การโคลนและลักษณะสมบัติของตัวยับยั้งเซอรีนโปรติเนส *Pm*SERPIN6 จากกุ้งกุลาดำ *Penaeus monodon*

นางสาวธีรดา หอมวิเศษ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CLONING AND CHARACTERIZATION OF *Pm*SERPIN6, A SERINE PROTEINASE INHIBITOR, FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	CLONING AND CHARACTERIZATION OF *Pm*SERPIN6, A SERINE PROTEINASE INHIBITOR, FROM THE BLACK TIGER SHRIMP *Penaeus monodon*
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ธีรดา หอมวิเศษ: การโคลนและลักษณะสมบัติของตัวยับยั้งเซอรีนโปรติเนส *Pm*SERPIN6 จากกุ้งกุลาดำ *Penaeus monodon*. (CLONING AND CHARACTERIZATION OF *Pm*SERPIN6, A SERINE PROTEINASE INHIBITOR, FROM THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร.กุลยา สมบูรณ์วิวัฒน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ดร.อัญชลี ทัศนาขจร, 140 หน้า.

ตัวยับยั้งเซอรีนโปรติเนสในกลุ่มเซอร์ปิน ทำหน้าที่ควบ คุมกระบวนการทางชีวภาพและพบได้ในสิ่งมีชีวิตหลากหลายชนิด ในกุ้งสกุล Penaeid มีรายงานการจัดจำแนกเซอร์ปินเพียงเล็กน้อยและยังไม่มีการศึกษาลักษณะสมบัติอย่างชัดเจน งานวิจัย นี้สามารถจำแนกเซอร์ปินจากฐานข้อมูลห้องสมุดซีดีเอ็นเอของกุ้งกุลาดำได้ทั้งสิ้น 8 ชนิด โดยให้ชื่อว่า *Pm*SERPIN1-8 จากการเปรียบเทียบลำดับกรดอะมิโนของโปรตีนพบว่า *Pm*SERPIN6 มีความคล้ายคลึง กับเซอร์ปิน 6 จาก *Manduca sexta* มากที่สุด ซึ่งมีรายงานการศึกษาแล้วว่าเกี่ยวข้องกับการยับยั้งระบบโพรฟีนอลออกซิเดส ซึ่งเป็นกระบวนการที่สำคัญในระบบภูมิคุ้มกันของสิ่งมีชีวิตจำพวกอาร์โทรพอด ดังนั้นในงานวิจัยนี้จึงสนใจศึกษาลักษณะสมบัติของ *Pm*SERPIN6 จากการศึกษาการกระจายตัวของ *Pm*SERPIN6 ในเนื้อเยื่อของกุ้งกุลาดำ พบยีน *Pm*SERPIN6 แสดงออกมากในอวัยวะน้ำเหลือง เซลล์เม็ดเลือด หัวใจ และเหงือก แต่ไม่พบในตับ จากการศึกษาระดับการแสดงออกของยีน *Pm*SERPIN6 ในการตอบสนองต่อการติดเชื้อจุลชีพก่อโรคในช่วงเวลา 0 - 48 ชั่วโมงหลังการกระตุ้นด้วยเชื้อ โดยใช้เทคนิค RT-PCR พบว่าระดับการแสดงออกของยีน *Pm*SERPIN6 ลดลงเล็กน้อยหลังการฉีดเชื้อไวรัส WSSV แต่ไม่พบการเปลี่ยนแปลงหลังการฉีดเชื้อแบคทีเรีย *Vibrio harveyi* นอกจากนี้ได้ทำการตรวจวัดจำนวนเม็ดเลือดที่ผลิตโปรตีน *Pm*SERPIN6 โดยใช้เทคนิค Immunocytochemistry พบว่าหลังการฉีดเชื้อทั้งไวรัสและแบคทีเรีย จำนวนเม็ดเลือดที่ผลิตโปรตีน *Pm*SERPIN6 เพิ่มขึ้นอย่างชัดเจน แสดงให้เห็นว่าโปรตีน *Pm*SERPIN6 มีการตอบสนองต่อการติดเชื้อจุลชีพก่อโรคในระยะท้าย ในงานวิจั ยนี้ได้ทำการผลิตโปรตีนรีคอมบิแนนท์ *Pm*SERPIN6 (r*Pm*SERPIN6) เพื่อศึกษาแอกทิวิตีในการยับยั้งเอนไซม์โปรติเนส จากการทดลองบ่มโปรติเนสชนิดต่างๆกับ r*Pm*SERPIN6 ที่อัตราส่วนโดยโมลประมาณ 1:400 พบว่าแอกทิวิตีของเอนไซม์ Trypsin, Subtilisin A, Chymotrypsin และ Elastase เหลืออยู่เพียง 13, 13, 39 และ 66 เปอร์เซ็นต์ตามลำดับ แสดงให้เห็นว่า r*Pm*SERPIN6 สามารถยับยั้งการทำงานของเอนไซม์ได้ทั้ง 4 ชนิดที่ระดับต่างๆกัน และเมื่อบ่ม r*Pm*SERPIN6 กับสารสกัดหยาบจากเม็ดเลือด และทดสอบความสามารถ ในการยับยั้งระบบโพรฟีนอลออกซิเดส พบว่า r*Pm*SERPIN6 ไม่ออกฤทธิ์ยับยั้งการกระตุ้นระบบ โพรฟีนอลออกซิเดส ดังนั้น *Pm*SERPIN6 จึงไม่น่าจะมีส่วนเกี่ยวข้องในการควบคุมระบบโพรฟีนอลออกซิเดส

ภาควิชา : ชีวเคมี ลายมือชื่อนิสิต ธีรดา หอมวิเศษ

สาขาวิชา : ชีวเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก กุลยา สมบูรณ์วิวัฒน์

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TEERADA HOMVISES: CLONING AND CHARACTERIZATION OF *Pm*SERPIN6, A SERINE PROTEINASE INHIBITOR, FROM THE BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: KUNLAYA SOMBOONWIWAT, Ph.D., THESIS CO-ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., 140 pp.

Serine proteinase inhibitors (SERPINs or serpins) have been found as a regulator of various biological processes in a diverse range of organisms. In Penaeid shrimp, a few serpins have been identified and their functions have not been clearly characterized. Herein, eight serpin genes, namely *Pm*SERPIN1 - 8, were identified from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/home.jsp). Protein sequence alignment revealed a high similarity of *Pm*SERPIN6 to the serpin-6 from *Manduca sexta* which are an inhibitor in prophenoloxidase (proPO) system, an important defense mechanism in arthropod immunity. Among those, *Pm*SERPIN6 was selected for further characterization. Tissue distribution analysis revealed that *Pm*SERPIN6 transcripts mainly expressed in the lymphoid organ, hemocyte, heart and gill, but not in the hepatopancreas. To investigate the participation of *Pm*SERPIN6 in response to pathogen challenge, semi-quantitative RT-PCR analysis of *Pm*SERPIN6 at 0 - 48 h after pathogen challenge was performed. The *Pm*SERPIN6 transcript expression levels in hemocytes was slightly decreased after systemic white spot syndrome virus (WSSV) injection but remained unchanged upon *Vibrio harveyi* injection. Interestingly, immunocytochemistry showed that the number of *Pm*SERPIN6 producing hemocyte was increased at 72 h after both *V. harveyi*- and WSSV- infection indicating the response of *Pm*SERPIN6 in the late phase of pathogen challenge. Furthermore, the recombinant protein of *Pm*SERPIN6 (r*Pm*SERPIN6) was produced in order to assay for its proteinase inhibitory activity. After incubation of various commercial protinases with r*Pm*SERPIN6 at the mole ratio of about 1:400, the remaining activity of trypsin, subtilisin A, chymotrypsin, and elastase decreased to 13, 13, 39, and 66%, respectively, implying that r*Pm*SERPIN6 could inhibit the activity of all tested proteinases at different strength. *In vitro* assay for inhibition of prophenoloxidase activation revealed that *Pm*SERPIN6 might not involve in regulation of the proPO system.

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LIST OF ABBREVIATIONS

A	absorbance
bp	base pair
CFU	colony forming units
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EST	expressed sequence taq
EtBr	ethidium bromide
h	hour
HLS	hemocyte lysate supernatant
kb	kilobase
kDa	kilodalton
L-DOPA	L-3, 4-dihydroxyphenylalanine
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
ml	millilitre

mM	millimolar
ng	nanogram
nm	nanometer
O.D.	optical density
^{o}C	degree celcius
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAP, PPAE, ppA	prophenoloxidase activating enzyme
PCR	polymerase chain reaction
PO	phenoloxidase
PPAF	prophenoloxidase activating factor
proPO	prophenoloxidase
RNA	ribonucleic acid
RT	reverse transcription
sec	second
WSSV	white spot syndrome virus
YHV	yellow head virus
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I

INTRODUCTION

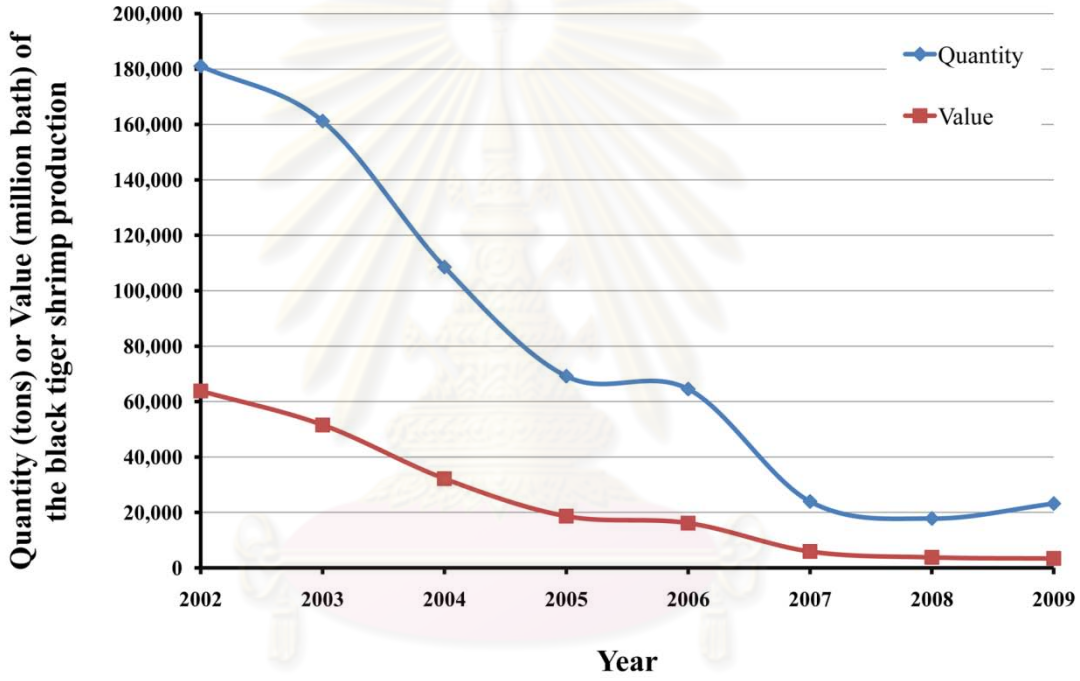
1.1 General introduction

A commercial business shrimp farm which encouraged for human consumption began in the 1970s. The world shrimp production reached more than 1.6 million tonnes and a value of 9 billion U.S. dollars in 2003. About 75% of farmed shrimp is produced in Asia, in particular in China and Thailand (Source: FAO databases, 2007).

Shrimps have been one of the most economically important aquaculture in Thailand for over 20 years. Mostly, shrimp farms in Thailand are spread along the areas of Southern coast such as in the Nakorn Sri Thammarat and Surat Thani provinces. The shrimp farming has been a multi-billion dollar industry as a result shrimps have become a major export product of Thailand. Each year, Thailand supplies about 20 percent of the world shrimp trade with the two major species of the black tiger shrimp *Penaeus monodon*, and the white shrimp *Litopenaeus vannamai*. In the past, Thailand was the world's leading exporter and the largest producer of *P. monodon*.

At the beginning, *P. monodon* culture in Thailand expanded quite slowly than that of Taiwan because of lacking suitable farm practices. After 1985, the shrimp production in Thailand increased rapidly leading to switching of the top shrimp supplier from Taiwan to Thailand. Since 2003, the black tiger shrimp production was significantly declined because of the outbreak of serious viral or bacterial diseases (Figure 1.1). Accordingly, *L. vannamai* has been imported and become a popular cultured species. The major advantages of *L. vannamai* over *P. monodon* are that the domesticated stocks of specific pathogen free and specific pathogen resistant shrimp are currently commercially available. The favorable traits of *L. vannamai* are high growth rate, disease resistant, and rapid maturation. Thus, it gives a higher production yield per crop.

Advantages of *P. monodon* are that it can be grown to a large size (40-60 g) to fetch a higher price in international markets, and it is fast growing shrimp. The black tiger shrimp can tolerate a wide range of salinity, 0.2 to 70 ppt. Salinity within the range of 10 to 25 ppt has no considerable effect on growth when food is sufficient. Shrimp growth is found to be slower at very low salinity. Moreover, it can tolerate to temperature up to at least 37.5 ^{o}C, and the mortalities occur at the temperatures below 12 ^{o}C only. The most preference of *P. monodon* over *L. vannamei* is that it is a native species in Thailand.



Source: Office of Agricultural Economics (www.oae.go.th/oae_report/export_import/export.php)

Figure 1.1 The black tiger shrimp export of Thailand during the year 2002 – 2009.

The statistic record revealed that Thailand's major shrimp product markets are USA and Japan. In 2008-2009, USA and Japan imported Thai's shrimp products for about 49.05% and 20.55% of the total shrimp production, respectively. This inferred a high demand for shrimp consumption. Not only for export, there is also a strong demand on shrimp in the local markets. Therefore, it is necessary to sustain the shrimp farming production. Because *L. vannamai* is the alien species and the broodstock has to be imported, maintaining of the native species *P. monodon* by developing the domesticated broodstock and the farming process, learning how to protect them from the microbial infection, and understanding in shrimp immune system will lead to sustainable shrimp aquaculture in Thailand.

