42 °C for 1 h and terminated at 70 °C for 15 min.

2.6.3. Tissue distribution of *Pm*SERPIN8 by RT-PCR

This experiment was to study the level of expression of *Pm*SERPIN8 in tissues from the black tiger shrimp. The *Pm*SERPIN8 gene was amplified from cDNA, as a template using the specific primer. A pair of specific primers, forward primer *Pm*SERPIN8-F2 and reverse primer *Pm*SERPIN8-R2 (Table 2.1), was used for RT-PCR. The PCR reaction contains 3 µl of cDNA, 0.5 µl 5 µM *Pm*SERPIN8-F2 primer, 0.5 µl 5 µM *Pm*SERPIN8-R2 primer, 1.25 µl of 10× buffer (RBC), 0.25 µl 10 mM dNTP, 0.1 µl *Taq* DNA polymerase (RBC) and water to a total volume of 12.5 µl. The expression of elongation factor 1-α (EF-1α) gene was carried out as an internal control using specific primers, EF-1α-F and EF-1α-R (Table 2.1). The PCR was started with denaturation step at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 min. The PCR product was analyzed by 1% agarose gel electrophoresis.

2.6.4. Expression of *Pm*SERPIN8 in response to *V. harveyi* and YHV challenge

The real-time PCR was used to quantitative the expression of *Pm*SERPIN8 in hemocytes of black tiger shrimp after infection. The total RNA from hemocytes

was extracted and the cDNA was synthesized. The cDNA was used as a template in real-time PCR. The *Pm*SERPIN8 gene was amplified using a pair of primer, *Pm*SERPIN8-F3 and *Pm*SERPIN8-R3 (Table 2.1). The reaction mixture consisted of 10 µl 2× iQ™SYBR® Green Supermix (Bio-Rad), 0.3 µl 5 µM forward primer, 0.3 µl 5µM reverse primer, 3 µl of 5-fold diluted cDNA as template and water to a total volume of 20µl. The PCR profile was 95 °C for 8 min followed by 40 cycles of 95 °C for 10 sec, 58 °C for 20 sec and 72 °C for 10 sec. Each sample was done in triplicate. The data were analyzed by a mathematical model described by Livak and Schmittgen (2001).

2.6.5. Expression of *Pm*SERPIN8 in black tiger shrimp at different stages of development

This experiment studied the expression of *Pm*SERPIN8 in shrimp at different stages of development: nauplii, protozoae, mysis larvae and postlarvae. The cDNA synthesized from the shrimp total RNA was used as a template for real-time PCR. The PCR was carried out using a pair of primers *Pm*SERPIN8-F3 and *Pm*SERPIN8-R3 (Table 2.1). The EF-1α gene was used as an internal control. The 10- µl reaction consisted of 10 µl 2X iQ™SYBR® Green Supermix (Bio-Rad), 0.3 µl 5 µM forward primer, 0.3 µl 5 µM reverse primer, 3 µl of 5-fold diluted cDNA and water to a total volume of 20µl. The PCR profile was 95 °C for 8 min followed by 40 cycles of 95 °C for 10 sec, 58 °C for 20 sec and 72 °C for 10 sec. Each sample was done in triplicate. The data were analyzed by a mathematical model described by Livak and Schmittgen (2001).

2.7. Recombinant expression of *Pm*SERPIN8

The *PmSERPIN8* gene was amplified by PCR using Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes) from a singleton SG5654 (EST clone GL_H_SO1_0891_LF) (GenBank accession GU358488). The primers were designed to contain restriction sites, *Bam*HI and *Xho*I for the cloning of *PmSERPIN8* gene into an expression vector pVR600 (Table 2.1). The PCR amplification was performed in a final volume of 50 µl containing 5 µl of SG 5654 (100 ng), 5 µl 10× buffer, 2 µl *Pm*SERPIN8-F2, 2 µl *Pm*SERPIN8-R2, 1 µl 10 mM dNTP, 0.5 µl *T4* DNA polymerase Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes) and water to 50 µl. The PCR was started with a denaturation step at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 2 min. The PCR product was analyzed by 1% agarose gel electrophoresis and eluted using the NucleoSpin® Extract II Kit (MACHEREY-NAGEL).

The eluted DNA was ligated with T&A vector (RBC) in a total volume of 10 µl. The ligation mixture was incubated at room temperature overnight, transformed into an *E. coli* strain XL-1 Blue by $CaCl_2$ method. The transformants were spread on a LB agar plate containing 100 µg/ml ampicillin, 100 mM IPTG and 50 µg/ml X-gal. The recombinant plasmid DNA was prepared using the High-speed Plasmid Mini Kit (Geneaid). The correct gene sequence was verified by DNA sequencing (Macrogen Inc., Korea). For cloning into pVR600, 3 µl of the recombinant plasmid (pTA-*Pm*SERPIN8) (300 ng) was mixed with 1 µl of pVR600 and digested with *Bam*HI and *Xho*I in a total volume of 20 µl containing 2 µl 10× buffer, 0.5 µl *Bam*HI, 0.5 µl *Xho*I and water to 20 µl at 37 °C overnight. The mixture was extracted with phenol-chloroform and precipitated with ethanol. The ligation reaction was carried out in a

total volume of 10 µl containing 5 µl of digestion mixture, 1 µl T4 DNA ligase buffer, 1 µl T4 DNA ligase and water to 10 µl. The ligation reaction was incubated at room temperature overnight and stopped by heating at 75 °C for 15 min. The ligation mixture was, then, digested with *Hin*dIII for 3 h in 15-µl digestion reaction.

The mixture reaction was finally transformed into an *E. coli* strain XL-1 Blue by $CaCl_2$ method. The recombinant plasmid was extracted from the transformant, digested with *Bam*HI and *Xho*I and analyzed by 1% agarose gel electrophoresis to verify the insert. The correct recombinant plasmid was transformed into an *E. coli* strain BL21(DE3) as an expression host. The BL21(DE3) transformant was used to over-produce the protein *Pm*SERPIN8.

2.8. General technique for protein method

2.8.1. Concentration of protein

The concentration of protein was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard protein. The protein was added into a microcentrifuge tube and a total volume was made up to 100 µl with buffer. Then, 1 ml of Bradford working solution was added, mixed and incubated at room temperature for 10 min. The A_{595} was measured. By comparing to the standard curve, the concentration of protein was calculated.

2.8.2. SDS-PAGE analysis

The protein was analyzed by SDS-PAGE. Appropriate percentage of SDS-polyacrylamide slab gel was prepared. The gel was run using 1× running buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS). The samples were loaded into the wells. Electrophoresis was set at 25 mA for 50 min per gel. After completion,

the gel was stained with staining solution (0.1% Coomassie brilliant blue R250, 10% (w/v) acetic acid and 45% (w/v) methanol) at room temperature for 1 h with shaking. The gel was de-stained in de-staining solution (10% (w/v) acetic acid and 10% (w/v) methanol) to remove excessive stain for 3-5 h.

2.8.3. Western blot detection of protein

The gel, nitrocellulose membrane and filter paper were soaked in a transfer buffer (48 mM Tris-base, 39 mM Glycine, 20% (v/v) methanol) for 30 min. The filter paper was placed on the anode platform. The air bubbles were removed by rolling a glass pipette on the filter paper. The nitrocellulose membrane was, then, placed on the filter paper. The gel was placed onto the nitrocellulose membrane and rolled over by a glass pipette to remove air bubbles. A filter paper was placed on top of the gel and rolled over by a glass pipette. The power was set at 90 mA for 90 min to transfer the protein to the nitrocellulose membrane.

To detect the protein, the nitrocellulose membrane was soaked in a blocking solution (5% (w/v) skim milk in 1× PBS buffer containing 0.05% (v/v) Tween 20) at room temperature for 3 h. The nitrocellulose membrane was washed 3 times with washing solution (1× PBS buffer containing 0.05% (v/v) Tween 20) for 5 min and incubated with anti-His antibody diluted 1:5000 in hybridization buffer (1% (w/v) skim milk in 1× PBS buffer containing 0.05% (v/v) Tween 20) for 1 h at room temperature. The nitrocellulose membrane was again washed 3 times with washing solution and incubated with secondary antibody conjugated with alkaline phosphatase diluted 1:10000 in hybridization buffer for 1 h at room temperature. The nitrocellulose membrane was finally washed 3 times with washing solution. The

protein was detected by color development using an alkaline phosphatase substrate BCIP/NBT.

2.9. Over-expression of recombinant protein *Pm*SERPIN8

The recombinant plasmid was transformed into *E. coli* BL21(DE3) as an expression host. A single colony was cultured in LB medium containing 30 µg/ml of kanamycin at 37 °C overnight with 250 rpm shaking as a starter .The starter was diluted 1:100 with LB medium containing the antibiotic. The culture was grown at 37 °C with 250 rpm shaking until the OD_{600} reached 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 1 mM for induction. Aliquots of culture were collected at 1–4 h after induction and the cells were pelleted by centrifugation at 8,000 rpm for 5 min. The cell samples were analyzed by 12% SDS-PAGE. The recombinant protein was confirmed by western blot analysis using anti-His antibody (GE Healthcare).

2.10. Protein purification

The recombinant *Pm*SERPIN8 protein was produced in an *Escherichia coli* expression system. The cell pellet was suspended in phosphate buffered saline pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), broken by sonication and centrifuged at 8,000 rpm for 10 min. The supernatant was removed and the pellet was washed twice with 0.5 M NaCl contain 2% Triton X-100 in PBS pH 7.4, twice with 0.5 M NaCl and twice with water. The pellet was solubilized with 100 mM NaOH for 3 h. The supernatant was separated by centrifugation at 10,000 rpm for 10 min and dialyzed against 20 mM Tris-HCl pH 8 overnight. The dialyzed protein was purified by nickel affinity chromatography (GE Healthcare).

The column was equilibrated with a binding buffer (20 mM Tris-HCl pH 8, 0.3 M NaCl, 20 mM immidazol). The soluble protein was applied into the column, washed with a washing buffer (20 mM Tris-HCl pH 8, 0.3 M NaCl, 20 mM immidazol) and eluted with an elution buffer (20 mM Tris-HCl pH 8, 0.3 M NaCl, 0.3 M imidozol). The eluate was dialyzed against 20 mM Tris-HCl pH 8. The purified protein was analyzed by 12% SDS-PAGE and the concentration was determined.

2.11. Inhibition assay

The inhibitory activity of r*Pm*SERPIN8 against proteinases; α-chymotrypsin (type II bovine pancreas, Sigma), trypsin (bovine pancreas, Sigma), subtilisin Carlsberg (*Bacillus licheniformis*,Sigma) and elastase (porcine pancreas, Pacific science) was assayed in 50 mM Tris-HCl pH 8 in a total volume of 100 µl using 290 mM and 147 mM *N*-saccinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as a substrate for subtilisin and α-chymotrypsin, 147 mM *N*-benzoyl–Phe-Val-Arg-*p*-nitroanilide for trypsin and 886 mM *N*-saccinyl-Ala-Ala-Ala-*p*-nitroanilide for elastase. The final concentration of trypsin, subtilisin and α-chymotrypsin were 0.04 mM and elastase was 0.08 mM. The proteinase to inhibitor ratio of 1:12.5, 1:25, 1:50, 1:100, 1:200 were used to determine the inhibition. The reaction was incubated at 30 °C for 15 min and stopped by adding 50 µl 50% acetic acid. The absorbance of *p*-nitroaniline formed was measured at 410 nm using a microplate reader (Costar Clear). The remaining activity was calculated and plotted against the molar ratios of inhibitor to proteinase.

In this experiment, we also study the affect of heparin on the inhibitory activity of r*Pm*SERPIN8. The assay reactions was added 25 IU of heparin sodium salt from porcine intestinal mucosa (Sigma) and conducted as described above.

2.12. Growth inhibition assay

2.12.1. Inhibition of *Bacillus subtilis*

A single colony of *Bacillus subtilis* was cultured in LB medium (1% (w/v) peptone, 1% (w/v) NaCl and 0.5 % (w/v) yeast extract) at 37 °C overnight as a starter (Patat et al., 2004). The starter was inoculated in fresh LB medium at 37 °C shake until the OD_{600} reached 0.1. The cells were diluted 1:100 in LB medium containing 4 and 8 µM r*Pm*SERPIN8 in a total volume of 150 µl and shaken at 37 °C. The growth of bacterial cell was monitored at 595 nm using a microplate reader Spectro*MAX* M5 at 0, 2, 4, 6, 8, 12 and 14 h. The buffer, 20 mM Tris-HCl pH 8, was used as a control.

2.12.2. Inhibition of *V. harveyi*

A single colony of *V. harveyi* was cultured in TSB medium at 37 °C for an overnight as a starter (Patat et al., 2004). The starter was inoculated in fresh TSB medium and shaken at 30 °C until the OD_{600} reached 0.1. The cells were diluted 1:100 in TSB medium containing 4 and 8 µM r*Pm*SERPIN8 in a total volume of 150 µl and shaken at 37 °C. The bacterial cell growth was monitored at 595 nm by a microplate reader Spectro*MAX* M5 at 0, 2, 4, 6, 8, 12 and 14 h. The buffer, 20 mM Tris-HCl pH 8, was used as a control.

2.13. Immunofluorescence and Immunocytochemistry

2.13.1. Sample preparation

2.13.1.1. Sample of immunofluorescence

The hemocytes were collected from normal shrimp of 15 g body weight and fixed with 400 µl 4% (w/v) paraformaldehyde for 10 min. The hemocytes were centrifuged at 800 × g for 10 min at 4 ºC and suspended with 100 µl 1× PBS. The cell suspension was fixed onto the poly-L-lysine coated slide (1×10^5 cells/400 µl/slide) by centrifugation at 1,000 × g for 5 min.

2.13.1.2. Sample of immunocytochemistry

The black tiger shrimp were injected with 100 µl of 10^5 CFU/ml *V. harveyi*. The hemolymph was collected and fixed with 400 µl 4% (w/v) paraformaldehyde for 10 min at 6, 24, 48 and 72 h post injection. The hemocytes were centrifugated at 800 × g for 10 min at 4 °C and suspended with 100 µl 1× PBS. The cell suspension was fixed onto the poly-L-lysine coated slide (3×10^5 cells/400 µl/slide) by centrifugation at 1,000 × g for 5 min.

2.13.1.3. Primary antibody Probe

The fixed hemocytes were washed in PBS for 5 min and permeabilized using 400 µl 0.1% Triton X-100 in 1× PBS at room temperature for 5 min and washed 3 times using 400 µl of 1× PBS for 5 min.

The slides were, then, blocked with 400 µl 10% FBS (fetal bovine serum) in 1× PBS at room temperature for 1 h and washed 3 times with 400 µl 1× PBS containing 0.05% Tween 20 for 5 min. The slides were incubated with 1:1000 dilution of rabbit anti-*Pm*SERPIN8 antiserum in 1× PBS containing 1% FBS at 37 °C for 1 h and washed 3 times with 400 µl 1× PBS, 0.05% Tween 20 for 5 min.

2.13.2. Immunofluorescence

This was to analyze the type of hemocyte producing *Pm*SERPIN8 protein from normal shrimp by immunofluorescence technique. The slides were incubated with anti-*Pm*SERPIN8 antiserum. After that the slides were incubated with 1:500 dilution of fluorescence dye, Alexa 488-conjugated anti-rabbit (goat anti-rabbit antibodies) (Invitrogen) in 1× PBS containing of 1% FBS at 37 °C for 1 h and washed 3 times with 400 µl of 1× PBS, 0.05% Tween 20 for 5 min. The cells were incubated with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) in mounting medium to stain the nuclear DNA and incubated at room temperature 15 min. The fluorescence cells were detected under fluorescence microscope (Olympus).

2.13.3. Immunocytochemistry

To analyze the expression of *Pm*SERPIN8 in response to pathogen infection, immunocytochemistry was used. The slides were incubated with anti-*Pm*SERPIN8 antiserum. After that the slides were incubated with 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG (goat anti-rabbit antibodies) (Invitrogen) in 1× PBS containing 1% FBS at 37 °C for 1 h and washed 3 times with 400 µl 1× PBS, 0.05% Tween 20 for 5 min. The positive cells were detected using a detection solution (375 mg/ml NBT, 188 mg/ml BCIP and 1 mM levamisole in 0.1 M Tris-HCl pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl). The reaction was stopped by washing with Tris buffered saline (TBS). The positive cells were detected under light microscope (Olympus). At least, three hundred cells were counted per slides.

2.14. PO assay

Hemolymph was collected from approximately 30 shrimp of 10 g body weight using 200 µl 10% sodium citrate as an anticoagulant per 1 ml hemolymph. The hemocytes were separated by centrifugation at 800 × g for 10 min at 4 °C and lysed in 500 µl CAC buffer (0.01 M sodium cacodylate, 10 mM $CaCl_2$ pH 7) (Hung et al., 1997). The hemocyte lysate supernatant (HLS) was separated by centrifugation at 12,000 rpm for 10 min at 4 °C. The protein concentration of HLS was determined by Braford method (1976) using bovine serum albumin as a standard.

The reaction of PO assay contained 40 µl HLS (56 µg protein), 35 µl r*Pm*SERPIN8 (2.3 µM) or 45 µl r*Pm*SERPIN8 (3 µM) and CAC buffer to 85 µl. The controls were added 35 µl BSA (2.3 µM) or 45 µl BSA (3 µM). The negative control was added an inhibitor of phenoloxidase, 10 µl 17 mg/ml PTU (phenylthiourea) (Sigma). The reactions were activated with 40 µl 1 mg/ml lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma) at room temperature for 5 min and started by adding 25 µl 3 mg/ml of L-3,4-dihydroxyphenylalanine, L-DOPA (Sigma). The PO activity was monitored by measuring the absorption of dopachromes formed at 490 nm at 0, 5, 10, 15, 20, 30 and 40 min using a microplate reader.

2.15. In *vitro* clotting assay

Hemolymph from 3 individuals was collected using 200 µl 10% sodium citrate as an anticoagulant per 1 ml of hemolymph. The hemolymph was pooled into new tube. The clotting reaction consisted of 40 µl of 20 mM Tris-HCl pH 8 or r*Pm*SERPIN8 (21.96 µg) or BSA (21.96 µg) as a control and 200 µl of fresh hemolymph. The reaction was started by adding 10 µl 40 mM $CaCl_2$. Negative control

was not added the calcium chloride solution. The solutions were mixed and incubated at room temperature. The clot formation was observed in 1-2 min for the positive control and 1-10 min for r*Pm*SERPIN8 and BSA.

2.16. Agar diffusion assay of *Pm*SERPIN8 inhibitory activity against the hemocyte proteinases from *V. harveyi*-infected shrimp

Three black tiger shrimp of 10 g body weight were injected with 100 µl of *V. harveyi* (10^5 CFU/ml) and the hemolymph was collected at 6 h after injection using 200 µl of 10% sodium citrate as an anticoagulant. The hemocytes were separated by centrifugation at 800 g for 10 min. The hemocytes were added with 100 µl of 50 mM Tris-HCl pH 8 and homogenized. The hemocyte lysate supernatant (HLS) was separated by centrifugation at 12,000 rpm at 4 °C for 10 min.

The inhibition reaction consisted of 50 µl HLS, 5 µl of 1 M Tris-HCl pH 8 or 17.6 µg r*Pm*SERPIN8 or 8.8 µg of r*Pm*SERPIN8 and water to a total volume of 100 µl. The mixtures were added into the wells on a skim milk agar plate (0.6% (w/v) agarose, 0.75% (w/v) skim milk and 50 mM Tris-HCl pH 8) (Chen et.al., 2008). The plate was incubated at room temperature overnight. Clear zone of proteinase digestion around the well was observed for positive control.

CHAPTER III

RESULTS

3.1. Exon-intron structure of *PmSERPIN8* gene on genomic DNA

To study the genomic structure of *PmSERPIN8* gene in the genomic DNA of the black tiger shrimp, the genomic DNA was extracted from the shrimp pleopods and used as a template for PCR using Advantage *Taq* DNA polymerase and gene-specific primers, *Pm*SERPIN8-F1 and *Pm*SERPIN8-R2 (Table 2.1). The PCR product was analyzed by 1% agarose gel electrophoresis shown in (Fig 3.1). The DNA fragment of 3106 bp was cloned into T&A vector. The correct plasmid was screened with restriction enzyme digestion and subjected to DNA sequencing. The nucleotide sequence from the genomic DNA was compared with that of the cDNA. Fig. 3.2 shows the nucleotide sequence of *Pm*SERPIN8 gene in genomic DNA. The full-length *PmSERPIN8* gene was derived from 5 exons of 320, 139, 244, 239 and 312 bp separated by 4 introns of 447, 657, 326 and 479 bp.