1.2 Taxonomy of *Penaeus monodon*

Penaeid shrimp can be classified into the largest phylum, the Arthropoda, in Kingdom of Animalia, characterized by jointed appendages and an exoskeleton or cuticle that is periodically molted. The major characteristic appearance to the species of *P. monodon* is the black bands over their carapace and abdomen giving a tiger-striped. The taxonomic definition of *P. monodon* is as follows (Baily-Brock and Moss, 1992):

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Subclass Eumalacostraca
Order Decapoda
Suborder Natantia
Infraorder Penaeidea
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1985
Genus Penaeus Fabricius, 1798
Subgenus Penaeus
Species monodon

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

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

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

1.3 Shrimp diseases

Shrimp farming in many countries crashed from the serious problem of pathogen infection (Moriarty, 1999). The infection of wild shrimp has disseminated since contamination of waste from infected aquaculture processing near coast. Although shrimp pathogens show no danger to human health, there is a history of causing economic losses on shrimp farms. Moreover, stressful conditions to shrimp such as poor management practices, poor water and soil quality conditions, and unexpected changes in climatic conditions trigger the disease outbreak in farms. Therefore, appropriate management of farming practices is required for sustainable shrimp aquaculture (Duraiappah et al., 2000).

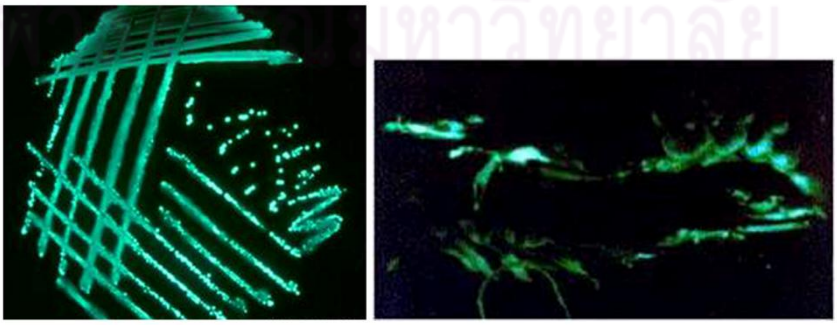
In the past, many chemicals were used to control shrimp diseases such as chlorine compounds, formalin, providone-iodine, quarternary ammonia compounds, furazolidone, malachite green, glutaraldehyde, potassium permanganate, peroxides, and copper sulfate (Boyd, 2002). The large amounts of biocides treatment in shrimp farms were toxic to the environment, workers and consumers. Now, consumers have a strong concern on food qualities and safety because these affect human health. Importer countries have banned chemically contaminated shrimp especially of antibiotics, pesticides and other harmful chemical residues.

Many viral diseases generally cause serious problems in every aquaculture leading to severe economic losses (Meyer, 1991). The viral outbreaks often result from several stress factors such as overcrowding, unsuitable culture temperature, and low dissolved oxygen. The outbreaks of bacterial diseases also result from extreme environmental stresses and cause acute problems (Pullin et al., 1992).

1.3.1 Bacterial diseases

Vibriosis is a shrimp disease caused by infection of bacteria belonging to the *Vibrio* species. Vibriosis occurs in the culture of marine shrimp in salt or brackish water and is a cause of shrimp mortality.

Vibrio is a genus of gram-negative, motile, bioluminescent bacteria with morphology of rod shape, 0.5-0.8 μm in width and 1.4-2.6 μm in length. *Vibrio* is anaerobic bacteria usually found in seawater and do not form spores. There are several pathogenic strains including *Vibrio harveyi*, *Vibrio splendidus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio vulnificus*, *Vibrio campbelli*, *Vibrio fischeri*, *Vibrio damsella*, *Vibrio pelagicus*, *Vibrio orientalis*, *Vibrio ordalii*, *Vibrio mediterrani*, and *Vibrio logei* (Rao, 2002). The major shrimp pathogenic species is *V. harveyi*. Among the *V. harveyi* isolates, some are virulent and some are not, suggesting a great deal of genetic variation in this species. The *Vibrio* spp. infection in shrimp can occur at all life stages, but mostly in hatcheries. The clinical signs of this disease in adult shrimp are appear hypoxic, show reddening of the body with red to brown gills, reduce feeding and may be observed swimming lethargically at the edges and surface of ponds. The bacteria are able to emit a blue-green color light by luciferase catalysis reaction. The luminescence can be visible on the head and pectoral part of infected shrimp (Figure 1.2). *Vibrio* infection alone may cause shrimp mortality for up to 95%. It was also evidenced that shrimps co-infected with *Vibrio* and white spot syndrome virus has faster and higher mortality rates than shrimp infected with *Vibrio* alone (Phuoc et al., 2008).



Source: www.thailandshrimp.com

Figure 1.2 Luminescence of *Vibrio* on selective medium plate and infected shrimp.

1.3.2 Viral diseases

One of the most important problems of cultured penaeid shrimp causing seriously losses in the world production including Thailand is the pandemic viral diseases. Eight types of virus including Baculovirus penaei (BP), *P. monodon*-type baculovirus (MBV), type C baculovirus of *P. monodon* (TCBV), hemocyte-infecting baculovirus of *P. monodon* and *P. esculentus* (HB), infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvo-like virus (HPV), lymphoidal parvo-like virus (LOPV), and type III Reo virus (REO-III) were reported to be effected to the cultivated *P. monodon* (For review: Lightner, 1992). The two major concerns to *P. monodon* aquaculture are white spot syndrome virus (WSSV) and yellow-head virus (YHV).

1.3.2.1 White spot syndrome (WSS)

White spot syndrome was firstly found in the Northeast Asia in 1992-1993, and rapidly spread to many shrimp farming countries in Asia and Indo-Pacific (Stokstad, 2010).

WSSV is a bacilliform, non-occluded enveloped virus with 210 - 380 nm in length and 70-167 nm in width of intact enveloped virions. More than 40 proteins have been characterized from this virus. Some non-structural proteins are involved in transcriptional regulation such as VP9 and in DNA replication regulation such as WSSV477. Other proteins which are the structural have been located in the virion or nucleocapsid (Escobedo-Bonilla et al., 2008). WSSV genome is a circular, double-stranded DNA molecule with about 292,967 bp in length (van Hulten et al., 2001).

Because white spot syndrome is highly lethal and quickly contagious in shrimp, it is the major causative of the severe disease outbreaks in many countries. The disease is from the infection of virus in the family of related to white spot syndrome virus (WSSV), a viral pathogen of crustaceans. It can infect both freshwater and marine species such as shrimp, crab, and crayfish. The infection of WSSV has been observed in several commercial penaeid species, including *P. monodon*, *L.*

vannamei, *Marsupenaeus japonicas*, and *Fenneropenaeus chinensis*. WSSV is now recognized as the most serious for shrimp aquaculture worldwide (Escobedo-Bonilla et al., 2008).

WSSV is highly virulent leading to 100% shrimp mortality within a few days after infection. The clinical signs of WSS are rapid reduction in food consumption, lethargy, reddish discoloration of their cuticle, and appearing of white spot of 0.5-2 mm diameter on the inside of their carapace and cuticle over the abdominal segments (Afsharnasab et al., 2009). The exact mechanism of white spot formation is not known. It is possible that a WSSV infection may induce the dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle and giving rise to white spots (Escobedo-Bonilla et al., 2008). Histopathological evidence has shown that WSSV are able to infect a wide variety of host cells from epidermal and mesodermal origin only. Because WSSV can transmit between the crustaceans, this virus can speedily spread along the culture within a day.

According to experimental data on feeding shrimp with WSSV-infected tissues as determined by *in situ* hybridization (ISH), the primary sites of WSSV replication in early juvenile *P. monodon* are the subcuticular epithelial cells of the stomach and cells in the gills, in the integument and in connective tissue of the hepatopancreas (Escobedo-Bonilla et al., 2008).

Source: National Institute of Animal Health
(http://www.dld.go.th/niah/AnimalDisease/aquatic_WhiteSpot.htm)

Figure 1.3 White spot appearing after WSSV infection on shrimp carapace.

1.3.2.2 Yellow-head disease (YHD)

Yellow-head disease is a viral infectious disease occurring in a number of crustaceans, particularly in *P. monodon*. Like white spot syndrome, this disease causes the massive losses in shrimp production in many areas. The first occurrence of this disease was in *P. monodon* ponds in Eastern Thailand in 1990 (Limsuwan, 1991).

The pathogenic virus of YHD is yellow head virus (YHV) which is a positive-sense single-stranded RNA virus (Ongvarrasopone et al., 2008). The yellow head virus particle possesses a bacilliform morphology consisting of structural units, the nucleocapsid, and envelope (Boonyaratpalin et al., 1993). The yellow head virus is classified in the virus family *Roniviridae* (Order *Nidovirales*) and in the genus *Okavirus* (Walker et al., 2005). Major clinical signs of the disease are high feed consumption in early phase of infection, turning yellow of shrimp cephalothorax with yellowish hepatopancreas, and then increasing of shrimp mortality reaching 100% within 3-5 days (Figure 1.4). The primary mechanism of spread of YHV in pond culture appears to be from infected crustacean carriers which appear to have latent infections, for example; *P. merguiensis*, *Metapenaeus ensis*, and *Palaemon styliferus* (Briggs et al., 2004).

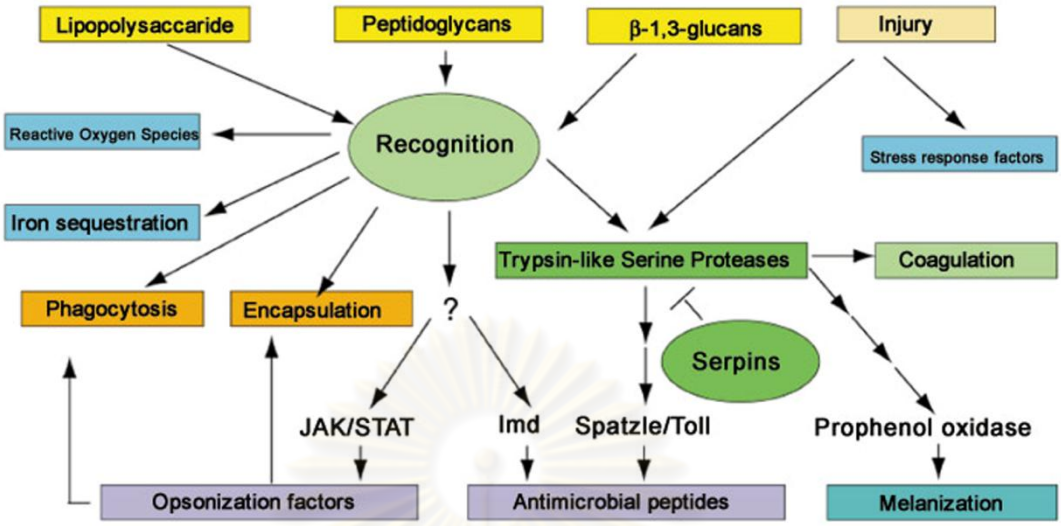
Source: www.sc.mahidol.ac.th/tha/award/ttsf43.htm

Figure 1.4 Yellow head of shrimp infected with yellow head virus comparing to the normal.

1.4 Invertebrate immunity

All living organisms are continuously exposed to the harmful substances, and most of them can protect themselves by more than one way. The first shield of the protection is a body barrier such as their exoskeleton, which prevents the body from pathogen infection, and mucous which can trap some particles or microorganisms. For the second shield of protection, the common immune system can be classified into two groups, innate and adaptive (acquired) immunity. While vertebrates possess both systems, the invertebrates have only one system, innate immunity. The key hallmark of acquired immune system is the ability to generate the recognition molecules which is specific to the unique antigen, therefore, this immune system is said to have both specificity and memory, capability to rapidly respond in case of re-exposure of an antigen.

The innate immunity composes of several mechanisms which defend the host from infection by other organisms via non-specific manner. The cells in innate immune system have pattern recognition molecules immediately responding to foreign substances. The innate immune responses are thought to be an evolutionary older defense strategy and the dominating system in many organisms. Figure 1.5 shows the overview of the immune system of the *Drosophila* by detection of microbial pathogens by recognition proteins and activation of a large array of interconnected and synergetic host defense mechanisms.



Source: CGM - Department Cellular Dynamics and Development

Figure 1.5 Schematic overview of the innate immune system of the *Drosophila*.

1.5 The crustacean immune response

Like most other arthropods, crustaceans have an innate defense system which is considered to be non-specific mechanism. The innate response is mediated by pattern recognition receptors which recognize conserved components of microbes or the signal sending out from injuries or damages.

This defense can be divided into the large groups of humoral and cellular mediated responses (Jiravanichpaisal et al., 2006). Hemocytes play important roles in immunity by mediating cellular defenses and synthesizing humoral effectors which are stimulatory signals regulating several immune cascades. Cell-mediated immunity actions in; for example wound repair, blood coagulation, phagocytosis, nodule formation, encapsulation, and cytotoxicity system. The humoral immunity refers to the production of soluble component playing roles in the defense system. These soluble substances, proteins and enzymes, are considered to be associated with immune reactions such as prophenoloxidase (proPO), antimicrobial action and clotting system.

1.5.1 Cellular response

The cellular response is the cell-mediated reaction against pathogen invasion including phagocytosis, encapsulation, and nodule formation. Circulating hemocytes are important effector cells in crustacean immunity and can be grouped into three types, hyaline, semigranular, and granular cells. Each cell type involves in different immune reactions.

Phagocytosis is the cellular process of engulfing solid particles, closing up, and pinching of the membrane to be an internal phagosome which internalizes the solid particles such as bacteria (Jeon et al., 2010). It is a major mechanism of removing the pathogens and cell debris. The mechanism of phagocytosis includes three stages that are recognition the microbes with lectins, ingestion, and killing (For review: Sharon and Lis, 2004).

Cellular encapsulation is an immune response, occurs when a microbe is too large to phagocytose (Gillespie et al., 1997). It protects host by attracting the host hemocytes to a foreign invader and aggregating on its surface. The microbes in capsule occurring from cell encapsulation process are harmless. This reaction occurs against a wide range of pathogens and parasites resulting in the formation of multiple layers of dead hemocytes.

When the number of invader is high, nodule formation occurs from cellular aggregation involving in cell-cell co-operation. The forming nodule includes central core of entrapped foreign particles. The nodule trapped microorganisms will be killed by physiological stresses such as oxygen and nutrients deficiences.

1.5.2 Pattern recognition proteins

Pattern recognition proteins (PRPs) are proteins which can recognize the specific non-self or altered-self molecular patterns. When the host was invaded by foreign organisms or substances, the first line of response is recognition of factors that present at surface of invader. Previously, there were many reports on recognition protein identification in crustacean (For review: Vazquez et al., 2009). The target

molecular patterns varying among groups of pathogens include the lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acids (LTA) of the Gram-positive bacteria, the β-1,3-glucan of fungi, and double-stranded RNA of virus. Moreover, carbohydrate recognition molecules such as peptidoglycan recognition proteins are important because carbohydrate molecules are a common composition of microbial cell wall. In the black tiger shrimp, glucan binding proteins (GBP) were identified and characterized for their functions (Sritunyalucksana et al., 2002).

1.5.3 Prophenoloxidase (proPO) system

Prophenoloxidase system or melanisation is an important immune response in many invertebrates involving in the activation of many proteinases in the cascade to produce a final product of melanin. It is evidenced that the melanisation cascade is stimulated by the appearance of the stimuli factors which can also affect the cellular responses such as phagocytosis and encapsulation reactions.

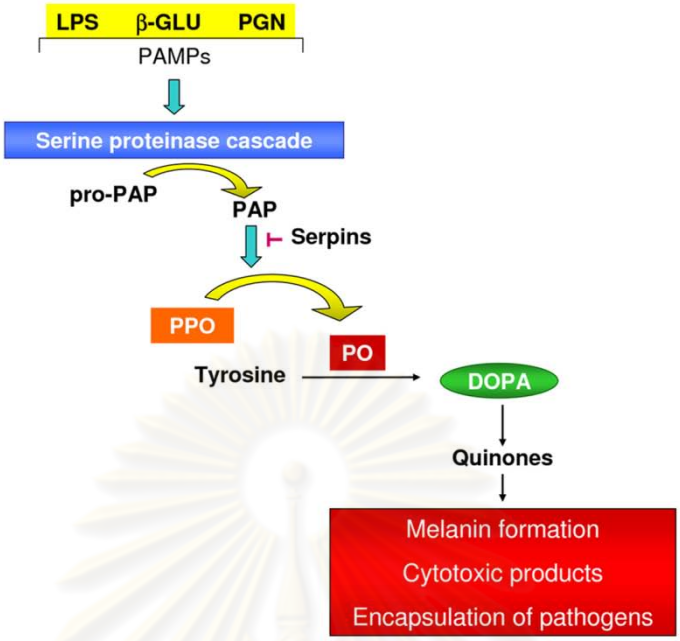
In vitro studies have been shown that the phenoloxidase (PO) exists as a zymogen, prophenoloxidase (proPO), which can be activated by a cascade of several proteinases especially serine proteinases. The serine proteinase, an activator of proPO system, can be stimulated by many microbial components such as lipopolysaccharide or peptidoglycan and β-1,3-glucans via the pattern recognition proteins.

The enzymes that are able to activate the proPO are termed prophenoloxidase activating enzyme or factor such as ppA, PPAE, and PPAF. In crayfish, ppA is a trypsin-like proteinase and presents as an inactive form in the hemocyte granules (Aspán and Söderhäll, 1991; Wang et al., 2001). The ppA enzyme is released with proPO and become an active form after degranulation in the presence of microbial elicitors. The active ppA is used in the conversion process of proPO to an active phenoloxidase, a key enzyme in melanin synthesis. Phenoloxidase catalyzes the conversion of *O*-hydroxylation of monophenols to diphenols and converts oxidized diphenols to quinones, which can non-enzymatically polymerise to form melanin.

Occurance of melanisation can be observed by blackening of the parasite in the hemolymph of host or black spot on the cuticle especially at the wound site. The production of insoluble melanin deposits involving in the process of wound healing and encapsulation of foreign substances which can effect to inhibit growth of microbial parasites. To prevent an excessive of melanisation, it is necessary to have the inhibitors to regulate the proteinase activities (Kanost, 1999).

In penaeid shrimp, enzymes involving in proPO system are identified in semigranular and granular cells. In the black tiger shrimp *P. monodon*, two genes of clip domain serine proteinase which are the member of proPO system designated as *Pm*PPAE1 and *Pm*PPAE2 were identified from shrimp hemocyte (Charoensapsri et al., 2009; Charoensapsri et al., 2011). The functions of *Pm*PPAE1 and *Pm*PPAE2 were investigated using double stranded RNA (dsRNA)-mediated RNA interference technique. From the result, they concluded that both *Pm*PPAE1 and 2 are the proteinases participating in the shrimp proPO system. For phenoloxidase, they also found that the proPO mRNA of *P. monodon* is expressed only in the shrimp hemocytes. Currently, two phenoloxidases (*Pm*proPO1 and *Pm*proPO2) have been identified (Amparyup et al., 2009). The previous study reported that both are important for shrimp in fighting against bacterial infection.

Because the toxicity of quinone produced in the melanisation system harms the animal, an inhibitor is required for regulation of this process. In crustacean, one of the inhibitor, called melanisation inhibition protein (MIP) was firstly found in crayfish and classified as an inhibitor specific to directly control proPO system. *Pl*MIP is an inhibitor identified from the hemolymph of crayfish *P. leniusculus* and functions to inhibit quinones production and also affect the proPO activating enzyme activity (Söderhäll et al., 2009). In *P. monodon*, *Pm*MIP protein was also highly detected in shrimp hemolymph and found that the protein was degraded or removed after microbial infection or proPO activation to allow the melanisation to occur (Angthong et al., 2010).



Source: Garcia E S, Castro D P, Figueiredo M B, Genta F A, Azambuja P. (2009). "*Trypanosoma rangeli*: a new perspective for studying the modulation of immune reactions of *Rhodnius prolixus*". *Parasit Vectors* 2(1):33.

Figure 1.6 Overview of the prophenoloxidase (proPO) system.

1.5.4 Antimicrobial peptides (AMPs)

The evolutionary conserved components of the innate immunity called antimicrobial peptides or AMPs have been found in all classes of life. Most of AMPs are small in size, about less than 150–200 amino acid residues with amphipathic structure and cationic property, whist a little of anionic peptides are also exist. Antimicrobial peptides have been demonstrated to kill a large spectrum of microorganisms such a Gram-negative and Gram-positive bacteria including conventional antibiotics resistance strains, mycobacteria, enveloped viruses, fungi, and even transformed or cancerous cells. Other than direct killing of the pathogen, AMPs also have ability to enhance an immunity of host by functioning as an immune-modulator.

Because of its small size, it is easy to be synthesized and rapidly diffuses to the point of infection. The action of AMPs to kill bacteria includes disrupting membranes by attaching and inserting to form pores and interfering with metabolism

by binding to intracellular molecules which are crucial to cell living. The initial contact between the peptides and target organisms would be by electrostatic force, as most bacterial surface is anionic. For mechanism of peptide to break down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the organism by rupturing the membrane and allowing the leakage of the certain cellular components.

There are many reports on antimicrobial peptides in penaeid shrimp (For review: Tassanakajon et al., 2010). Penaeidins, a family of antimicrobial peptides acting against Gram-positive bacteria and fungi, have been identified in several penaeid shrimp. Several isoforms of crustins have been also identified in several species. The crustins from shrimp showed no homology with other known antibacterial peptides, but possessed sequence identity with an inhibitory protein family that is the whey acidic protein (WAP).

Another major group of AMPs found in penaeid shrimp is anti-lipopolysacccharide factors or ALFs. Several isoforms of ALF (ALF*Pm*) have been identified from the *P. monodon* EST database (Tassanakajon et al., 2006). Previous researches implied that ALF*Pm*s was important in shrimp immune defense against bacterial and viral infections.

Peptides derived from hemocyanin of *L. vannamei*, *P. stylitostris*, and *P. monodon* possessing an antifungal (Destoumieux-Garzon et al., 2001) or antiviral activity (Zhang et al., 2004) has been also identified. According to Gram-positive bacterial growth inhibitory activity, histones and histone derived peptides of *L. vannamei* have been recently reported as innate immune effectors (Patat et al., 2004).

1.5.5 Clotting / Coagulation system

In general, coagulation in crustacean is an essential complex process by which blood forms clots and a damaged blood vessel wall is covered by a fibrin-containing clot to stop bleeding. The hemolymph coagulation response is for preventing host from loss of hemolymph through injury vessel, and dissemination of bacteria

throughout the body (Martin et al., 1991). It is a proteolytic cascade containing a series of reactions which are activated by microbial cell wall components leading to fibrin formation.

The process of coagulation in horseshoe crab is regulated by a proteolytic cascade activated by the elicitors through specific recognition proteins (Muta et al., 1993). In the past, this cascade system has been investigated composing of three serine proteinase zymogens and one clottable protein or coagulogen. In the presence of an elicitor, one of the zymogen proteinase is autocatalytically activated. The active proteinase changed another zymogen to the active form by limited proteolysis. Then, the proclotting enzyme is activated to be clotting enzyme, catalyzing the transformation of coagulogen to insoluble coagulum gel covering at the open circulatory blood system or trapping the invading microorganism in plasma.

Coagulation in crayfish is based on the transglutaminase-mediated crosslinking of a specific plasma clotting protein (Hall et al., 1999). The crayfish clotting protein (CP) is a very high density lipoprotein (VHDL) consisting of two identical 210 kDa subunits linking by disulfide bonds. Each of CP subunit contains both lysine and glutamine side chains, which are recognized and become covalently linked to each other by transglutaminases (TGases). Clotting process is induced when TGase is released from hemocyte or tissue and become activated by Ca^{2+} leading to large plasma CP molecules aggregates by crosslinking.

Like crayfish, coagulation in other crustaceans such as lobster and shrimp is based on crosslinking of the clottable protein by transglutaminase. The clottable proteins from the spiny lobster *Panulirus interruptus* have been identified to be a homodimeric lipoglycoproteins of about 400 kDa (Fuller and Doolittle, 1971). In *P. monodon*, a clottable protein was identified, purified, and characterized. In the presence of Ca^{2+} and transglutaminase in hemocyte lysate, the clottable protein with molecular mass of 380 kDa formed stable clots (Yeh et al., 1998).

1.6 Serine proteinase

Serine proteinase family hydrolyzes the peptide bond of substrate via a nucleophillic serine residue at the active site, Ser, arranged with Asp and His into a catalytic triad (Hedstrom, 2002). Serine proteinases are widely distributed in nature and are found in all kingdoms including viral genome. Proteinases play crucial roles in various biological processes not only in food digestion but also in metamorphosis, wound healing, innate immunity, and in the pathogenesis of the microorganisms.

Initiation of proteinase activity is controlled by the activation of the zymogen triggering serine proteinase cascades leading to amplification of other reactions. Beneficial functions of the proteinase reactions in organisms are tremendous, but deadly if unmanaged. Several of the proteinase processes are tightly controlled by the proteinase inhibitors to prevent excessive activations that are detrimental to the cells or organisms (Kanost, 1999).

1.7 Serine proteinase inhibitor

A number of proteinase inhibitors with difference specificity function in regulation of proteolysis. Hemolymph of insects and other arthropods contains a number of serine proteinases, and also proteinase inhibitors from several protein families. In arthodpods, the serine proteinase inhibitors have been identified depending on their 3D structure and function such as families of Kazal, Kunitz, α-macroglobulin, Serpins, and pacifastin (Liang et al., 1997).

1.7.1 Small size proteinase inhibitors

Small proteinase inhibitors with low molecular weight can be classified into three families including Kazal, Kunitz, and pacifastin.

Proteinase inhibitors from the Kunitz family have been characterized from the horseshoe crap, lepidopteran, and dipteran. The proteins in this family are single

domain with approximately 60 amino acid residues and are the inhibitor of trypsin and chymotrypsin (Kanost, 1999). Kunitz-type domains are common functional elements found in extracellular protein. Kunitz domains appear not only as a single-domain such as bovine pancreatic trypsin inhibitor (BPTI), but also as multiple tandem repeats. Crystal structure of trypsin-BPTI complex shows that the Kunitz domain inserts a protruding reactive site loop into the active cleft of its cognate proteinase (Macedo-Ribeiro et al., 2008).

The pacifastin family is classified into the low molecular mass proteinase inhibitor with 35-36 amino acid residues in length. The rigid protein structure is conserved with six cysteine residues involving in three disulfide bridges (Simonet et al., 2002). The pacifastin-type proteinase inhibitor was found to be a regulator in biological process. For example, pacifastin from the crayfish *Pacifastacus lunousculus* acts as an inhibitor in the endogenous proPO-activating proteolytic cascade (Hergenhahn et al., 1987; Liang et al., 1997).

Kazal-type serine proteinase inhibitors or KPIs in invertebrate can be a single or multiple domain protein which are linked by peptide spacers. A feature of this protein family is that it is composed of about 40-60 amino acid residues including some spacer amino acids. The Kazal motif has a general amino acid sequence of C_1-X_a-C_2-X_b-PVC_3G-X_c-Y-X_d-C_4-X_e-C_5-X_f-C_6 where the subscripts *a*, *b*, *c*, *d*, *e* and *f* are integral numbers of amino acid residues. Six conserved cysteine residues in a Kazal domain forms three intra-domain disulfide bridges between C_1-C_5, C_2-C_4, and C_3-C_6 resulting in characteristic three-dimensional structure. One of the extended out loop peptide segment providing for easy access of the active proteinase called the reactive site loop contains the proteinase-specificity determination P_1 residue and the scissile peptide bond. The inhibitory domain of KPI inhibits the proteinase by a standard mechanism. The reactive site loop of each domain acting as a substrate analogue binds to the active site of the proteinase by competitive inhibition manner forming a relatively stable enzyme-inhibitor complex (For review: Rimphanitchayakit and Tassanakajon, 2010). The KPIs are believed to play a role as a regulator of the serine proteinases in several biological processes. In invertebrates, the KPIs are implied their potential roles in immune system from the up-regulation in responses to microbial challenges but the actual functions of this protein need further determination.