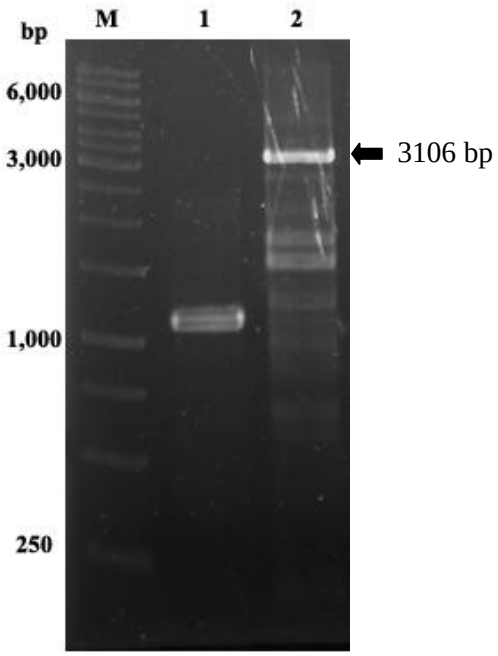


Fig.3.1. Analysis of amplified DNA fragments from the genomic DNA by 1% agarose gel electrophoresis. The genomic DNA was used as a template for PCR using specific primer, *Pm*SERPIN8-F1 and *Pm*SERPIN8-R2. Lane M: GeneRuler™ 1 Kb DNA ladder (Fermentas); lane 1: *PmSERPIN8* gene fragment amplified from the cDNA clone; lane 2: the PCR products amplified from the genomic DNA.

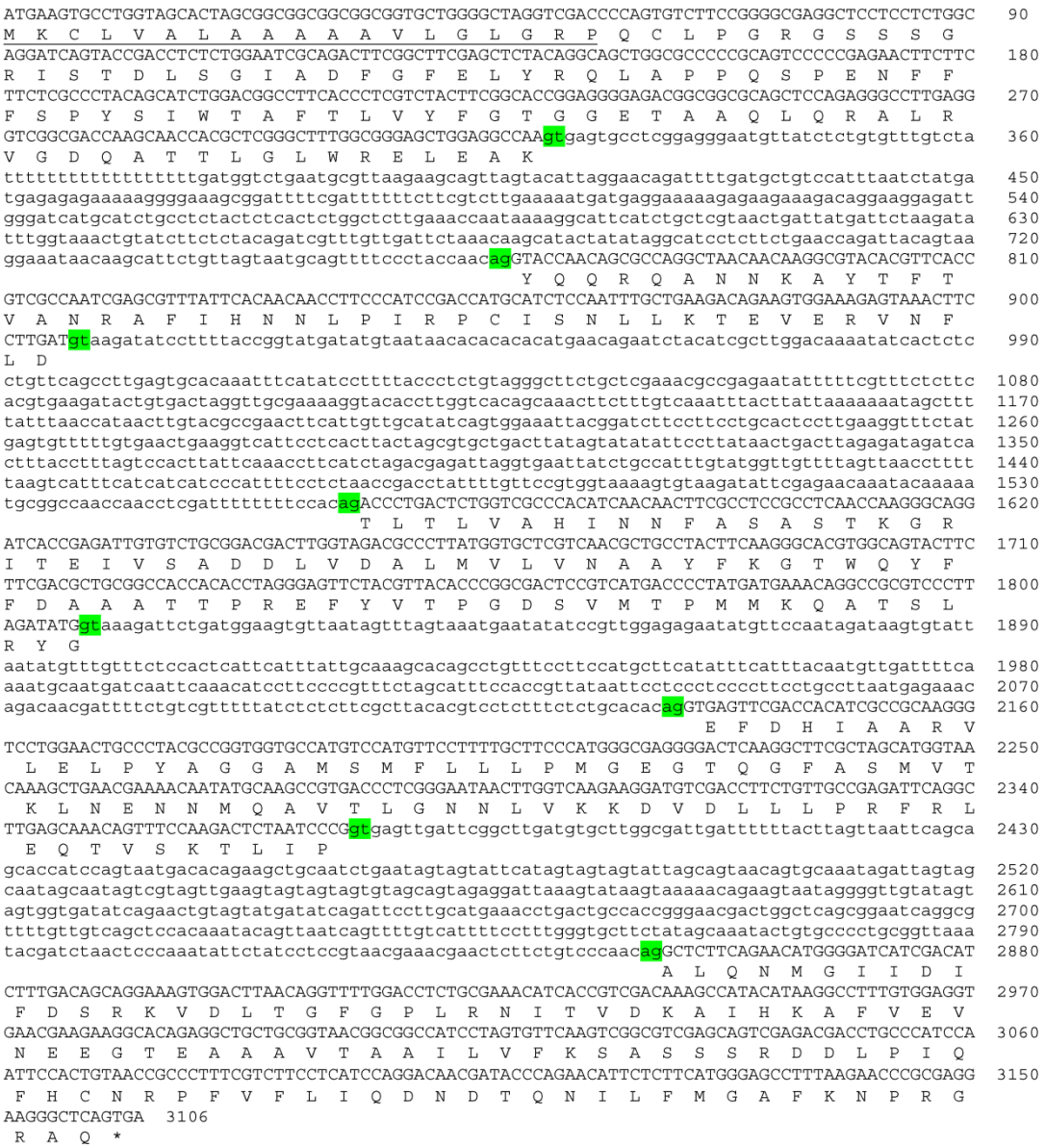


Fig. 3.2. Genomic organization of *PmSERPIN8* gene. The full-length gene of *PmSERPIN8* was derived from 5 exons of 320, 139, 244, 239 and 312 bp (uppercase letters) separated by 4 introns of 447, 657, 326 and 479 bp (lowercase letters). The putative signal is underlined. The conserved 5′ and 3′ spliced junction sequences were shaded.

3.2. Tissue distribution

Expression of *PmSERPIN8* gene in tissues from the black tiger shrimp *Penaeus monodon* was investigated. Ten tissues; hepatopancreas, lymphoid organ, eye stalk, epipodite, heart, hemocytes, antennal gland, stomach, intestine and gill were collected, extracted the total RNAs and synthesized the cDNAs. The cDNAs were analyzed by RT-PCR. The expression of elongation factor 1-alpha (EF-1α) was used as an internal control. The specific primers were used performed the PCR, the *Pm*SERPIN8-F2 primer, *Pm*SERPIN8-R2 primer (table 2.1) to amplified a 1,209 bp. The products were analyzed by 1% agarose gel electrophoresis as shown in (Fig. 3.3). The results show that *PmSERPIN8* transcript is expressed primarily in epipodite and hemocytes. Lesser transcription is in eye stalk, heart, antennal gland, lymphoid organ, stomach, intestine and hepatopancreas. No expression is observed in gill.

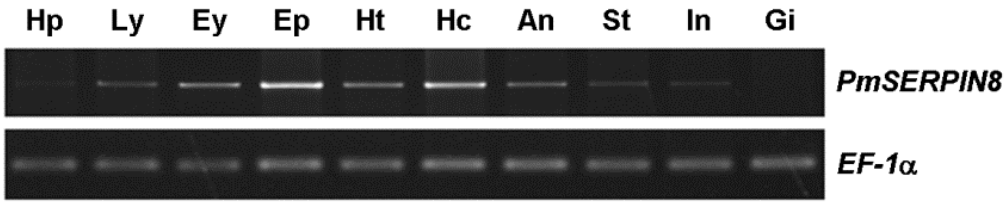


Fig. 3.3. Expression of *PmSERPIN8* gene in various shrimp tissues. Total RNAs were prepared from hepatopancreas (Hp), lymphoid organ (Ly), eyestalk (Ey), epipodite (Ep), heart (Ht), hemocyte (Hc), antennal gland (An), stomach (St), intestine (In) and gill (Gi) and subjected to RT-PCR analysis. Expression of *EF-1α* gene was used as an internal control.

3.3. Expression analysis of *Pm*SERPIN8 after *V. harveyi* and YHV challenge in shrimp hemocytes.

The expression of *PmSERPIN8* after *V. harveyi* and yellow head virus (YHV) challenge in hemocytes of the black tiger shrimp was studied by quantitative real-time PCR. The shrimp were separated into 3 groups: the *V. harveyi* 639-injected group, the YHV-injected group and the control normal saline-injected group. The total RNAs were extracted from the hemocytes at 0, 6, 24, 48 and 72 h after infection. The cDNAs were synthesized and analyzed by quantitative real-time PCR using the specific primers, *Pm*SERPIN8-F3 and *Pm*SERPIN8-R3 (Table 2.1). The EF-1α gene was an internal control.

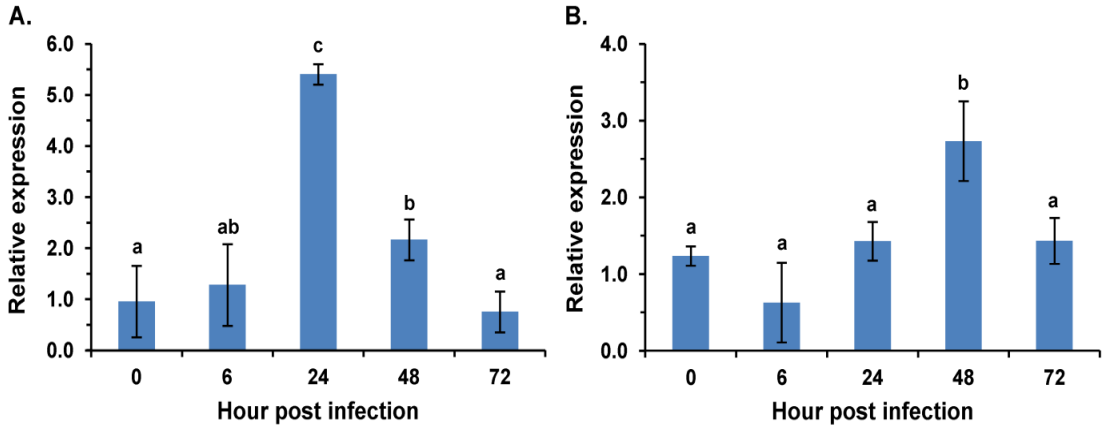


Fig. 3.4. Expression profiles of *PmSERPIN8* gene transcripts upon bacterial and virus infection. (A) Expression of *PmSERPIN8* gene in the hemocytes of *V. harveyi*-infected shrimp at 0, 6, 24, 48 and 72 h. (B) Expression of *PmSERPIN8* gene in hemocytes of YHV-infected shrimp at 0, 6, 24, 48 and 72 h. The control shrimp were injected with 0.85% NaCl. The expression of *PmSERPIN8* gene of infected group was normalized with that of an internal control elongation factor 1-α (EF-1α) and calculated relative to that of the control group. Letters indicate significant difference among the data ($P < 0.05$).