1.7.2 α-Macroglobulin

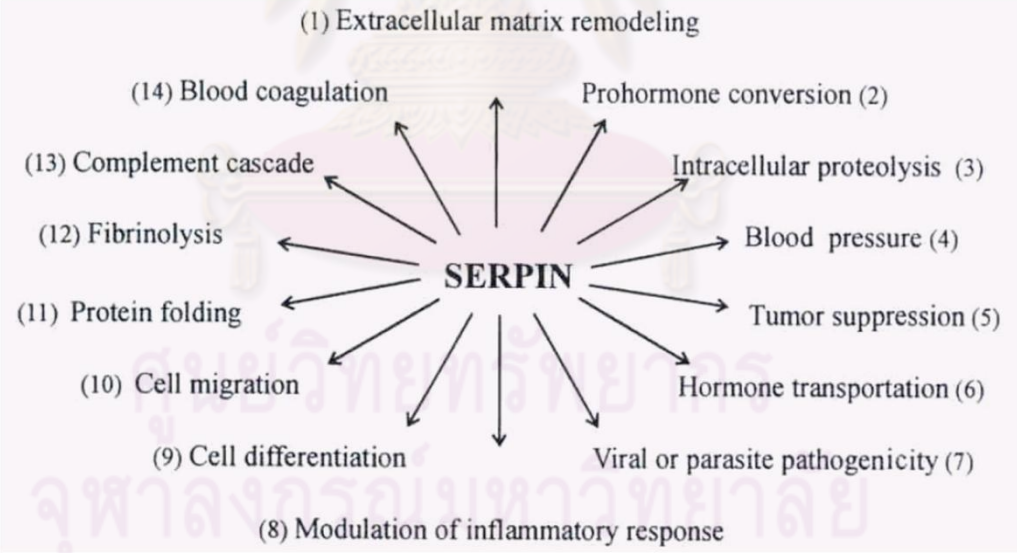
α-Macroglobulins (αMs) are a family of proteinase inhibitors whose size is much larger than the small size proteinase inhibitors described above. They have been identified in a wide range of vertebrate and invertebrate species. Most of them are thiol ester region containing proteinase inhibitors. Each subunit of this protein family contains an exposed bait region that is susceptible for proteolytic cleavage. Clevage of the protein inhihibitor bait region leads to a conformational change that traps the proteinase into inhibitor cavity by forming α-macroglubulin dimer. The conformational change also leads to covalent crosslink between proteinase and α-macroglobulin (For review: Sottrup-Jensen, 1989; Kanost, 1999).

In crustacenas, alpha-2-macroglobulin (A2M) genes have been characterized in many organisms such as horseshoe crab *Limulus* sp. (Iwaki et al., 1996), sea scallop *Chlamys farreri* (Ma et al., 2005) and the crab *Scylla serrata* (Vaseeharan et al., 2007). In shrimp, A2Ms have also been cloned and characterized from several species including, *M. japonicuss* (Rattanachai et al., 2004), *P. monodon* (Lin et al., 2007), and *L. vannamei* (Lin et al., 2008) and *Fenneropenaeus chinensis* (Ma et al., 2010). In *P. monodon* and *M. japonicus*, it has been found that the A2M mRNA levels showed significant increase after peptidoglycan challenge. Three forms of A2M reported in *F. chinensis*. They have different pattern of reponses upon pathogen challenge. The FcA2M-1 mRNA level observed in both hemocytes and lymphoid organ after WSSV or *Vibrio* challenge was increased. Whereas, those of FcA2M-2 showed up-regulation in lymphoid organ but not in hemocytes.

1.7.3 Serpin-type serine proteinase inhibitor

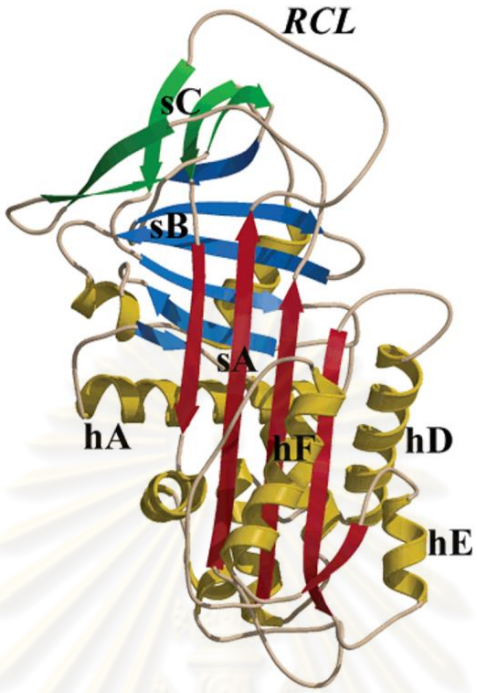
Serpins were found in most organisms except fungi with molecular weigth of 40-60 kDa and about 400 amino acid residues in length. Serpin is primarily found to be an irreversible inhibitor acting as substrate of its target proteinase. From previous reports, serpin functions as a regulator of various biological processes (Figure 1.7) (Potempa et al., 1994). While many serpins have an inhibitory activity, they have some serpins without inhibitory activity called non-inhibitory serpins (Gettins, 2002).

3D structure of serpins is conserved with divergent in amino acid sequences consisting of 3 beta-sheets and 8-9 alpha-helices in its core domain (Figure 1.8). A typical feature of serpin is the reactive center loop or RCL which is an exposed protein motif of about 20 amino acids, located near its C-terminus. This motif contains a scissile bond between residues called P_1 and P_1', which is cleaved by the target proteinase (Wilczynska et al., 1995). Serpin with proteinase inhibitory activity always show the conserved hinge region at the position of P_{17} to P_8 in RCL region (P_{17}:E, P_{16}:E/K/R, P_{15}:G, P_{14}:T/S, P_{12}-P_9:A/G/S, and P_8:T). The mechanism of proteinase inhibition like a suicide substrate of serpin is the formation of serpin-proteinase complex upon cleavage of serpin by its target proteinase which brings about the large conformational change of serpin (Stratikos and Gettins, 1999). The very stable serpin-proteinase complex formed results in the inactivation of the proteinase activity. Specificity of serpin to the target proteinase depends mainly on the amino acid at the P_1 residue such as alanine (A) is specific to elastase, phenylalanine (F) to chymotrypsin, and lysine (K) or arginine (R) to trypsin.



Source: Potempa J, Korzus E, Travis J. (1994). "The serpin superfamily of proteinase inhibitors: structure, function, and regulation". *J Biol Chem* 269(23):15957-15960.

Figure 1.7 Overview of serpin function in biological processes.

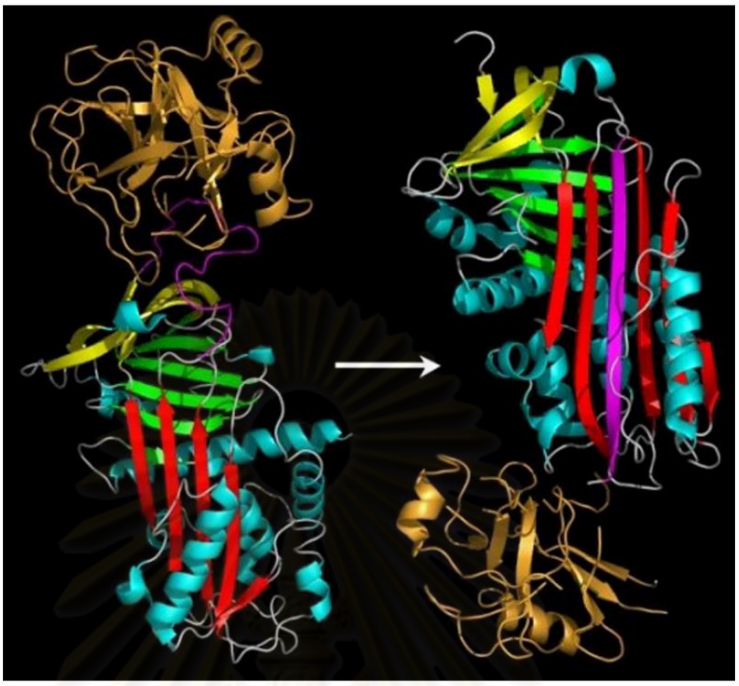


Source: Gettins P G. (2002). "Serpin structure, mechanism, and function". *Chem Rev* 102(12):4751-4804.

Figure 1.8 Three-dimensional structure of serpin-type serine proteinase inhibitor.

To date, several serpins in invertebrates have been extensively studied for their functions. In *Drosophila melanogaster*, several serpins have been identified as a regulator of different immune reactions (Garrett et al., 2009). Serpin-28D regulates hemolymph phenoloxidase (PO) involved in proPO system, a melanisation process (Scherfer et al., 2008). An extensive melanisation in tissue exposed to air of spn28D-deficient fly has been reported. Serpin-5 is involved in development of fruit fly by forming a stable 67 kDa complex with proteinase resulting in wing unfolding and expansion (Charron et al., 2008). Serpin-27A functions as a negative inhibitor of proPO activation by inhibition of proPO activating enzyme (PPAE) (Nappi et al., 2005). For tobacco hornworm *Manduca sexta*, there have been many serpins reported so far. Serpin-3 has been identifed from hemolymph with a low concentration and it was increased after microbial challenge. Serpin-3 regulates proPO system by inhibiting proPO activating proteinases (PAPs) (Zhu et al., 2003). Serpin-4 and -5 control proPO system by inhibiting the proteinases upstream to PAPs (Tong and Kanost, 2005). Serpin-6 has been identified as complexes with PAP-3 and hemolymph proteinase 8 (HP8) implying the involvement in defense mechanism (Zou and Jiang, 2005).

In shrimp, very few serpins have been reported. In 2006, a serpin called *Pm*SERPINB3 from *P. monodon* has been found to be up-regulated after bacterial infection (Somboonwiwat et al., 2006). In 2009, a serpin from Chinese shrimp, *F. chinensis*, (*Fc*-serpin) has been discovered (Liu et al., 2009). *Fc*-serpin gene expression profile showed differences after bacterial and viral stimulation. However, so far, there is no report on characterization of other serpins in shrimp. Also, as found in other organisms that several serpins exist and play role in many biological processes, therefore, study of serpin in shrimp is still needed.



Source: www.wikidoc.org/index.php/Serpin

Figure 1.9 Mechanism of inhibition of serpin molecule.

1.8 Objectives of this thesis

This research aimed to identify new shrimp serpins and to study the expression of an interesting serpin from *P. monodon* in response to pathogen infection. All serpins from the *Penaeus monodon* EST datbase were identified, and an intersting serpin named *Pm*SERPIN6 was further analyzed. Expression analysis of the *Pm*SERPIN6 gene and protein after viral or bacterial infection was carried out using RT-PCR and immunocytochemistry techniques, respectively. Moreover, distribution of *Pm*SERPIN6 transcript in shrimp tissues was determined. To study function of *Pm*SERPIN6, the recombinant protein was produced, purified, and determined for its inhibiting effect on proteinase and proPO.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments and Chemicals

2.1.1 Equipments

Amicon Ultra-15 50K concentrators (Millipore)

Autoclave Model # LS-2D (Rexall Industries Co. Ltd., Taiwan)

Automatic micropipettes P2, P10, P100, P200 and P1000 (LioPette / Select BioProduct)

Balance Satorius 1702 (Satorius)

-20^{o}C Freezer (Whirlpool)

-80^{o}C Freezer (ThermoForma)

Gel Documention System (GeneCam FLEX1, SynGene)

Gene Pulser (Bio-RAD)

Incubator 37^{o}C (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets Class II Model NU-440-400E (NuAire, Inc., USA)

Microcentrifuge tubes 0.6 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Microtiter plate reader (BMG Labtech)

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

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

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

PD-10 column (GE Healthcare)

pH meter model # SA720 (Orion)

Pipette tips 10, 20, 100 and 1000 µl (Axygen® Scientific, USA)

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

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Touch mixer Model # 232 (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

Whatman® 3 MM Chromatography paper (Whatman International Ltd., England)

96-well plate (Bio-RAD)

2.1.2 Chemicals and reagents

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

2-Mercaptoethanol, C_2H_6OS (Fluka)

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

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

Absolute ethanol, C_2H_5OH (BDH)

Absolute methanol, CH_3OH (Scharlau)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide (Plus one)

Adenosine-5'-triphosphate potassium salt (ATP) (Sigma)

Agarose (Sekem)

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

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

Amplicillin (BioBasic)

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (MERCK)

Bovine serum albumin (Fluka)

Bromophenol blue (MERCK)

Calcium chloride ($CaCl_2$) (MERCK)

Chloramphenicol (Sigma)

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)

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

Dithiothreitol (Pharmacia)

Ethidium bromide (Sigma)

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

GeneRuler™ 100bp DNA ladder (Fermentas)

GeneRuler™ 1kb DNA ladder (Fermentas)

Glycerol, $C_3H_8O_3$ (Scharlau)

Glycine, NH_2CH_2COOH (Scharlau)

Hydrochloric acid (HCl) (MERCK)

Imidazole (Fluka)

Isoamylalcohol (Merck)

Isopropanol, C_3H_7OH (MERCK)

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

Laminarin (Sigma)

Magnesium chloride ($MgCl_2$) (MERCK)

Methanol, CH_3OH (MERCK)

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

N, N', methylenebisacrylamide (Fluka)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Nytrans® super charge nylon membrane (Schleicher&Schuell)

Paraformaldehyde (Sigma)

Phenol, saturated (MERCK)

Prestained protein molecular weight marker (Fermentas)

RNase A (Sigma)

Skim milk powder (Mission)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium cacodylate trihydrate (CAC), $(CH_3)_2AsO_2Na \cdot 3H2O$, (Sigma)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium chloride, NaCl (BDH)

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

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

Sodium hydroxide, NaOH (Eka Nobel)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

Triton® X-100 (MERCK)

TriReagent® (Molecular Research Center)

Tween™-20 (Flula)

Unstained protein molecular weight marker (Fermentas)

Urea (Fluka, Switzerland)

Xylene cyanol FF, C25H27N2O6S2Na (Sigma)

2.1.3 Kits

RevertAID™ first strand cDNA synthesis kit (Fermentas)

T&A cloning vector kit (RBC Bioscience)

NucleoSpin® Extract II kit (Macherey-Nagel)

2-D Quant kit (GE healthcare)

QIAprep spin miniprep kit (Qiagen)

2.1.4 Proteinases and Substrates

Trypsin (Sigma)

Subtilisin A (Sigma)

Chymotrypsin (Sigma)

Elastase (USBiology)

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)

2.1.5 Bacterial strain

Escherichia coli strain XL-1-Blue

E. coli strain BL21(DE3)

E. coli strain Rosetta(De3)pLysS

Vibrio harveyi strain 639

2.1.6 Software

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

ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

ClustalX (Thompson, 1997)

ExPASy ProtParam (http://au.expasy.org/tools/protparam.html)

GENETYX version 7.0 program (Software Development Inc.)

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

Penaeus monodon EST database (http://pmonodon.biotec.or.th/home.jsp)

PHYLIP (Felsenstein, 1993)

SECentral (Scientific & Educational Software)

SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/)

2.1.7 Vector

pET-22b(+) (Novagen)

pET-32a(+)(Novagen)

pVR500

T&A cloning vector (RBC Bioscience)

2.2 Data mining of serpin sequences from *P. monodon* EST database

The *P. monodon* EST database (http://pmonodon.biotec.or.th/home.jsp) was searched for nucleotide sequence clusters corresponding to the serpin genes. The appropriate representative clones from the contigs or singletons were re-sequenced to confirm the correctness of sequence information. The BLASTx program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the sequences against the GenBank database (Altschul and Lipman, 1990). The open reading frames (ORFs) and the deduced amino acid sequences were predicted using the Genetyx program. The online ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to compare the amino acid sequences among the serpins (Larkin et al., 2007). The signal sequences were predicted using the online SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004).

2.3 Phylogenetic analysis

The amino acid sequences of mature proteins of *Pm*SERPIN6-8, *Pm*SERPINB3 and selected serpins from other invertebrate organisms reported in the GenBank database (Table B1, Appendix B) were aligned using ClustalX program (Chenna et al., 2003). Phylogenetic analysis was performed using Phylip (Felsenstein, 1993) and Treeview program.

2.4 Animals

The black tiger shrimp, *P. monodon*, each weighing of about 15 - 20 grams were purchased from a local shrimp farm at Suratthani province, Thailand. Shrimps were acclimatized in the laboratory aquaria at a temperature of 28±4 °C and at the salinity of about 15 ppt for at least 1 week before used in each experiment.

2.5 Pathogenic challenged shrimp

2.5.1 Preparation of *V. harveyi* strain 639 and WSSV for injection

2.5.1.1 *V. harveyi* strain 639

V. harveyi strain 639, a shrimp pathogen, was grown on a tryptic soy agar (TSA) plate containing 2% (w/v) NaCl at 30 ^{o}C for overnight. A single colony was inoculated in tryptic soy broth (TSB) containing 2% (w/v) NaCl and cultured at 30 ^{o}C, with 250 rpm for 12-16 h. After that, the culture was inoculated into fresh TSB containing 2% (w/v) NaCl at a dilution of 1:100 and grown at 30 ^{o}C with shaking until A_{600} reached 0.6 where it was 10^8 CFU/ml cell densities monitoring by plate count method. The culture was diluted 1:100 in 0.85% (w/v) NaCl, making cell suspension to be 10^6 CFU/ml.

2.5.1.2 WSSV

The WSSV stock was firstly prepared. The hemolymph of the moribund WSSV-infected shrimp was collected using syringe pre-loaded with 1:1 volume of lobster hemolymph medium (LHM: 486 mM NaCl, 15 mM $CaCl_2$, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 8.1 mM $MgSO_4$, 36 mM $NaHCO_3$, 0.05% (w/v) dextrose in Minimum Essential Medium (Invitrogen)). The solution was mixed in microcentrifuge tube and kept at -80 ^{o}C until used for WSSV injection. The WSSV solution was diluted in LHM at 1:8000 dilutions. One hundred microliter of diluted WSSV was used for injection into each shrimp.

2.5.2 Pathogen challenge

In each challenge experiment, shrimps were separated into 2 groups, challenged and non-challenged group. The shrimp were injected with each solution at the fourth abdominal segment. Three individual experiments were performed.

2.5.2.1 *V. harveyi* challenge

One hundred microliters of 10^6 CFU/ml *V. harveyi* strain 639 in 0.85% (w/v) NaCl were injected into the shrimp for the challenged group. For the control group, sterile 0.85% (w/v) NaCl was used instead.

2.5.2.2 WSSV challenge

One hundred microliters of 1:8000 dilution of WSSV in LHM were injected into the shrimp for the challenged group. For the control group, LHM was used instead of virus solution. This dosage of WSSV used in this experiment was empirically determined to be sufficient to kill the shrimp in about 4 days.

2.5.3 Detection of *V. harveyi* or WSSV infection

The experimental shrimps were determined for the infection of WSSV or *V. harveyi* before and after challenged in each experiment.

2.5.3.1 DNA extraction

To detect the pathogenic infection in shrimp, PCR technique was used. The gills of *V. harveyi* or WSSV challenged shrimp were individually collected, and then DNA was extracted by homogenizing tissues in 200 µl of lysis buffer (2.5 N NaOH and 10% (w/v) SDS). The homogenized samples were boiled for 10 min and chilled on ice for 3 min. The samples were centrifuged for 10 min at 8,000 x g at 4 ^{o}C. The supernatant was collected and diluted 1:100 in sterile H_2O. The diluted DNA was used as a template for PCR amplification.

2.5.3.2 *V. harveyi* detection

A primer pair specific to the *gyr*B gene of *V. harveyi* (forward primer FA2: 5' TCTAACTATCCACCGCGG 3' and reverse primer RB3: 5' AGCAATGCCATCTTCACGTTC 3') was used for amplification a product fragment of 363 bp. The PCR reactions consisted of 2 µl of 10X PCR buffer, 2 µl of diluted DNA template, 1.6 µl of 2.5 mM dNTPs, 2 µl of 2.5 mM $MgCl_2$, 5 µl of each 2 µM primers, 2.3 µl of PCR-grade water, and 0.1 µl of 5U/µl *Taq* polymerase (Fermentas). The amplification profile was 30 cycles of denaturation at 94 ^oC for 1 min, annealing at 63 ^oC for 1 min, and extension at 72 ^oC for 2 min, followed by final extension at 72 ^oC for 7 min. The PCR product was detected after run on 1% TBE-agarose gel electrophoresis (TBE buffer: 89 mM Tris-HCl, 8.9 mM boric acid, and 2.5 mM EDTA, pH 8.0) using ethidium bromide staining. The DNA band was observed under UV transilluminator.

2.5.3.3 WSSV detection

A primer pair of forward primer FWSSV: 5' AGAGCCCGAATAGTGTTTCCTCAGC 3' and reverse primer RWSSV: 5' AACACAGCTAACCTTTATGAG 3' was used in amplification of 250 bp fragment. The PCR reaction was performed including 2.5 µl of 10X PCR buffer, 0.5 µl of 10 mM dNTPs, 2.5 µl of 2.5 mM of $MgCl_2$, 0.5 µl of each 10 µM primers, 1 µl of diluted DNA template, and 0.2 µl of 5U/µl *Taq* polymerase (Fermentas). The reaction volume was adjusted to 25 µl with PCR-grade water. The amplification profile was 36 cycles of denaturation at 90 ^oC for 30 sec, annealing at 60 ^oC for 30 sec, and extension at 72 ^oC for 30 sec, before final extension at 72 ^oC for 5 min. Again, the PCR product was run on 1% agarose gel electrophoresis as above.

2.6 Shrimp tissues collection

To determine tissue distribution of *Pm*SERPIN6 gene, eye stalk, epipodite, gill, heart, hepatopancreas, stomach, lymphoid organ, intestine, and antennal gland were separately dissected from individual unchallenged shrimp and immediately frozen in liquid nitrogen (N_2) until used.

To get the hemocyte samples, shrimp hemolymph was collected from the ventral sinus of shrimp using 24 G / 1 inch needle fitted onto a 1.0 ml sterile syringe pre-filling with an anticoagulant, either 100 µl of 10% (w/v) trisodium citrate or 500 µl of MAS solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose and 9 mM EDTA, pH 7.0). Hemolymph was centrifuged at 800 x g for 15 min at 4 ^{o}C to separate the hemocyte pellet.

2.7 RNA preparation and first-stranded cDNA synthesis

Total RNA was extracted from shrimp tissues following by the first stranded cDNA synthesis. The cDNA was used as a template for PCR amplification.

2.7.1 Total RNA extraction

The shrimp tissues were homogenized by a pestle in 1 ml of ice-cold TriReagent® (Molecular Research Center), and then 200 µl of chloroform was added. The samples were vigorously shaken for 15 min and incubated at room temperature for 2-5 min before centrifugation at 12,000 x g 4 ^{o}C for 15 min. The aqueous colorless upper phase was transferred to the new 1.5 ml microcentrifuge tube. The chloroform extraction step was repeated once. The total RNA in the suspension was precipitated by adding 500 µl of isopropanol, leaved at room temperature for 5-10 min and centrifuged at 12,000 x g, 4 ^{o}C for 15 min. After that, the supernatant was discarded. The pellet of total RNA was washed in 1 ml of 75% ethanol and stored at -80 ^{o}C until used.

The total RNA pellet in 75% ethanol was centrifuged at 12,000 x g 4 ^{o}C for 10 min, and the clear solution was removed. The pellet was briefly air-dried for 5-10 min before adding an appropriate volume of diethyl pyrocarbonate (DEPC)-treated water and leaving it on ice until it was completely dissolved.

2.7.2 Quantification and qualification of total RNA

The quantity of total RNA was spectrophotometrically measured at 260 nm based on the specific property of UV absorption. The integrity of RNA was analyzed by 1.5% TBE-agarose gel electrophoresis and the gel was stained with EtBr and visualized under UV light.

The concentration of total RNA could be determined in the unit of ng/µl using the following formular: [RNA] = A_{260} × dilution factor × 40 (Sambrook et al., 1989).

The quality of RNA samples were investigated by the ratio of A_{260}/A_{280}. Because maximum wavelength of protein absorption which may be contaminated in RNA sample is at 280 nm, the purity of RNA should be given a ratio of A_{260}/A_{280} in range of above 1.7.

2.7.3 DNase treatment of total RNA

To remove contaminated chromosomal DNA in the total RNA preparation, the samples were treated with RQ1 RNase-free DNase (Promega). The reaction contains 5 µg of total RNA in 1x RNase-free DNase buffer and 1 unit of enzyme per 5 µg of total RNA. The DNase treatment reactions were incubated at 37 ^{o}C for 30 min. Then, the total RNA was purified by phenol/chloroform extraction, followed by isopropanol precipitation. The reactions of DNase-treated RNA were adjusted the volume to 40 µl with DEPC-treated water. Then, 250 µl of TriReagent® were added and mixed vigorously. Fifty microliters of chloroform was then added, shaken, and incubated at room temperature for 2-5 min. After centrifugation, the aqueous upper phase was mixed with an equal volume of isopropanol to precipitate RNA. RNA pellet collected

by centrifugation was washed with 1 ml of 75% ethanol. After centrifugation at 12,000 x g, 4 ^{o}C for 15 min, RNA pellet was air-dried and resuspended in an appropriate volume of RNase-free water. The quantity and quality of total RNA was examined as described in the section 2.7.2.

2.7.4 First-strand cDNA synthesis

After purification, total RNA was used as template for the first-stranded cDNA synthesis using RevertAID^{TM} first strand cDNA synthesis kit (Fermentas). According to the kit's instruction, one microgram of DNA-free total RNA was incubated with 1 µl of oligo(dT)$_{18}$ primer in a total volume of 12 µl. The mixture of RNA and primers was incubated at 65 ^{o}C for 5 min and then chilled on ice. The master mix containing 4 µl of 5X reaction buffer, 1 µl of 20 U/µl RiboLock^{TM} RNase inhibitor, 2 µl of 10 mM dNTP mix, and 1 µl of 200 U/µl RevertAid^{TM} M-MuLV Reverse Transcriptase was then added and the reaction was incubated at 42 ^{o}C for 1 h and finally heated at 70 ^{o}C to terminate the reaction. The cDNA was stored at -20 ^{o}C until used.

2.8 Cloning of the full-length *Pm*SERPIN6 cDNA

To obtain the full-length *Pm*SERPIN6 cDNA, a specific primer pair (forward: 5´ ATGAGGCTCCTGGTAGCTAT 3´ and reverse: 5´ CTACGAACTGGCCTTCAC 3´), were designed from the nucleotide sequences of the EST clone (clone nos. CT1604 and CT2832). The complete ORF of *Pm*SERPIN6 was amplified by PCR from the cDNA of unchallenged shrimp hemocyte at annealing temperature of 54 ^{o}C. The 50 µl total volume reaction contained 5 µl of 10X buffer, 4 µl of 2.5 mM dNTP, 5 µl of 10 µM primer each, 2.5 µl of cDNA template, 27.5 µl of ultrapure water, and 1 µl of 50X Advantage2 DNA polymerase (Clontech). The PCR product was purified and cloned into the T&A vector (Figure 2.1) using the T&A cloning vector kit (RBC Bioscience) following the product protocol. Blue-white screening facilitates screening of the positive clones on the LB plate supplemented with ampicillin, IPTG, and X-

Gal. The selected clones were sequenced in both directions using M13 forward and reverse primers with an automated sequencer by a commercial service (Macrogen Inc., Korea).

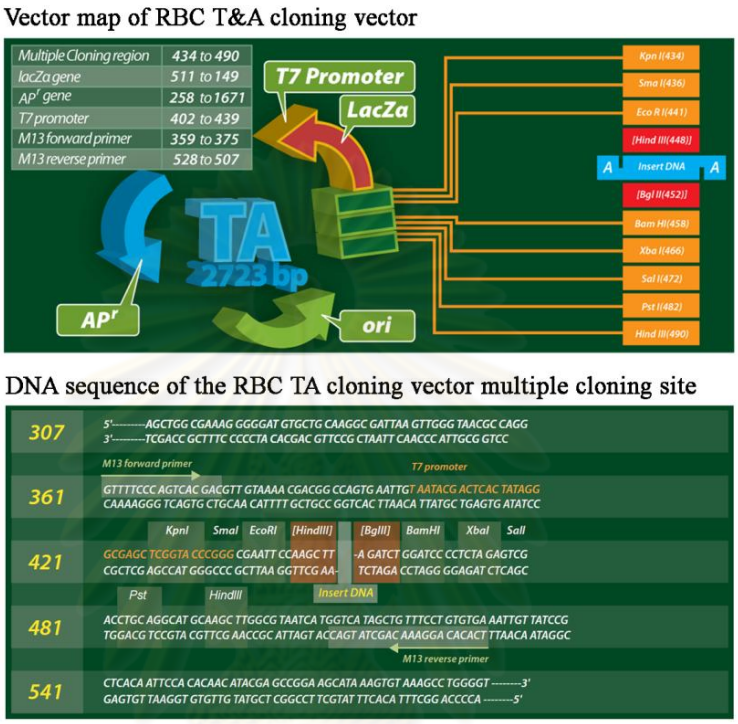


Figure 2.1 Vector map and DNA sequence of RBC T&A cloning vector (RBC Bioscience)

2.9 *Pm*SERPIN6 mRNA expression analysis by RT-PCR

To investigate the response to microbial infections (*V. harveyi* and WSSV infections) and distribution of *Pm*SERPIN6 gene in shrimp tissue, semi-quantitative reverse transcription-PCR (RT-PCR) was carried out.