The results in Fig. 3.4 A show that, upon *V. harveyi* infection, the *PmSERPIN8* gene is up-regulated at 24 h. The expression is decreased at 48 and 72 h. Similarly, the *PmSERPIN8* is up-regulated in response to YHV infection at 48 h.

3.4. Expression of *PmSERPIN8* in Shrimp at different developmental stages

The total RNAs were extracted from shrimp at 4 developmental stages; nauplius, protozoea, mysis and postlarva, and used for cDNA synthesis. The cDNAs were analyzed by quantitative real-time PCR. The expression of elongation factor 1-alpha (EF-1α) was an internal control. The expression of *PmSERPIN8* gene in all stages was compared relative to that of nauplius stage. The results are shown in Fig. 3.5. The *PmSERPIN8* gene was expressed in all stages but the levels of expression were different. The *PmSERPIN8* was highly expressed in nauplius stage and decreased in protozoea, mysis and postlarva.

3.5. Construction of a *Pm*SERPIN8-expression plasmid

The *PmSERPIN8* gene consists of an open reading frame of 1,254 bp coding for a protein of 417 amino acid residues with signal peptide of 19 amino acid residues. A pair of primers, *Pm*SERPIN8-F2 and *Pm*SERPIN8-R2 (Table 2.1), were designed to amplify the gene coding for mature protein. The EST clone GL_H_SO1_0891_LF was used as a template for PCR using Phusion® Hot Start High-Fidelity DNA Polymerase (Finnzymes). The PCR product of 1209 bp in size was isolated and cloned into pVR600 as an expression vector. The recombinant plasmid was screened and digested with *Bam*HI and *Xho*I (Fig. 3.6).

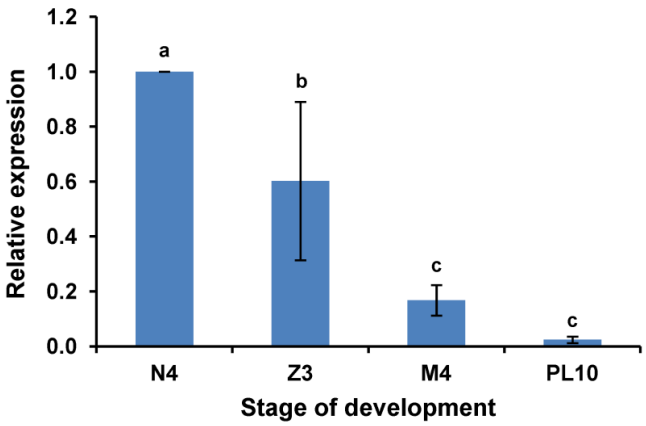


Fig.3.5. Expression analysis of *Pm*SERPIN8 in developmental stages of black tiger shrimp by quantitative real-time PCR. The total RNA was prepared from nauplius (N4), protozoae (Z3), mysis (M4) and postlarva (PL10). The gene expression of elongation factor 1-α (EF-1α) was used as an internal control. The levels of expression of *Pm*SERPIN8 in all stage shrimp were compared relatively with nauplius stage which was set to 1. Letters indicate significant difference among the data (P <0.05).

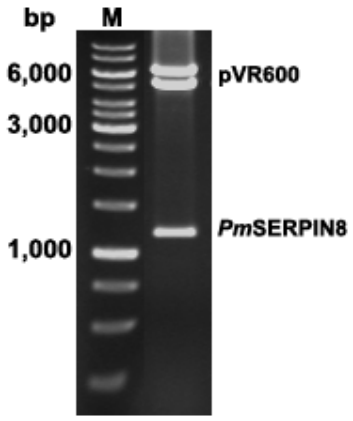


Fig. 3.6. The recombinant plasmid pVR600_*Pm*SERPIN8 digested by *Bam*HI and *Xho*I. The digestion was analyzed by 1% agarose gel electrophoresis. Lane M: GeneRuler™ 1 kb ladder (Fermentas); the second lane: pVR600_*Pm*SERPIN8 digested by *Bam*HI and *Xho*I

3.6. Expression of the recombinant protein r*Pm*SERPIN8

The expression of r*Pm*SERPIN8 from pVR600_*Pm*SERPIN8 plasmid was carried out in an *E. coli* system using BL21(DE3). The cells were induced with 1 mM IPTG, and 1-ml aliquots were collected at 0, 1, 2, 3, 4 h after induction. Cells was harvested by centrifugation, solubilized with the SDS-PAGE loading buffer and analyzed by 12% SDS-PAGE. The Coomassie Brilliant Blue-stained gel revealed that a protein band of about 43 kDa was increased after induction time (Fig. 3.7 A). Since the r*Pm*SERPIN8 was fused to the His·Tag at its C-terminus, it is verified by western blot analysis using anti-His antibody as a primary antibody. A band of protein was detected by color development using the NBT/BCIP as a substrate.

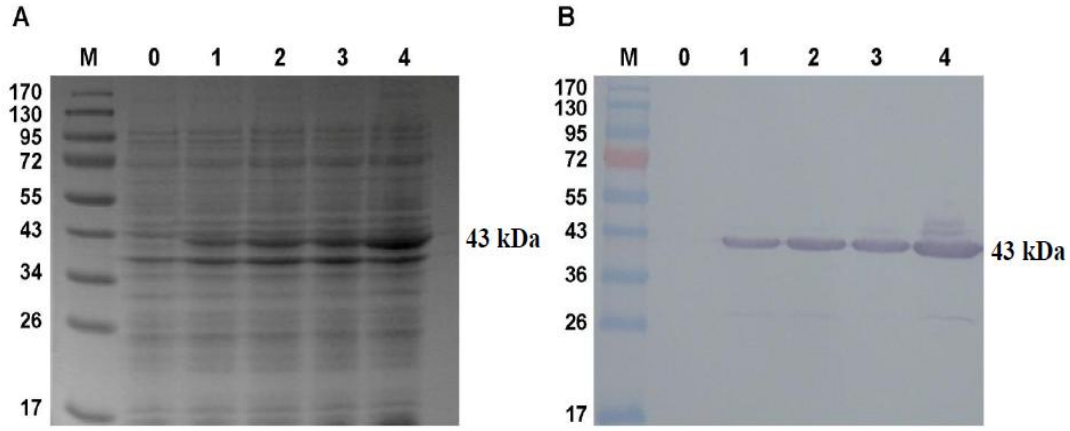


Fig. 3.7. The expression of r*Pm*SERPIN8 by 12% SDS-PAGE. (A**)** Expression of r*Pm*SERPIN8 at various time points after IPTG induction and (B) western blot analysis of the expression.

3.7. Purification of r*Pm*SERPIN8

The 4-h IPTG-induced cells were harvested by centrifugation, broken by sonication and centrifuged to separate the cell lysate and pellet. SDS-PAGE analysis showed that the recombinant protein r*Pm*SERPIN8 was mainly expressed in the insoluble inclusion bodies (Fig. 3.8 A). The inclusion bodies were washed and solubilized with 100 mM NaOH for 3 h. The solubilized protein was dialyzed against 20 mM Tris-HCl pH 8 and purified by nickel-NTA column (Fig. 3.8 B). A band of purified r*Pm*SERPIN8 with an approximately 43 kDa in size was purified to homogeneity.

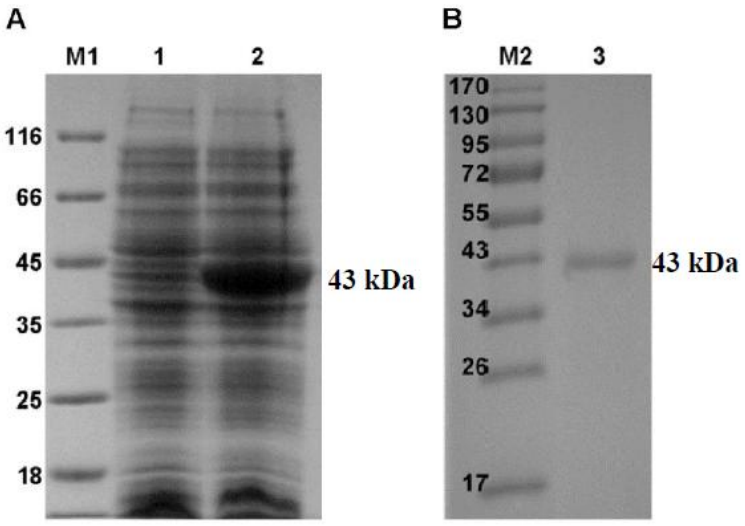


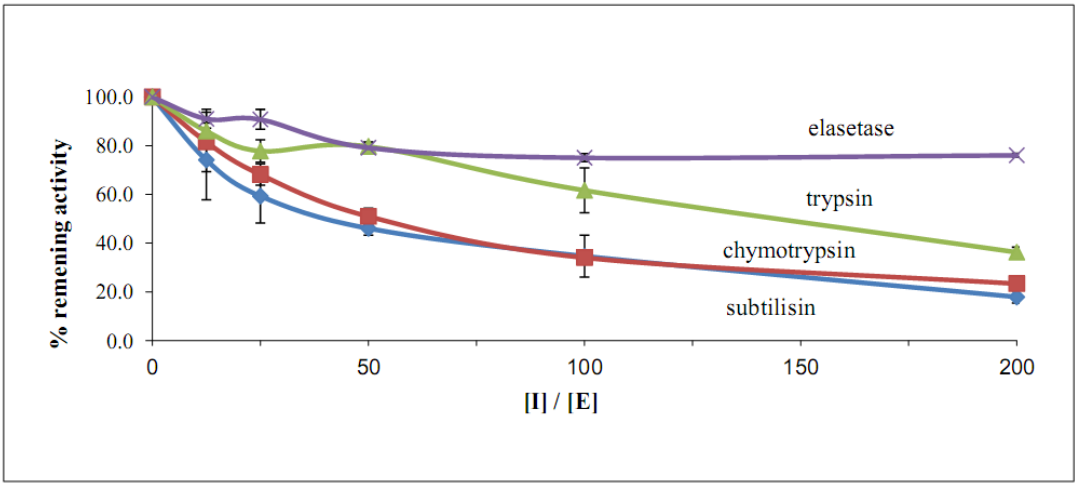
Fig. 3.8. The recombinant protein of *Pm*SERPIN8 was produced from *E. coli* stain BL21(DE3), analyzed in 12% SDS-PAGE (A) The r*Pm*SERPIN8 was expressed inclusion body, lane 1 supernatant, lane 2 pellets. (B) The purified protein of *Pm*SERPIN8.