2.9.1 cDNA sample

To determine the distribution of *Pm*SERPIN6 transcript in shrimp tissues, cDNA from ten tissues consisting of hemocyte, gill, hepatopancreas, lymphoid organ,

heart, epipodite, eye stalk, antennal gland, intestine and stomach were used as template for RT-PCR analysis.

To determine the alteration of *Pm*SERPIN6 transcript upon WSSV and *V. harveyi* challenges, hemocyte cDNA from experimental shrimp at each time point after pathogen infection was used as a template for RT-PCR.

The hemolymph of five shrimps from both challenged and non-challenged groups was individually collected at 0, 12, 24, and 48 h after injection (hpi) for WSSV challenge and at 0, 6, 12, 24, and 48 hpi for *V. harveyi* challenge using 1:10 volume of 10% (w/v) tri-sodium citrate as an anticoagulant. Hemocyte of each sample was collected and extracted for total RNA. The total RNA from 3 samples at each time point of the challenged or non-challenged shrimp was pooled. The cDNA samples were synthesized and then used as a template for RT-PCR.

2.9.2 Primers design

According to the multiple nucleotide sequence alignments of many serpins (data not shown), the conserved region was located at the 3´-end of serpin sequences, whilst variation in sequences was found at the 5´-end. Therefore, *Pm*SERPIN6 specific primers to be used in RT-PCR experiment were designed near the 5´-end cDNA sequence. The forward and reverse primers were 5´ GTCGATGATCAAGTCGCCACGCTCAA 3´ and 5´ TATCGATGTAGGCGCGGT TAGCGATG 3´, respectively. The expected size of the amplification product obtained from cDNA template was 118 bp.

2.9.3 Amplification of *Pm*SERPIN6 gene fragment

The fragment of *Pm*SERPIN6 transcript was amplified by RT-PCR. Five microliters of 10 fold diluted cDNA was used as a template for PCR amplification. The PCR reaction in a 25 µl total volume was carried out. The reaction contained 2.5 µl of 10X PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM each dNTP, 0.25 µl

of 10 µM primer each, 5 µl of 10-fold diluted cDNA, 14.75 µl of ultrapure water, and 0.25 µl of 5 U/µl *Taq* polymerase (Fermentas). PCR profile was started with pre-denaturation at 94 ^{o}C for 2 min and followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 60 ^{o}C for 30 sec, and extension at 72 ^{o}C for 30 sec.

Ten microliters of PCR product were mixed with one-tenth volume of 10X DNA loading dye (50 mM Tris-HCl, 2.5 mg/ml bromophenol blue, 2.5 mg/ml xylene cyanol, 60% glycerol at pH 7.6) and analyzed on 1.5 % agarose gel electrophoresis preparing with 1x TBE. The gels were stained with ethidium bromide solution for a while and de-stained in water for about 10-15 min. The positive DNA products were visualized as fluorescent bands under a UV transilluminator.

2.9.4 Amplification of β-actin gene fragment

For internal control of RT-PCR, β-actin gene (GenBank accession no. DW042525) was used. The specific primers of shrimp actin amplifying 337 bp product are actinF: 5´ GCTTGCTGATCCACATCTGCT 3´ and actinR: 5´ ATCACCATCGGCAACGAGA 3´. The PCR reaction mixture of 25 µl total volume consisted of 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM each dNTP, 0.25 µl of 10 µM primer each, 1 µl of 10-fold diluted cDNA of interest, 17.75 µl of ultrapure water, and 0.25 µl of 5 U/µl *Taq* polymerase (Fermentas). The PCR profile was same as *Pm*SERPIN6 amplification (section 2.9.3) but optimized to only 25 cycles. The five microliter of PCR products was mixed with 10X DNA loading dye and analyzed on 1.5% agarose gel electrophoresis as described above (section 2.9.3).

2.9.5 Analysis of *Pm*SERPIN6 expression ratio

The positive DNA bands of *Pm*SERPIN6 and β-actin gene were determined for their intensity using GeneTools program (Syngene). The expression level of *Pm*SERPIN6 transcripts were normalized to that of β-actin gene of the same cDNA template. *Pm*SERPIN6 expression level of challenged shrimp was subsequently

normalized to that of the control group at each time point of infection. The resulting relative expression ratios of *Pm*SERPIN6 gene were calculated from three independent experimental groups.

2.9.6 Statistic analysis

Statistical analysis was carried out from three sets of experiment using a One-Way ANOVA and Post Hoc test, with significance accepted at $P < 0.05$.

2.10 Expression of recombinant *Pm*SERPIN6 protein

The recombinant *Pm*SERPIN6 (r*Pm*SERPIN6) was produced in the *E. coli* expression system. Moreover, the recombinant protein was used to raise a specific polyclonal antibody which was of great benefit to further experiments.

2.10.1 Competent cell preparation and transformation

There are two types of *E. coli* competent cells used in this study, $CaCl_2$-treated cells and electrocompetent cells.

The overnight culture of *E. coli* XL-1-Blue, BL21(DE3), or Rosetta(DE3)pLysS was inoculated into fresh LB media (1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl) containing an appropriate antibiotic and incubated at 37 ^{o}C with 250 rpm shaking until OD_{600} reached about 0.5.

2.10.1.1 Competent cell for electro-transformation

The cell suspension was cooled down by chilling on ice for 30 min, before centrifugation at 4,000 x g for 15 min. The cell pellet was washed twice using 1 and 0.5 volume, respectively, of sterile pre-cooled distilled water. Then, cell pellet was

resuspended in an appropriate volume of iced-cooled 10% (v/v) glycerol. Forty microliters of cell suspension were aliquoted and immediately frozen at -80 ^{o}C until used.

2.10.1.2 Competent cell for $CaCl_2$-transformation

The 10 min pre-chilled culture was centrifuged at 4,000 x g for 5 min. Then, the cells were washed once with 0.5 volume of 10 mM $CaCl_2$ solution. The final cell pellet was resuspended in an appropriate volume of 100 mM $CaCl_2$ solution supplemented with 10% (v/v) glycerol and chilled on ice for about 30 min. One hundred microliters of competent cells were taken into an aliquot and immediately frozen at -80 ^{o}C until used.

2.10.1.3 Transformation

For electro-transformation, plasmid solution at the maximum volume of 2 µl per 40 µl cells was transformed into electro-competent cells. The plasmid was incubated with competent cells on ice for 1 min, and then the mixture was transferred into cooled and cleaned 0.2 cm electrode gap cuvette for electroporation. The mixture was pulsed using Minipulser electroporation system (Biorad) at constant 2.5 kV. The mixture was immediately transferred into 1 ml fresh LB media.

For $CaCl_2$ transformation, up to 20 µl plasmid was mixed with 100 µl of competent cells. The plasmid and competent cells were mixed and chilled on ice for at least 30 min. After that, the reaction was incubated at 42 ^{o}C for 1 min and optional on ice for 3 min. One milliliter of fresh LB media was subsequently added to the mixture.

Afterward, 1 ml culture containing recombinant cell was incubated at 37 ^{o}C with shaking for 1 h. The cell suspension was spread onto LB agar plate supplemented with appropriate selective substances.

2.10.2 Expression vector preparation

Two sets of expression plasmid which are pET-22b(+) (Figure 2.2) and pVR500, were used for construction of r*Pm*SERPIN6 expression vector.

2.10.2.1 pET-22b(+) preparation

The advantage of pET-22b(+) vector over other pET vectors is that it contains *pelB* ladder sequence upstream the insertion site. The signal sequence of pelB ladder transfers the recombinant protein to periplasm of bacteria which help preventing the toxicity from the over produced recombinant protein to the host.

Stock solution of pET-22b(+) vector (Novagen) was transformed into *E. coli* strain XL-1-Blue. The positive clones were selected by plating the transformants onto LB plate containing 100 µg/ml ampicillin. The vectors were extracted and purified from a single positive clone which is resistant to ampicillin using QIAprep spin miniprep kit (Qiagen). The vector was determined for quality and concentration by spectophotometric method.

Restriction site of *Nco*I (CCATGG) and *Bam*HI (GGATCC) in multiple cloning site of vector were chosen for cloning of the *Pm*SERPIN6 gene. pET-22b(+) vector was digested with both *Nco*I and *Bam*HI in the same reaction (1X NEB buffer 4) by incubating at 37 ^{o}C for overnight. The linear vector was purified by agarose gel elution using NucleoSpin® Extract II kit (Macherey-Nagel). Briefly, the expected DNA band was excised from agarose gel with a clean sharp scalpel and with a minimize size of gel slice. The piece of gel slice was transferred to a clean microcentrifuge tube and weighed. Two hundred microliters of NT buffer was added into 100 mg of gel slice. The sample was incubated to 55 ^{o}C for about 10 min or until gel was completely melted. The solution was added to a NucleoSpin extract column and centrifuged at 11,000 x g for 1 min. The flow-through was discarded, and the column was washed with 600 µl of NT3 buffer. After centrifugation, the silica membrane in column was dried by centrifugation at 11,000 x g for another 2 min to completely remove NT3 buffer. To elute the purified plasmid, NE buffer was added to

the center of silica membrane, leaved at room temperature for 1 min to increase elution yield, and centrifuged for 1 min. The collected linear overhang vector was measured at A_{260} for its concentration and stored at -20 ^{o}C until used.

2.10.2.2 pVR500 preparation

pVR500, a derivative of pET-32a(+) vector (Figure 2.3), was transformed into *E. coli* strain XL-1-Blue. The purified vector was double digested with two restriction enzymes, *Nco*I and *Bam*HI. The linear DNA fragment was eluted by agarose gel elution, determined the concentration, and stored at -20 ^{o}C until used.

2.10.3 Expression of recombinant *Pm*SERPIN6 using pET-22b(+)/*Pm*SERPIN6

2.10.3.1 Construction of recombinant pET-22b(+)/*Pm*SERPIN6

2.10.3.1.1 Mature sequence amplification of *Pm*SERPIN6 gene

Two specific primers were designed to amplify sequence coding for mature *Pm*SERPIN6 protein with extension of *Nco*I restriction site at 5´-end of forward primer (bolded) and hexa-histidine tag (underlined) and *Bam*HI restriction site (bolded) at 5´-end of reverse primer. The forward primer was 5´ TATA**CCATGG**GCCAGTGCTTTTCGGAGCAG 3´ and the reverse primer was 5´ TATA**GGATCC**CTAATGATGATGATGATGATGCGAACTGGCCTTCAC 3´. The PCR was performed using T&A plasmid (RBC Bioscience) containing full-length of *Pm*SERPIN6 gene as a template. The PCR amplification was achieved by pre-denaturation at 94 ^{o}C for 2 min following with 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing temperature of 56 ^{o}C for 30 sec, and extension at 72 ^{o}C for 3 min, before final extension at 72 ^{o}C for 10 min. The PCR reaction of the total 50 µl contained 5 µl of 10X *Pfu* buffer with $MgSO_4$, 1 µl of 10 mM each dNTP mix, 1 µl of 10 µM primer each, 0.5 µl of plasmid template, 41 µl of nuclease-free water, and 0.5 µl of 3 U/ µl *Pfu* DNA polymerase (Promega).

The PCR product of about 1,230 bp was purified using NucleoSpin® Extract II kit (Macherey-Nagel). The concentration of the purified PCR product was examined.

2.10.3.1.2 Cloning into T&A cloning vector

To make cloning step easier, the insert gene of *Pm*SERPIN6 with *Nco*I and *Bam*HI restriction sites was cloned into T&A cloning vector. First, the A-overhang was added to the 3´-end of the insert fragment by preparing the reaction contained 5 µl of 10X *Taq* polymerase buffer, 8 µl of 25 mM $MgCl_2$, 10 µl of 10 mM dATP, 15 µl of purified PCR product for insertion, 11.6 µl of ultrapure water, and 0.4 µl of 5 U/µl *Taq* DNA polymerase. The reaction was incubated at 70 ^{o}C for 30 min. The product was purified using NucleoSpin® Extract II kit. The purified DNA was then ligated into T&A cloning vector following the kit's instruction by overnight incubating at 4 ^{o}C. Then, 2 µl of ligation mixture was transformed into *E. coli* strain XL-1-Blue. The transformants were screened on LB agar plate supplemented with ampicillin, IPTG, and X-Gal.

2.10.3.1.3 Detection of recombinant plasmids

According to the disruption of the β-galactosidase gene in case of complete insertion into T&A vector, IPTG and X-Gal on LB plate were used in blue-white screening. The blue color product will be observed if the vector is re-circularized by using IPTG as an inducer and X-Gal as a substrate of β-galactosidase gene product, while the colony will be white if the gene of β-galactosidase is disrupted by gene insertion. The white positive colonies were selected and detected for gene insertion by PCR using M13 forward and reverse primers. The product was analyzed by 1.2% agarose gel electrophoresis.

The recombinant clone was selected and inoculated into LB broth containing 100 µg/ml ampicillin. The plasmid was extracted from the culture using QIAprep spin miniprep kit (Qiagen).

2.10.3.1.4 pET-22b(+)/*Pm*SERPIN6 ligation and transformation

The T&A plasmid containing *Pm*SERPIN6 gene were double digested with *Nco*I and *Bam*HI. The expected product was purified by agarose gel extraction. The *Nco*I-*Bam*HI overhang *Pm*SERPIN6 gene was ligated into pET-22b(+) cutting with the same enzymes. The total 20 µl of ligation mixture contained 2 µl of 10X ligation buffer, 50 ng of linear vector, 65 ng of DNA insert, and 1.5 µl of 400 U/µl T4 DNA ligase. The reaction was incubated at 16 ^{o}C for 16 h.

Afterwards, 10 µl of ligation mixture was heat shock-transformed into *E. coli* XL-1-Blue. The transformants were selected on LB agar plate with 100 µg/ml ampicillin.

2.10.3.1.5 Recombinant pET-22b(+)/*Pm*SERPIN6 isolation

To confirm the true positive clone, the PCR amplification was performed using T7 promotor and terminator primers in which their sequences flanking the multiple cloning site of pET vector. The PCR products of 1,550 bp in size were analyzed by 1.2% agarose gel electrophoresis.

The positive clone with pET-22b(+)/*Pm*SERPIN6 plasmid was cultured in LB broth containing ampicillin. The recombinant plasmid was extracted and purified.

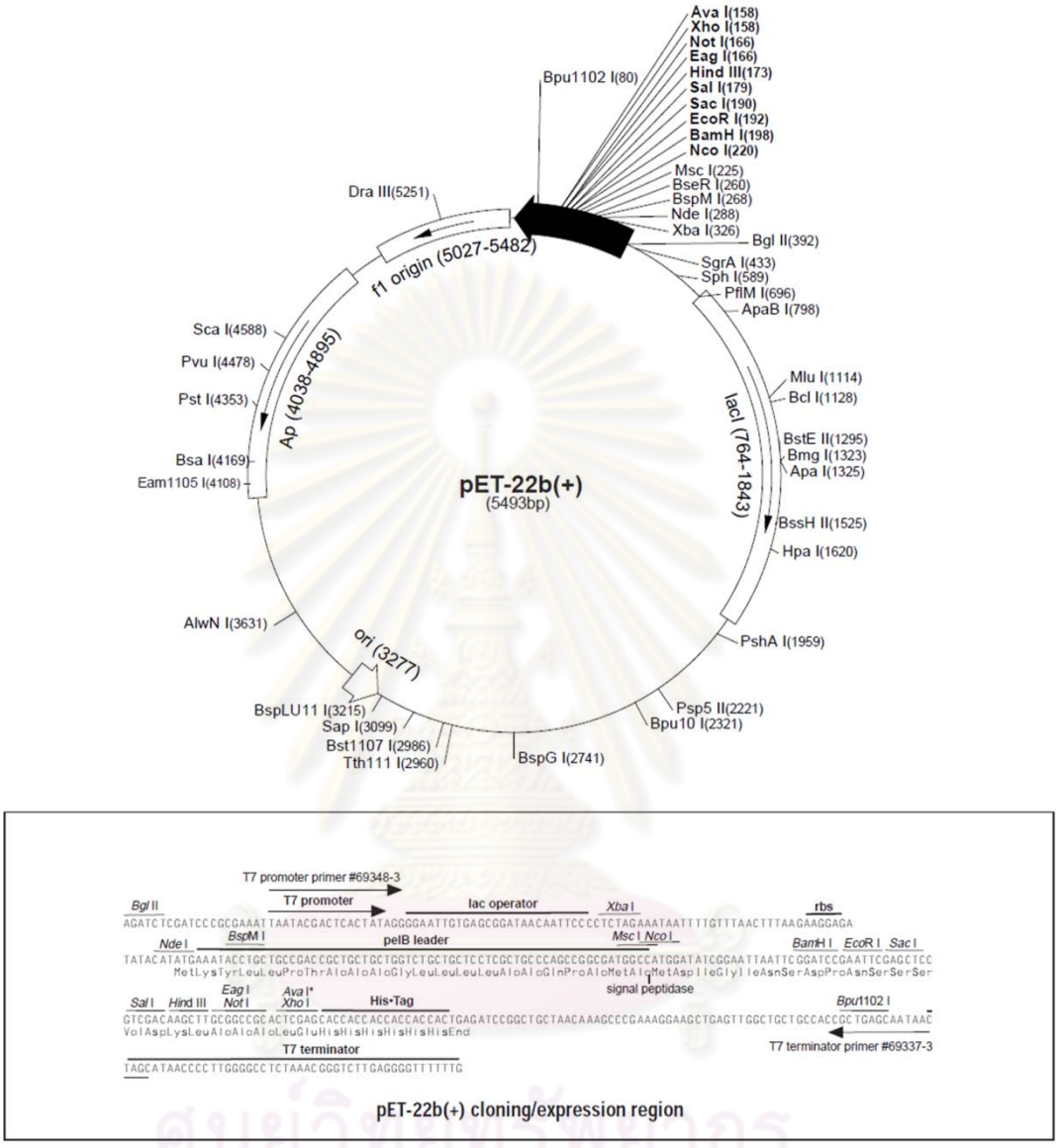


Figure 2.2 The pET-22b(+) vector map (Novagen®, Germany)

2.10.3.1.6 DNA sequencing

To confirm the correctness of inserted gene sequence, the purified pET-22b(+)/*Pm*SERPIN6 plasmid was sequenced in both direction using T7 promotor and T7 terminator primers with an automated sequencer by a commercial service (Macrogen Inc., Korea). The obtained sequences were analyzed using several bioinformatics programs such as ClastalW, Genetyx, and SECentral.

2.10.3.1.7 Transformation of pET-22b(+)/*Pm*SERPIN6 into *E. coli* BL21(DE3)

The pET-22b(+)/*Pm*SERPIN6 plasmid was transformed into expression host, *E. coli* strain BL21(DE3) by heat shock method. The positive clones were then screened on LB agar plate with 100 µg/ml ampicillin.

2.10.3.1.8 Small scale over-expression of recombinant *Pm*SERPIN6 (r*Pm*SERPIN6)

The single positive clone of *E. coli* BL21(DE3) carrying pET-22b(+)/*Pm*SERPIN6 plasmid was inoculated into LB media supplemented with 100 µg/ml ampicillin and incubated at 37 ^{o}C with 250 rpm shaking. The overnight cultured was inoculated at the dilution of 1:100 into fresh LB-ampicillin media. The density of culture was monitored by spectrophotometric assay at 600 nm until the OD_{600} reached 0.6. An inducer, IPTG, was added to a final concentration of 1 mM. One milliliter of cell culture was collected every hour during 6 h of induction. The culture was centrifuged to collect the cells. The pellets were lysed in 100 µl of 1X SDS-loading dye and analyzed by SDS-PAGE and Western blotting using monoclonal anti-His antibody (GE healthcare) as a primary antibody and mouse anti-rabbit IgG as a secondary antibody at the dilution of 1:3,000 and 1:5,000, respectively. The optimum time of induction and expression of r*Pm*SERPIN6 were then determined.

The small scale expression was performed again, and the cell pellet was collected at 6 h after induction by centrifugation at 8,000 x g for 10 min. The cell pellet was completely broken using sonicator. After centrifugation at 8,000 x g for 15 min, the pellet and supernatant were analyzed for recombinant protein expression by SDS-PAGE and Western blotting as above to determine whether the r*Pm*SERPIN6 was expressed in soluble or inclusion bodies forms.

2.10.3.2 Large scale over-expression of r*Pm*SERPIN6

The positive clone was cultured and expressed in 8 L of LB media supplemented with 100 µg/ml of ampicillin. After 6 h induction, cell pellet was collected by centrifugation at 8,000 x g for 10 min and resuspended in 1X phosphate buffer saline (PBS), pH 7.4. The cells were frozen and thawed three times before the cells were completely lysed under high pressure using a French Press. The inclusion bodies were collected by centrifugation and washed with 1% TritonX-100 in 1x PBS and with 1x PBS, pH 7.4. The inclusion bodies was solubilized in 20 mM sodium phosphate buffer, pH 8.0 containing 0.2 M NaCl, 20 mM imidazole, and 8M urea. The supernatant containing r*Pm*SERPIN6 was collected by centrifugation. The supernatant was kept at 4 ^{o}C until used.

2.10.4 Protein analysis

2.10.4.1 Analysis of recombinant protein by SDS-PAGE

To separate the proteins in mixture solution, discontinuous system of SDS-PAGE was used. After the polymerization of both separating and stacking gel is complete, the protein samples were prepared. The protein samples were diluted in 1X SDS loading buffer containing 12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 0.02% bromophenol blue, and 2.88 mM 2-mercaptoethanol. The samples were then boiled for 10 min and cooled down to the room temperature or kept on ice until loaded into gel. The samples must be spun down if they have some pellet occurred after boiling. Afterwards, either the protein samples or protein molecular weight

marker was loaded to each well. Electrophoresis was run in 1X SDS running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) at a constant current of 25 mA per gel. After electrophoresis, the gels were placed in Coomassie blue staining solution (0.1% (w/v) Coomassie brilliant blue R250, 10% (v/v) acetic acid, 45% (v/v) methanol) at room temperature with gentle shaking. Once the protein bands could be observed, the staining solution was discarded, and the gels were then immersed in destaining solution (10% (v/v) acetic acid, 10% (v/v) methanol) and shaken at room temperature. The solution of destaining was replaced regularly to assist the removal of staining until the gel background was clear.

2.10.4.2 Protein detection by Western Blot analysis

To detect whether expressed protein was the expected r*Pm*SERPIN6 or not, the Western blotting was performed. After running the SDS-PAGE, the gel was removed from the glass plates. The gels, nitrocellulose membrane, and filter papers were soaked in transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 15-30 min before they were sequentially laid on Trans-Blot® SD (Bio-Rad) as the blotting sandwich. The filter papers were placed on the platform of instrument, followed by the nitrocellulose membrane, the gel, and the filter papers, respectively. Protein transfer was carried out at a constant 110 mA for 90 min. After protein was transferred, the membrane was placed in a clean container. The membrane was blocked in blocking solution (5% (w/v) skim milk in 1X PBS buffer and 0.05% (v/v) Tween™-20 at pH 7.4 (PBS/Tween20)) at room temperature with orbital shaking for at least 3 h. After washing three times for 10 min with PBS/Tween20, the membrane was incubated with primary antibody in PBS/Tween20 containing 1% (w/v) skim milk for about 1-3 h at suitable dilution and temperature of incubation. Before incubation with secondary antibody at room temperature for an hour, the membrane was washed three times with PBS/Tween20. The secondary antibody with alkaline phosphatase (AP) conjugation was diluted in 1% (w/v) skim milk in PBS/Tween20. The membrane was washed again as above. The color development was performed by adding NBT and BCIP (Fermentas) at the final concentration of 375 and 188 µg/ml,

respectively, in 100 mM Tris- HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5 until the positive bands were detected.

2.10.4.3 Determination of protein concentration

2.10.4.3.1 Bradford assay

The protein content was measured based on the method of Bradford (Bradford, 1976) using bovine serum albumin (Fluka) as a standard protein. According to the binding of Coomassie brilliant blue G250 to proteins in sample, the red color of reaction was converted to the blue solution, and they were detected by monitoring the absorption at 595 nm. A sample solution of 100 µl was mixed with 1 ml Bradford working buffer and incubated for 10 min at room temperature before A_{595} was measured.

2.10.4.3.2 2-D quant kit

The protein solution was diluted to an appropriate concentration before aliquoted into 2 tubes with the volume of 20 and 40 µl, respectively. Five hundreds microliters of precipitant were added into samples or BSA standard solution at the different concentration, then mixed and leaved at room temperature for 3 min. After that, 500 µl of co-precipitant were added and mixed by inversion. The pellet was collected by centrifugation at 12,000 x g for 10 min and resuspended in 100 µl copper solution and 400 µl of DI-water. After mixing until there is no pellet in tube, 1 ml of working solution including 1:1 volume of solution A : solution B was added and immediately mixed. The reactions were incubated at room temperature for about 15 min and then monitored the absorbance at 480 nm.

2.10.5 Expression of r*Pm*SERPIN6 protein using pVR500/*Pm*SERPIN6

To prepare recombinant protein with a thioredoxin tag, pVR500 was used as an expression vector.

2.10.5.1 Construction of recombinant pVR500/*Pm*SERPIN6 clone

The mature sequence of *Pm*SERPIN6 was sub-cloned from pET-22b(+)/*Pm*SERPIN6 into pVR500 at the *Nco*I and *Bam*HI cloning site. After double digestion of pET-22b(+)/*Pm*SERPIN6 with *Nco*I and *Bam*HI, the overhang linear DNA was purified by agarose gel elution. The inserted gene was ligated into pVR500 digested with the same enzymes. The ten microliters of ligation reaction containing 22 ng of vector, 22.5 ng of DNA insert, 1 µl of 10X T4 DNA ligase buffer, and 0.2 µl of 400 U/µl T4 DNA ligase was incubated at 16 ^{o}C for 16 h. The ligation mix (2 µl) was electro-transformed into *E. coli* XL-1-Blue, and positive clones were screened on LB-ampicillin plate. The positive clone containing pVR500/*Pm*SERPIN6 plasmid was cultured, and the plasmid was purified and cut with *Nco*I and *Bam*HI to check for gene insertion. To confirm the correctness of sequence of the gene inserted, the plasmid was subjected to nucleotide sequencing.

The recombinant plasmid was transformed by heat shock method into expression host, *E. coli* strain Rosetta(DE3)pLysS and BL21(DE3). The recombinant clones were selected on LB agar plate supplemented with ampicillin and chloramphenicol for Rosetta(DE3)pLysS strain and only ampicillin for BL21(DE3) strain.

2.10.5.2 Over-expression of pVR500/*Pm*SERPIN6 in *E. coli* strain Rosetta(DE3)pLysS

The small-scale expression of r*Pm*SERPIN6 from a clone containing pVR500/*Pm*SERPIN6 plasmid was performed as described above in section 2.10.3.1.7. The cell suspension in LB broth with antibiotics was taken at 0, 2, 4, and 6

h after IPTG induction and analyzed by SDS-PAGE and Western blotting. The cell pellets at 6 h after IPTG induction was sonicated using microtip probe with 30% amplitude. The supernatant and pellet were separated and analyzed by SDS-PAGE and Western blotting. The aliquot pellets of inclusion bodies were dispersed in 50 mM of buffer at various pH including sodium acetate buffer pH 5.2, sodium phosphate buffer pH 8.0, sodium carbonate buffer pH 10.0, and sodium phosphate buffer pH 12.0. The suspensions were incubated at 4 ^{o}C for overnight with agitation. After centrifugation to separate supernatant and pellet, the samples were analyzed by SDS-PAGE and Western blotting.

For large scale expression, *E. coli* Rosetta(DE3)pLysS containing pVR500/*Pm*SERPIN6 plasmid was culture in 6 L of LB-ampicillin-chloramphenicol media. After 6 h of induction, cell pellet was separated by centrifugation and completely broken by sonication. The pellet was then washed, and resuspended in 50 mM phosphate buffer, pH 12.0, to solubilize proteins from inclusion bodies.

2.11 Recombinant protein purification

2.11.1 Non-denaturing condition

The Ni Sepharose 6 fast flow bead (GE Healthcare), the affinity bead specific to His-tag, was packed into PD-10 column and washed twice with sterile-distilled water. The column was equilibrated with 10 column volumes of binding buffer, 20 mM sodium phosphate buffer containing 20 mM imidazole, pH 8.0.

Crude recombinant protein from pVR500/*Pm*SERPIN6 vector which derived from solubilizing the inclusion bodies with 50 mM sodium phosphate buffer, pH 12.0 was dialyzed against 20 mM sodium phosphate buffer, pH 8.0 containing 20 mM imidazole. After dialysis, the precipitate was removed by centrifugation. The supernatant was incubated with the affinity bead pre-equilibrated with binding buffer for 1 h. After that, the bead was washed with 10 column volumes of binding buffer. The two steps elution was carried out using 20 mM sodium phosphate buffer, pH 8.0 containing 50 mM and 120 mM imidazole, respectively. The elution fractions were

analyzed on 12.5 % SDS-PAGE with coomassie staining. The fractions containing r*Pm*SERPIN6 were pooled and dialyzed against 50 mM Tris-Cl, pH 8.0.