3.8. Inhibition assay

The purified r*Pm*SERPIN8 was assayed against serine proteinases: α-chymotrypsin, trypsin, subtilisin and elastase, in the presence of chromogenic substrates. The hydrolyzed product was *p*-nitroaniline which could be measured at A_{410}. The assay was carried out using increasing amount of purified r*Pm*SERPIN8. The remaining activity of proteinase was determined and plotted against the molar ratios of inhibitor to proteinase. Fig. 3.9 A reveals that the r*Pm*SERPIN8 exhibits strong inhibitory activity against subtilisin and α-chymotrypsin. Much lower inhibitory activity is against trypsin and elastase.

The inhibitory activity of r*Pm*SERPIN8 against subtilisin was also tested with 25 IU of heparin as showed in Fig. 3.9 B. The inhibitory activity of r*Pm*SERPIN8 against subtilisin in the presence of heparin was not much different from that without heparin. Therefore, heparin had no effect on the inhibitory activity of r*Pm*SERPIN8.

A



B

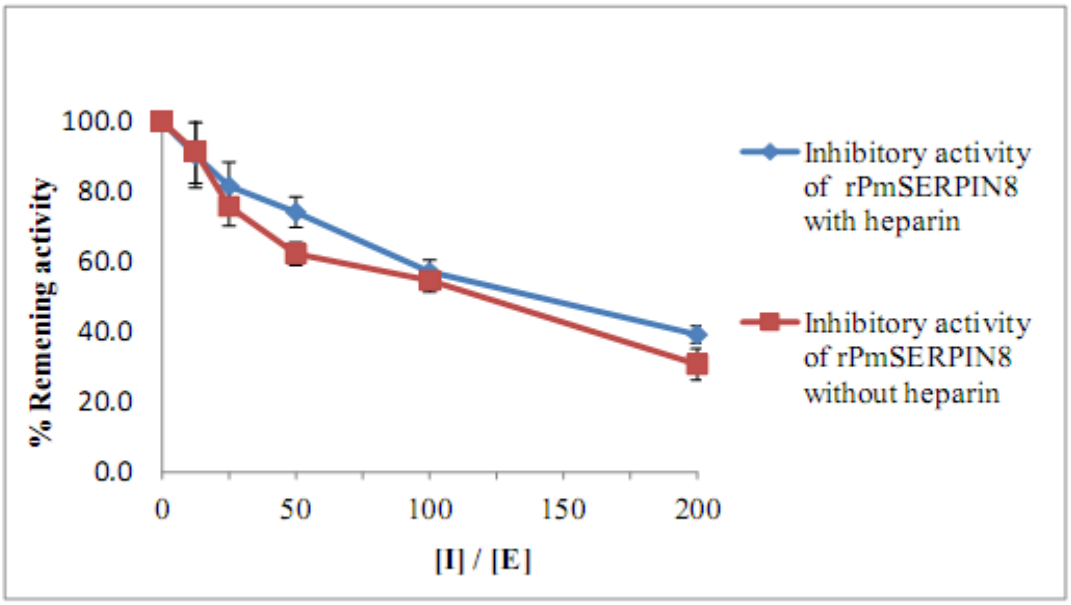


Fig. 3.9. Inhibitory activity of r*Pm*SERPIN8 against serine proteinases. (A) Elasetase (×), trypsin (▲), chymotrypsin (■), sutilisin (◆). (B) Inhibitory activity of rPmSERPIN8 against subtilisin with heparin (◆) and without heparin (■). Data are means with standard deviation of triplicate experiments.

3.9. Immunofluorescence and immunocytochemistry

Immunofluorescence technique was used to determine the type of hemocyte producing *Pm*SERPIN8 detected by rabbit anti-*Pm*SERPIN8 antiserum. The hemocytes were collected from normal shrimp and fixed with paraformaldehyde. The hemocytes were centrifuged onto a poly-L-lysine slide. After permeabilization with 0.1% Triton X-100. The anti-*Pm*SERPIN8-producing hemocytes were probed with a rabbit anti-*Pm*SERPIN8 antiserum followed by a secondary antibody, the fluorescence dye Alexa 488-conjugated anti-rabbit (goat anti-rabbit antibodies). The nuclear DNA were stained by DAPI. The hemocytes were, then, observed under fluorescence microscope. The result showed that the type of hemocytes having fluorescence were mainly semi-granular and granular cells (Fig. 3.10).

With *V. harveyi*-infected shrimp, the number of hemocytes that produced *Pm*SERPIN8 was determined by immumocytochemistry using rabbit anti-*Pm*SERPIN8 antiserum. The staining steps were similar to immunoflurescence but a different secondary antibody, an alkaline phosphatase-conjugated goat anti-rabbit IgG was used. The positive cells were detected by detection solution and observed under light microscope (Fig. 3.11 A). The results showed that the *Pm*SERPIN8-expressing hemocytes were increased at 48 h after infection (Fig. 3.11 B).

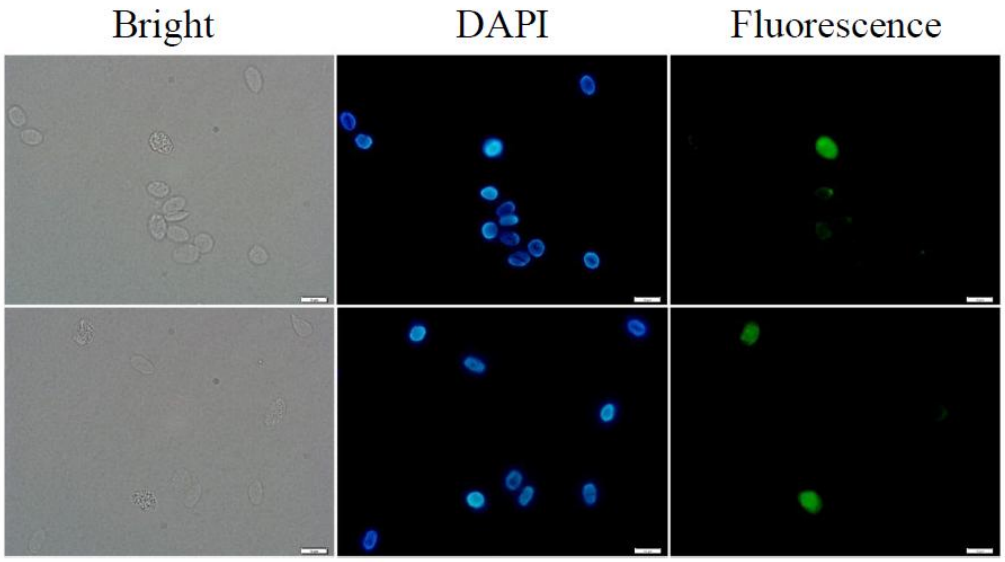


Fig. 3.10. The hemocytes produced *Pm*SERPIN8 in normal shrimp were determined by immunofluorescence using rabbit anti-*Pm*SERPIN8 antibody. The hemocytes were determined under fluorescence microscope. The cells were incubated with fluorescence dye, Alexa 488 conjugated anti-rabbit (goat anti-rabbit antibodies) and 4',6-diamidino-2-phenylindole,dihydrochloride (DAPI) in mounting medium to stain the nuclear DNA.

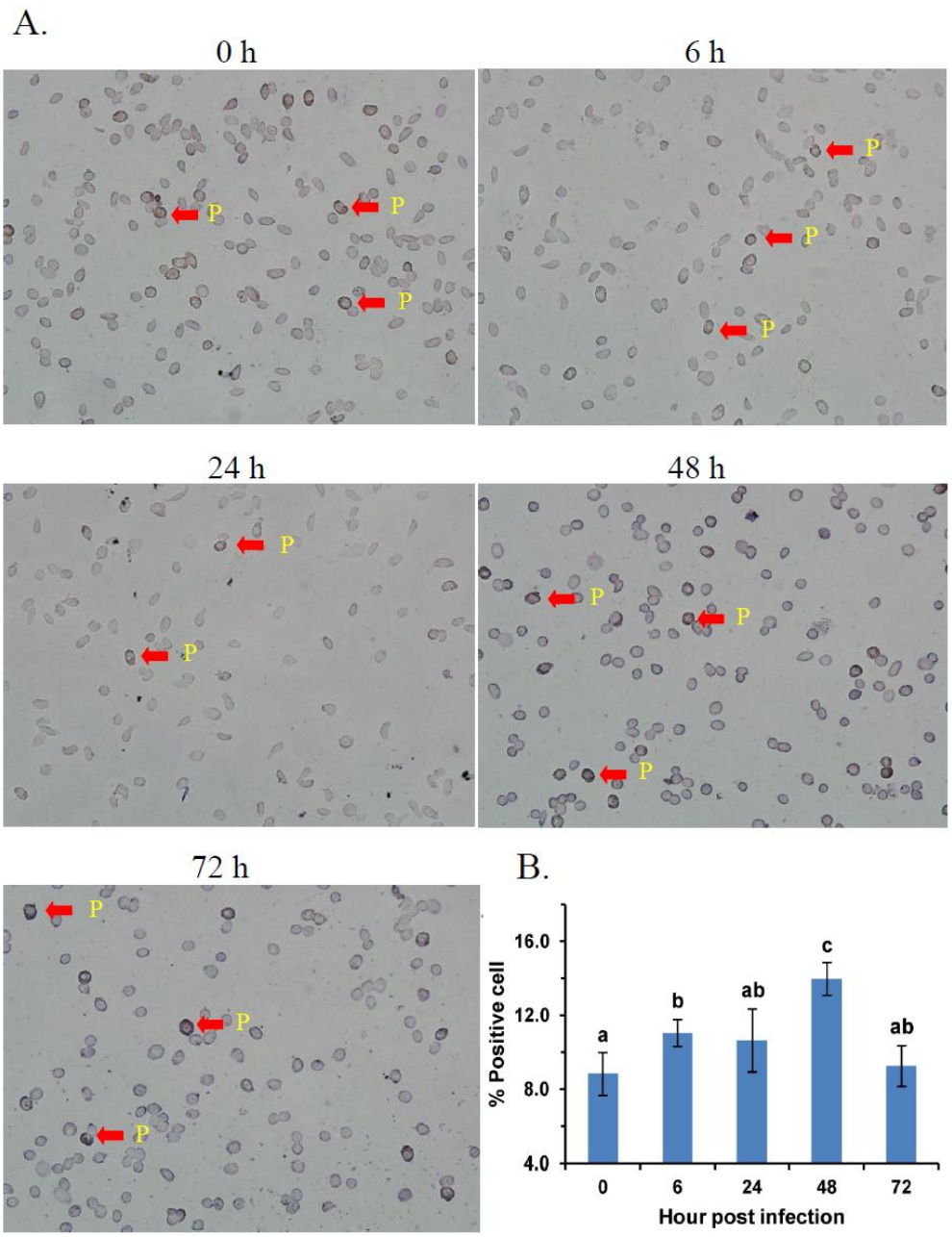


Fig. 3.11. The expression of r*Pm*SERPIN8 in hemocytes at 0, 6, 24, 48 and 72 h after *Vibrio harveyi* infection detected by immunocytochemistry using rabbit anti-*Pm*SERPIN8 antibody. The positive cells from a total cell count of 300 were calculated as % positive cells (A) and plotted against times after infection (B). The hemocyte at 0 h after infection was used as a control.

3.10. Inhibition growth of bacteria

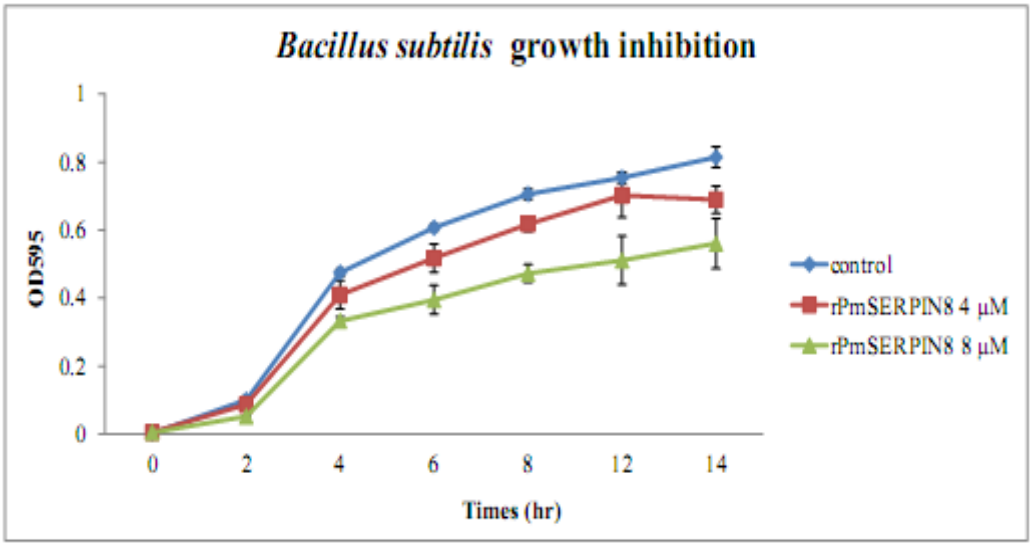
The growth inhibitory activity of r*Pm*SERPIN8 on bacteria was assayed. The growth of a Gram-positive bacterium *Bacillus subtilis* and a Gram-negative bacterium *V. harveyi* 639 at 30 °C with shaking was monitored by measuring OD_{600} in the presence of 4 and 8 µM r*Pm*SERPIN8. The buffer, 20 mM Tris-HCl pH 8, was used as a control. The results show that the growth of *B. subtilis* is inhibited by 4 and 8 µM r*Pm*SERPIN8 (Fig. 3.12 A) but not that of *V. harveyi* 639 (Fig. 3.12 B).

3.11. Phenoloxidase assay

The involvement of *Pm*SERPIN8 in prophenoloxidase (proPO) activating system was investigated by measuring the activity of phenoloxidase (PO) in the presence of *Pm*SERPIN8. The products of the reaction were dopachromes, products from the hydrolysis of 3,4-dihydroxyphenylalanine (L-DOPA) by PO that can be measured at A_{490}.

The hemocyte lysate supernate (HLS) was separated from the hemocytes and mixed with r*Pm*SERPIN8 to the final concentrations of 2.3 µM (Fig. 3.12 A) and 3 µM (Fig. 3.12 B) or PTU as an inhibitor of PO or BSA as a control. The system was activated by adding lipopolysaccharides (LPS). The reaction was started by adding L-DOPA. The activity was monitored by measuring the A_{490} at 0, 5, 10, 15, 20, 30 and 40 min. The results shown in Fig. 3.13 reveal that the r*Pm*SERPIN8 is able to inhibit the activation of proPO system while the BSA cannot. The phenoloxidase activity was down by as much as 50% at 40 min.

A



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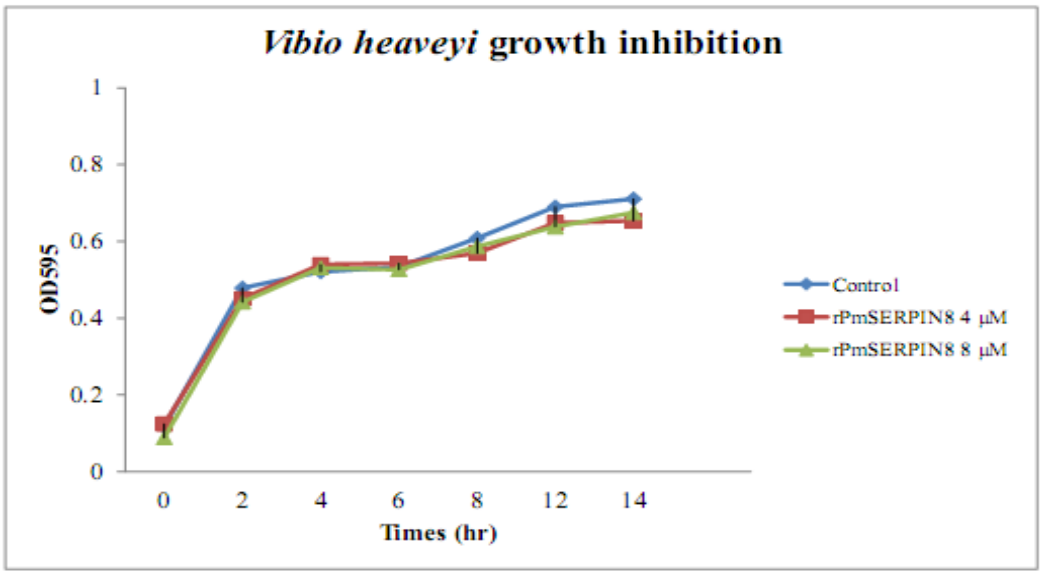
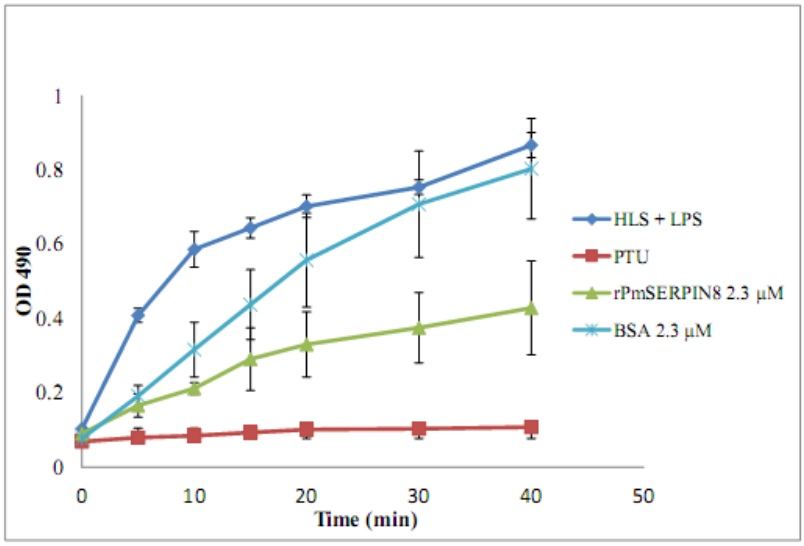


Fig. 3.12. Growth inhibition of bacteria. (A) *Bacillus subtilis*, (B) *V. harveyi* 639. The cultures were added 20 mM Tris-HCl pH 8 (◆) as a control, r*Pm*SERPIN8 to the final concentration of 4 µM (■) and 8 µM (▲).

A



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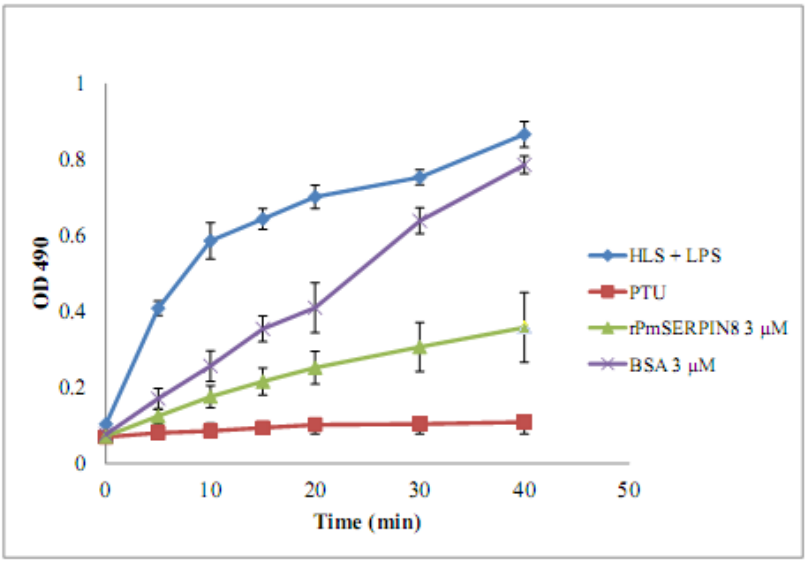


Fig. 3.13. Inhibitory activity of r*Pm*SERPIN8 against the activation of phenoloxidase (PO). The PO assay contained 56 µg of HLS, r*Pm*SERPIN8 to the final concentrations of 2.3 µM (A) and 3 µM (B). The system was activated by adding lipopolysaccharides (LPS). The 3,4-dihydroxyphenylalanine (L-DOPA) was added to start the reaction. The activity was monitored by measuring the A_{490}. BSA and PTU were added as a protein control and a negative control, respectively.

3.12. Clotting assay

To study whether the r*Pm*SERPIN8 was involved in anticoagulation process of shrimp blood, the whole blood clotting assay was employed. The shrimp hemolymph was mixed with the r*Pm*SERPIN8 to the final concentration of 2 µM. A cofactor $CaCl_2$ was added to start the process. BSA was used as a control. The positive control was added $CaCl_2$ whereas the negative control was not added. The clot formation was observed in 2 min for positive control and 4 min for hemolymph adding r*Pm*SERPIN8 or BSA. The result is shown in Fig. 3.14. Therefore, the r*Pm*SERPIN8 could not inhibit clot formation process in shrimp.

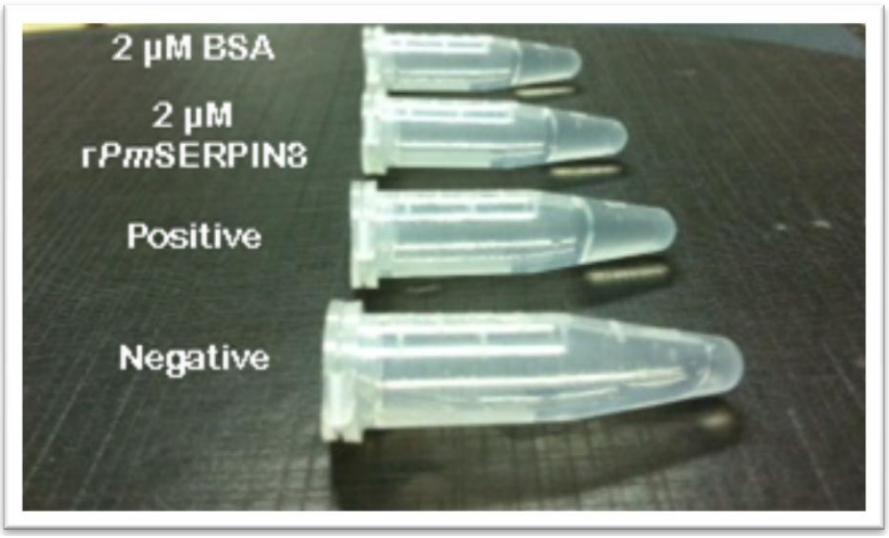


Fig. 3.14. The affect of r*Pm*SERPIN8 on the shrimp blood clotting system. Fresh hemolymph was mixed with 20 mM Tris-HCl pH 8 as a positive control, 2 µM BSA as a protein control and 2 µM r*Pm*SERPIN8. $CaCl_2$ was added to start the clotting process. The negative control was not added $CaCl_2$.

3.13. Inhibitory activity of *Pm*SERPIN8 against the hemolymph proteinases from *V. harveyi*-infected shrimp

The inhibitory activity of r*Pm*SERPIN8 against proteinases in the hemolymph from *V. harveyi* 639-infected shrimp was investigated using an agar diffusion assay. The hemolymph was collected at 6 h after infection. The hemolymph was mixed with, 2 and 4 µM r*Pm*SERPIN8. The mixtures were added into the wells in a skim milk agar plate. After incubation for an overnight, clear zone was observed around the well. It was found that the r*Pm*SERPIN8 was unable to inhibit the hemolymph proteinases for the diameters of clear zones were not much different from those of the hemolymph and the 20 mM Tris-HCl pH 8 buffer control.

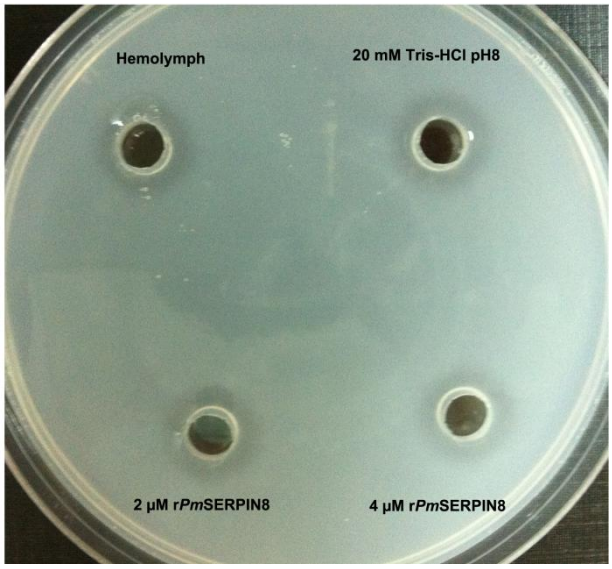


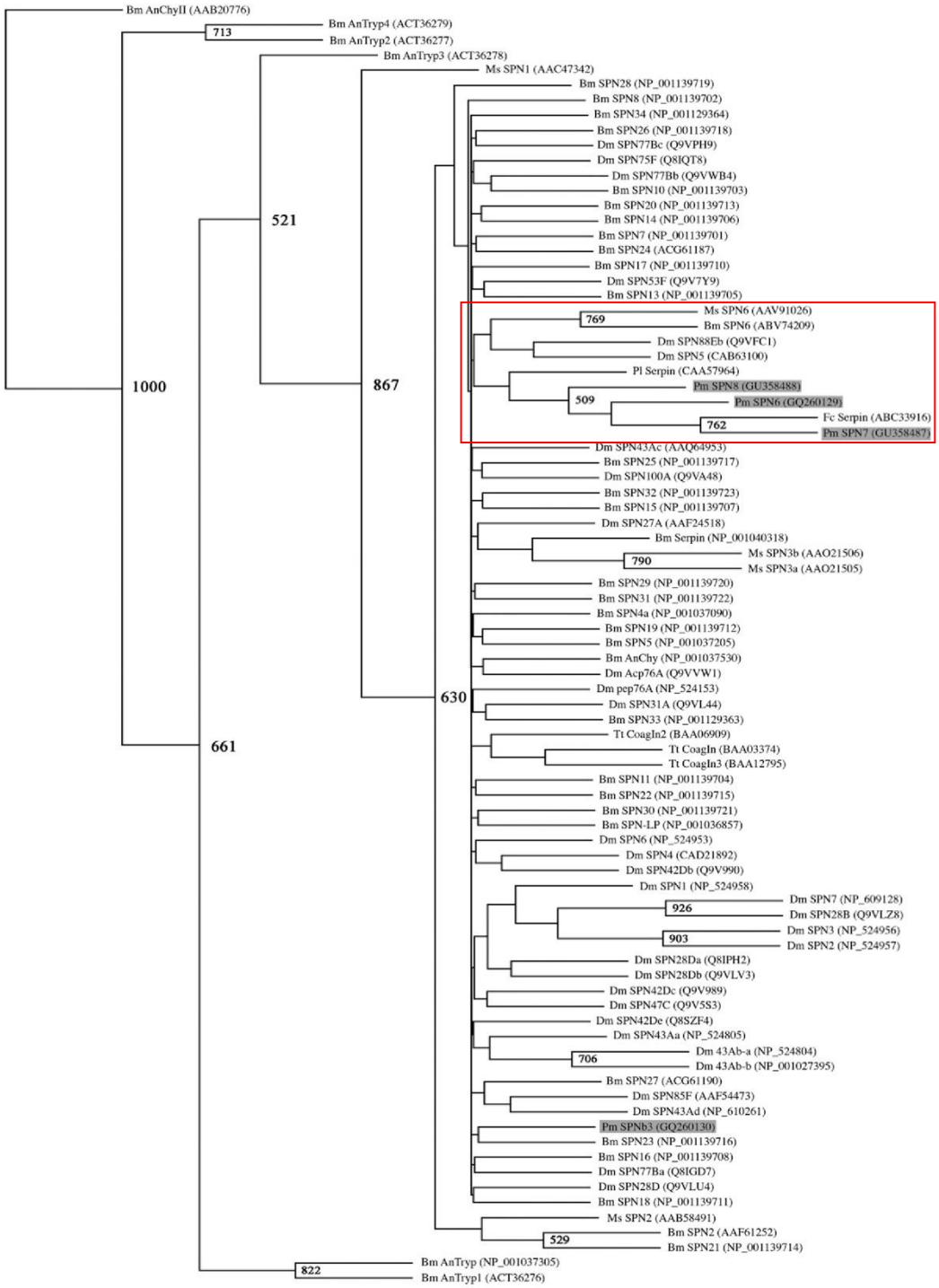
Fig.3.15. The inhibitory activity of r*Pm*SERPIN8 against proteinases in hemolymph from *V. harveyi* 639-infected shrimp. The hemolymph was mixed with r*Pm*SERPIN8 and added into the wells in the skim milk plate. The proteinase activity is seen as a clear zone surrounding the well. The hemolymph alone was a positive control. The buffer 20 mM Tris-HCl pH 8 was added as a buffer control.

CHAPTER IV

DISCUSSION

Serpin is a serine proteinase inhibitor, an essential regulator of a wide variety of biological processes such as complement system, blood coagulation, melanization, apoptosis etc. (Silverman et al., 2001). It has been found in a diverse range of organisms. Previously, serpins in other species were found to be involved in prophenoloxidase activating system (proPO). For examples, the serpin-3 of *Manduca sexta* inhibited PAP-1 and PAP-3 (Zhu et al., 2003). The serpin-4 and serpin-5 isolated from *M. sexta*, inhibited the activation of proHP8 and proPAP1 in proPO system (An and Kanost, 2010). In *Drosophilla*, the Spn28D was found to regulate PO activity in hemolymph (Scherfer et al., 2008).

By searching the serpins in *P. monodon* EST database, eight different *P. monodon* serpins with three serpins containing complete open reading frame were identified. Those having complete open reading frame were *PmSERPIN6*, *7* and *8* (Homvises et al., 2010). Amino acid sequence comparison among the related serpins revealed that the *Pm*SERPIN8 had 61%, 56%, 56% and 34% sequence identity to *Pm*SERPIN6, 7, *Fc*-serpin and *M. sexta* serpin-6, respectively. In phylogenetic analysis, *Pm*SERPIN8 was clustered with the insects such as *M. sexta* and *Drosophilla*. The serpin-6 of *M. sexta* was found to regulate the prophenoloxidase system (Zou and Jiang, 2005).



Source: Homvises et al., 2010

Fig. 4.1 Phylogenetic tree used the amino acid sequence and compared with serpins from other species in crustacean and insect. The phylogenetic analysis was using the neighbor-joining distance method.

In this thesis research, I was interested in *Pm*SERPIN8. The expression in shrimp tissues, in developing stages and in response to pathogenic infections was studied. The inhibitory activities of *Pm*SERPIN8 against the proteinases, the growth of bacteria, the coagulation process and the proPO system were elucidated.

The serpins of horseshoe crabs and crayfish are synthesized in hemocytes (Liang and Söderhäll, 1995). The RT-PCR was used to study the tissue distribution of *PmSERPIN8* to detect a specific *Pm*SERPIN8-producing tissue from normal black tiger shrimp. It was found out that the *PmSERPIN8* gene was expressed essentially in all tissues but mainly in hemocyte and epipodite. Similarly, the *Pm*SERPIN6 of *P. monodon* and *Bmserpin*-2 of *Bombyx mori* were also expressed in all tissues (Homvises 2010; Pan et al., 2009) suggesting their important roles in the organisms. In insects, the tissue of *M. sexta* and *D. melanogaster* that has important role in host immune response is fat body. In crustaceans, it is the hemocytes that are essential in immunity.

The first serpin reported in *P. monodon* is the *Pm*SERPINB3. It has been identified as differential expression gene in *V. harveyi*-infected *P. monodon* (Somboonwiwat et al., 2006). The *Pm*SERPIN8 gene was up-regulated in response to also *V. harveyi*-infection. The expression was up-regulated at 24 h post infection. Moreover, the expression was also up-regulated against viral infection, the yellow heard virus (YHV) at 48 h after infection. In *Fenneropenaeus chinensis*, the transcription of Fc-serpin mRNA was fluctuated and increased in hemocytes at 24 h post-injection with *V. harveyi* and *Staphylococcus aureus* mixed culture (Liu et al., 2009).

It was found that the serpins in insects, *Sphenophorus levi*s and *Bombyx mori*, were expressed in all developmental stages (Fonseca et al., 2011; Pan et al., 2009). It was interesting how the *Pm*SERPIN8 was expressed in the developmental stages of shrimp. By using quantitative RT-PCR, I found that the *Pm*SERPIN8 gene was also expressed in all stages. The expression of *Pm*SERPIN8 seemed to be constitutive in all stages but the higher transcriptional level was observed in nauplius stage and lower in later stages. Together with the up-regulated transcriptional level of *Pm*SERPIN8 after *V. harveyi* infection, I believed that the *Pm*SERPIN8 were involved in immune response.

In vertebrates and insects, the genomic organization of various serpin genes are different with variable number of exons and introns. Six patterns of intron-exon organization is found in vertebrates. In insects, the different patterns lead to the relation among serpins in terms of evolution. The genomic organization of *PmSERPIN8* was studied and found that the open reading frame of *Pm*SERPIN8 was derived from 5 exons separated by 4 introns. Its intron-exon pattern seemed to be unique different from those of vertebrates and insects.

The *Pm*SERPIN8 protein was recombinant expressed. The *PmSERPIN8* gene was cloned into a pVR600 expression vector and expressed in an *E. coli* system. The r*Pm*SERPIN8 was expressed as inclusion bodies, but could be solubilized in 0.1 M NaOH. The exposure of protein to strong alkali condition was kept as short as possible and the solubilized protein was dialyzed against a somewhat neutral buffer. The purified protein was detected in SDS-PAGE as approximately 43 kDa protein bands as opposed to the calculated figure of 45.5 kDa. The recombinant protein was purified for further used in several experiment afterwards.

Inhibitory serpin targets specific proteinase though recognition of the P_1 amino acid in the C-terminal reactive center loop (RCL) of serpin molecules (Huntington et al., 2000). The P_1 amino acid is a key amino acid residue that determines the target specificity (Potempa et al., 1994). For example, the P_1 Lys or Arg makes the serpin inhibitory to trysin (Gan et al., 2001). The P_1 of *Pm*SERPIN8 is Lys. It was found to inhibit moderately the trysin. It inhibited subtilisin and α-chymotrysin but weakly inhibited elasetase. The inhibitory activity was not as good as expected for the 50% inhibition was obtained at an inhibitor to proteinase ratio of 50. This was in contrast to other type of inhibitors, for example, the Kazal-type serine proteinase inhibitor SPI*Pm*2 that was able to inhibit subtilisin and elastase at the said ration of less than 5 (Donpudsa et al., 2009).

It was thought that the r*Pm*SERPIN8 might have specific target proteinase in the shrimp. It was then assayed its inhibitory activity against proteinases in the hemolymph from 6-h *V. harveyi* 639-infected shrimp using the agar diffusion assay. The r*Pm*SERPIN8 could not inhibit proteinases in the hemolymph.

The r*Pm*SERPIN8 was tested for its inhibitory activity on bacterial growth. The rSPI*Pm*2 has strong inhibitory activity against subtilisin and it can inhibit growth of subtilisin-producing *B. subtilis* (Donpudsa et al., 2009). With its inhibitory activity on subtilisin, the r*Pm*SERPIN8 was found to inhibit the growth of a Gram-positive bacterium *B. subtilis* but not that of a Gram-negative bacterium *V. harveyi* 639.

The expression of r*Pm*SERPIN8 protein could be detected by immunofluorescesce and immunocytochemistry using anti-*Pm*SERPIN8 antiserum. Previously, a close related *Pm*SERPIN6 was found to express more at 72 h after infection with *V. harveyi* (Homvises et al., 2010). In this case, the r*Pm*SERPIN8 was

up-regulated at 48 after infection with *V. harveyi* 639. The r*Pm*SERPIN8 expression at the translational level was about 24 h after the increase in transcriptional level of *Pm*SERPIN8. The *Pm*SERPIN8 was produced by all 3 types of blood cells, hyaline, semi-granular and granular hemocytes but preferentially by the latter 2 cell types. The semi-granular and granular hemocytes are known to produced and store secretory immune proteins.

The prophenoloxidase activating system (proPO) is an important component of innate immune response. The melanin was the final product of proPO system. The proPO was activated by bacterial cell wall components such as lipopolysaccharide (LPS), fungal β-1,3-glucan and peptidoglycan (PG). Then, a series of serine proteinases are activated sequentially which amplify the initial activation to the terminal proteinase in the proPO activation cascade (Söderhäll and Häll, 1984; Duvic et al., 1990; Yoshida et al., 1996; Gorman et al., 2007). The phenoloxidase (PO) is a final enzyme in this cascade. The serine proteinase cascade was regulated by serine proteinase inhibitors (Söderhäll and Cerenius, 1998). The serpin-3 of *M. sexta* inhibits PAP-1 and PAP-3 (Zhu et al., 2003). The serpin-4 and serpin-5 from *M. sexta* inhibit the activation of proHP8 and proPAP1 in proPO system (An et al., 2010). Morever, in *Drosophilla*, the Spn28D regulates PO activity in the hemolymph (Scherfer et al., 2008). The r*Pm*SERPIN8 of *P. monodon* was tested for this regulatory activity and found that it could impede the PO activity in proPO system. It is not clear which part of the proPO cascade is inhibited and remains to be studied further.

Another process in blood is the blood clotting process. The *Pm*SERPIN8 was also tested whether it could inhibit the blood clotting process. By using hemolymph from the black tiger shrimp, the clot formation was observed in the hemolymph in the

presence of r*Pm*SERPIN8 in 4 min which was not much different from the BSA control. Thus, the r*Pm*SERPIN8 is not involved in the blood coagulation in shrimp. In fact, the blood clotting process was caused by polymerization of clotting protein by transglutaminase. The proteolytic process does not appear to be necessary for blood clotting system in crustacean. The alpha-2 macroglobulin is one of serine proteinase inhibitor. In crayfish, the molecule of alpha-2 macroglobulin had free lysine and glutamine residues that might be used by transglutaminase to crosslink alpha-2 macroglobulin to the clotting protein. The alpha-2 macroglobulin is entrapped in clotting for inhibition of proteinases secreted from the microorganisms.

CHAPTER V

CONCLUSION

1. The open reading frame of *Pm*SERPIN8 gene is derived from a genomic gene containing 5 exons separated by 4 introns.
2. The *Pm*SERPIN8 is expressed highly in nauplius and decreased in protozoea, mysis and postlarva.
3. The *Pm*SERPIN8 are expressed in all tissues but mainly in hemocyte and epipodite.
4. The expression of *Pm*SERPIN8 in hemocytes is up-regulated in response to *V. harveyi*- and YHV-infection.
5. The *Pm*SERPIN8-producing hemocytes: hyaline, semi-granular and granular cells are up-regulated in *V. harveyi*-infected shrimp.
6. The r*Pm*SERPIN8 exhibits inhibitory activity against subtilisin and α-chymotrypsin. The inhibitory activity against subtilisin is not enhanced by heparin.
7. The r*Pm*SERPIN8 inhibits the growth of a Gram-positive bacterium *B. subtilis* but not a Gram-negative bacterium *V. harveyi*.
8. The *Pm*SERPIN8 inhibits the activation of phenoloxidase indicating its role in controlling the prophenoloxidase system.
9. The r*Pm*SERPIN8 is not involved in blood coagulation process.

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APPENDIX

1. **Preparation for SDS-PAGE electrophoresis**

Stock reagents

30 % Acrylamide, 0.8 % bis-acrylamide, 100 ml.

Acrylamide 29.2 g

N,*N*′-methylene-bis-acrylamide 0.8 g

Adjust volume to 100 ml with distilled water.

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 18.17 g

Adjust pH to 8.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 6.06 g

Adjust pH to 6.8 with 1 M HCl and adjust volume to 100 ml with distilled water.

5× Sample buffer

1 M Tris-HCl pH6.8	0.6	ml
50 % (w/v) Glyserol	5.0	ml
10 % SDS	2.0	ml
2-Mercaptoethanol	0.5	ml
1% Bromophenol blue	1.0	ml
Distilled water	0.9	ml

One part of sample buffer was added to four part of sample. The mixture was boiled for 5 min and loading to the gel.[i]

2. SDS-PAGE

12% Separating gel

H_2O	1.6	ml
30% (w/v) Acrylamide solution	2.0	ml
1.5 M Tris-HCl pH 8.8	1.3	ml
10% SDS	0.05	ml
10 % Ammonium persulfate	0.05	ml
TEMED	0.002	ml

5% Stacking gel

H_2O	1.4	ml
30% (w/v) Acrylamide solution	0.33	ml
1. M Tris-HCl pH 8.8	0.25	ml
10% SDS	0.02	ml

10% Ammonium persulfate	0.02	ml
TEMED	0.002	ml

3. Electrophoresis buffer, 1 litre

10× Stock buffer

Tris (hydroxylmethyl)-aminomethane	30.3	g
Glycine	144.0	g
SDS	10	g

Dissolve in distilled water to 1 litre. Do not adjust pH with acid or base.

1× Working buffer

25 mM Tris, 192 mM glycine

100 ml of 10× stock buffer was added in 900 ml of distilled water. Do not adjust pH with acid or base (final pH 8.3).

BIOGRAPHY

Miss Suphaphon Somnuk was born on December 14, 1985 in Rayong Province. She graduated with the degree of Bachelor of Science from the Department of Medical Technology, Faculty of Medical Technology**,** Huachiew Chalermprakiet University in 2007. Then, she worked in the Biochemistry Laboratory at Queen Sirikit National Institute of Child Health for one year. Since 2009, she has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University.

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