2.11.2 Denaturing condition

Crude protein from pET-22b(+)/*Pm*SERPIN6 vector in 20 mM sodium phosphate supplemented with 0.2 M NaCl, 8 M urea and 20 mM imidazole, pH 8.0 was purified under denaturing condition using pre-packed HiTrap chelating HP column (GE Healthcare) coated with Ni^{2+}. The column was equilibrated with binding buffer (20 mM sodium phosphate supplemented with 0.2 M NaCl, 8 M urea and 20 mM imidazole, pH 8.0). After sample application, the column was washed with 10 column volumes of binding buffer. The elution was performed with the 20 mM sodium phosphate supplemented with 0.2 M NaCl, 8 M urea, pH 8.0 buffer containing 200 mM and 300 mM imidazole, respectively. The elution fractions were run on 10% SDS-PAGE. The fractions containing expected recombinant protein were pooled and dialyzed against 20 mM Tris-Cl, pH 8.0.

2.12 Detection of *Pm*SERPIN6 protein in hemocyte and cell-free hemolymph

To detect the *Pm*SERPIN6 protein in normal shrimp hemocyte and cell-free hemolymph, SDS-PAGE and Western blotting techniques were used.

2.12.1 Hemocyte and cell-free hemolymph preparation

Hemolymph from 5 normal shrimps was collected under an equal volume of the MAS solution and centrifuged at 800 x g for 15 min to separate the hemocytes. The hemocyte pellet was washed three times with MAS solution, and then resuspended in 150 mM NaCl. The hemocyte lysate was prepared by homogenizing and then collecting the supernatant after centrifugation at 14,000 x g for 15 min. The

protein contents of cell-free hemolymph and hemocyte lysate were measured using the Bradford assay. BSA was used as a standard protein.

2.12.2 SDS-PAGE and Western blot analysis

Fifty micrograms of hemocyte lysate and 200 µg of cell-free hemolymph were separated on a 12.5% (w/v) acrylamide SDS-PAGE and transferred to nitrocellulose membrane using Trans-Blot® SD (Bio-Rad). The membrane was subjected to Western blot analysis using rabbit anti-*Pm*SERPIN6 polyclonal antiserum as a primary antibody at the dilution of 1:20,000 in PBS-Tween20 with 1% (w/v) skim milk and incubation at 37 ^{o}C for 3 h. After washing, the membrane was incubated with the alkaline phosphatase conjugated anti-rabbit IgG antibody at the dilution of 1:20,000 at room temperature for 1 h. The positive band was detected using NBT/BCIP solution as described above.

2.13 Immunocytochemistry

To determine the expression of *Pm*SERPIN6 protein in response to microbial infection in shrimp hemocyte, the immunocytochemistry was performed using anti-*Pm*SERPIN6 antiserum.

2.13.1 Hemocyte preparation

The hemocytes from the WSSV- and *V. harveyi*-infected shrimp at 0, 6, 24, 48 and 72 h after challenge were fixed with 4% (w/v) paraformaldehyde in MAS solution by incubating on ice for 10 min. The hemocyte was separated by centrifugation at 1,000 x g for 10 min at 4 ^{o}C, and the supernatant was discarded. Then, the hemocyte pellet was washed once with 200 µl of MAS solution and finally resuspended in 200 µl of MAS solution. The hemocytes were aliquoted at 10^5 cells and fixed onto the

poly-L-lysine coated slide by centrifugation at 1,000 x g for 10 min at 4 ^{o}C. The hemocytes from three individual shrimps at each time point were used.

2.13.2 Immunocytochemistry

After washing with TBS buffer for about 10 min, the fixed hemocytes slides were permeabilized by soaking in 0.2% (w/v) gelatin in TBS containing 0.5% (v/v) Triton X-100 at room temperature for 10 min. The slides were washed for three times of 10 min with PBS containing 0.1% Tween20 (PBS-T_{20}). After pre-incubation to block non-specific of antibody binding using PBS supplemented with 0.1% (v/v) Tween 20, 1% (w/v) BSA and 1% (v/v) normal goat serum, the slides were then incubated with a 1:2,000 dilution of the rabbit anti-*Pm*SERPIN6 antiserum in blocking solution at 37 ^{o}C for 3 h. After extensively wash with PBS-T_{20} for three times, alkaline phosphatase-conjugated anti-rabbit IgG antibody at the dilution of 1:2,000 was added and incubated at room temperature for 1 h. The slides were then washed 3 times for 10 min each. The positive cells were detected by adding the alkaline phosphatase substrate (NBT/BCIP solution). For negative control, the rabbit pre-immune serum was used instead of anti-*Pm*SERPIN6 polyclonal antiserum. The positive cells were counted and reported as the percentage of positive cells. For each sample, a minimum of 300 cells per slide were counted.

2.14 Recombinant *Pm*SERPIN6 protein activity assay

2.14.1 Preparation of thioredoxin protein for the control reaction

Due to the r*Pm*SERPIN6 from pVR500/*Pm*SERPIN6 vector contained the thioredoxin fusion tag, to assay for r*Pm*SERPIN6 activities *in vitro*, the thioredoxin protein was produced and used as a control.

The pET-32a(+) vector (Figure 2.3) containing gene of thioredoxin tag was transformed into *E. coli* Rosetta(DE3)pLysS by $CaCl_2$ transformation. The expression was performed as same as in r*Pm*SERPIN6 expression using pVR500/*Pm*SERPIN6

vector. After 6 h of IPTG induction, cell pellet was collected by centrifugation at 8,000 x g for 10 min and resuspended in 20 mM sodium phosphate buffer, pH 8.0. The cell suspension was frozen and thawed three times and followed by sonication to complete cell lysis. The soluble protein was purified under non-denaturing condition through Ni-affinity column. The fractions containing thioredoxin protein detecting by 15% SDS-PAGE and Western blotting analysis were pooled.

During dialysis against 50 mM Tris-Cl, pH 8.0 containing 150 mM NaCl and 2.5 mM $CaCl_2$ at room temperature for 3 h, the purified recombinant thioredixin was digested with thrombin (Sigma) using 6.7 units per mg fusion protein. To remove thrombin and small peptide fragment, the dialyzed fraction was further purified by Ni-affinity column. The elution fractions were run on 15% SDS-PAGE. The fractions containing the expected product of 14 kDa were pooled and dialyzed against 50 mM Tris-Cl, pH 8.0.

2.14.2 Proteinase inhibitory activity assay

To assay for inhibitory activity of r*Pm*SERPIN6, four commercial proteinases; trypsin (bovine pancreas, Sigma), subtilisin Carlsberg (*Bacillus licheniformis*, Sigma), α-chymotrypsin (bovine pancreas, Sigma) and elastase (porcine pancreas, USBiological) were tested. The proteinase inhibitory activity was assayed using a procedure of Hergenhahn et al. (Hergenhahn et al., 1987). The reaction mixture consisted of 50 mM Tris-HCl, pH 8.0, 0.04 µM each of poteinases, appropriate amount of recombinant protein tested, and 293.6 µM of N-benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride for trypsin assay, 1,107.59 µM of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide for elastase assay, or 147.28 µM of N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for chymotrypsin and subtilisin assay. After incubation at 30 ^{o}C for 20 min, the reactions were measured at the absorbance of 405 nm. The remaining proteinase activity was calculated as a percentage of the absorbance reduction comparing to the negative control which is the reaction without the recombinant protein. The control reactions were carried out by using recombinant thioredoxin instead of r*Pm*SERPIN6. All reactions were done in duplicate.

2.14.3 Phenoloxidase inhibitory activity assay

Hemolymph of unchallenged shrimps was collected using 10% (w/v) trisodium citrate as an anticoagulant. After centrifugation at 800 x g for 10 min, hemocyte pellet was pooled and resuspended in an appropriate volume of CAC buffer, pH 7.0 (10 mM sodium carcodylate buffer, pH 7.0 containing 0.45 M NaCl, 100 mM $CaCl_2$, and 26 mM $MgCl_2$). The suspension was mixed well using vortex and then centrifuged in the swinging bucket rotor at 5,000 x g for 20 min to collect the hemocyte lysate supernatant (HLS). The protein content of HLS was determined using Bradford assay.

To test for the prophenoloxidase inhibition activity, 40 µg of HLS were mixed with various concentration of r*Pm*SERPIN6 in 50 mM Tris-Cl, pH 8.0. The reaction volume was adjusted to 50 µl using 50 mM Tris-Cl, pH 8.0. Then, 50 µl of 1 mg/ml larminarin (Sigma) was added to activate the phenoloxidase system, before adding 50 µl of freshly prepared 3 mg/ml L-DOPA (Fluka). After incubation for 30 min in the dark, the PO activity was spectrophotometrically measured at 492 nm using a microplate reader (BMG Labtech). Remaining PO activity was then calculated as a percentage of A_{492} reduction comparing to the negative reaction without any inhibitor. The reactions were performed in triplicate.

The BSA and recombinant thioredoxin at the same concentration as r*Pm*SERPIN6 were used as controls. The 9 µl of 100X Proteinase inhibitor mix (GE Healthcare) was used instead of the r*Pm*SERPIN6 in the reaction as positive control. The control reactions were performed in duplicate.

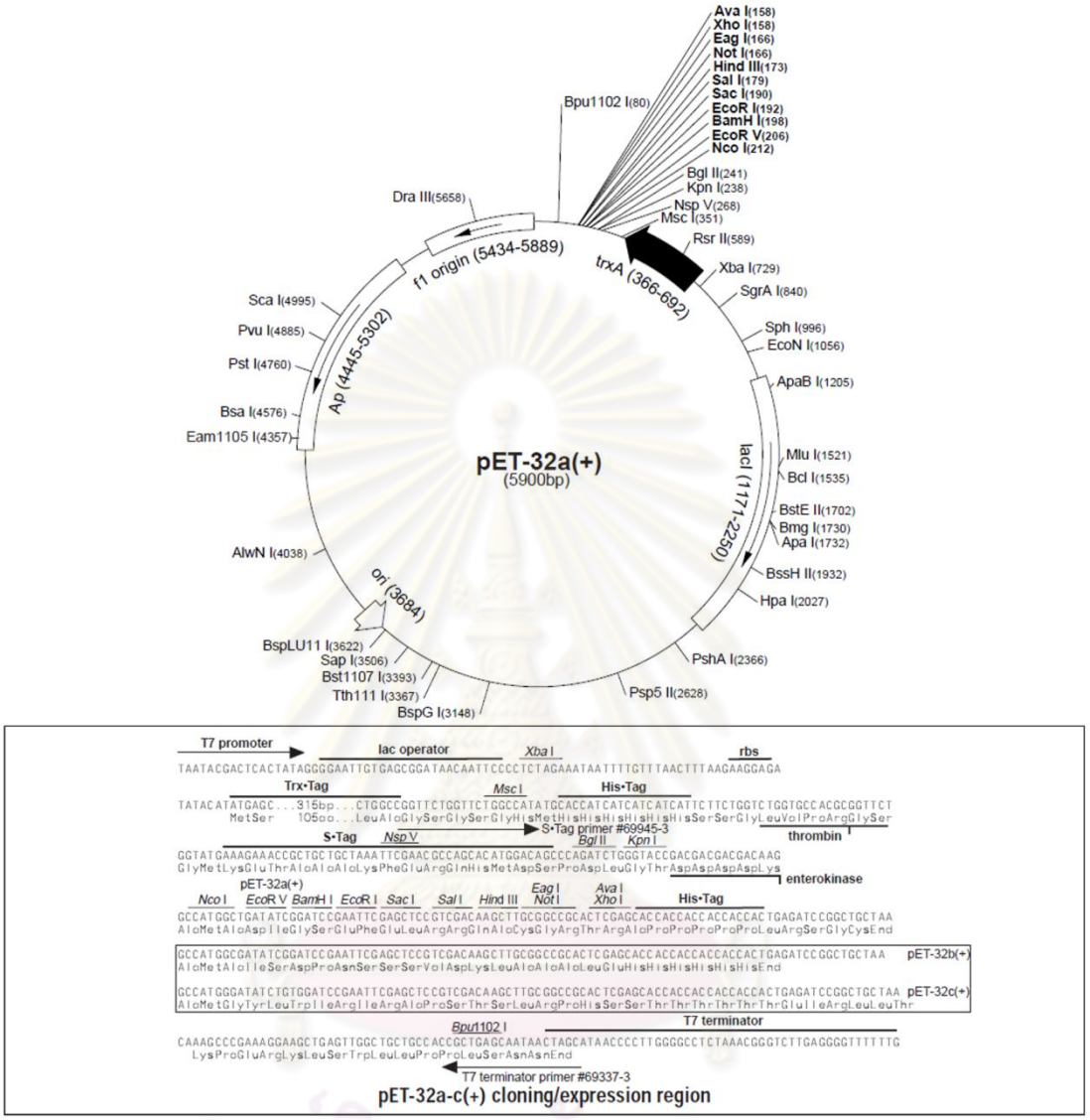


Figure 2.3 The pET-32a(+) vector map (Novagen®, Germany)

CHAPTER III

RESULTS

3.1 Data mining of the *P. monodon* EST database

To identify genes of *P. monodon*, belonging to serpin superfamily, we analyzed data from the *P. monodon* EST database. From the 10,536 unique clusters derived from 33,143 EST clones in the *P. monodon* EST database, six contigs (CT2488, CT1501, CT1116, CT1604, CT2832, and CT1087) and three singletons (SG5480, SG7094, and SG5654) corresponding to the genes of serpin superfamily were identified (Table 3.1). The representative clones of each contigs or singletons were re-sequenced to confirm the correctness of sequence information. The confirmed sequences were then searched against the Genbank database for similar proteins using BlastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Two contigs, CT1604 and CT2832, were found to be the same protein. Therefore, the data represented the total of 8 *P. monodon* serpins, and they were then named *Pm*SERPIN1 - 8.

It was found that the clone members of *Pm*SERPINs were mostly from the hemocyte, gill, and gill-epipodite cDNA libraries (Table 3.1). Other libraries were from hepatopancreas and heart cDNA libraries. According to the redundancy, the *Pm*SERPIN5, 6, and 7 are the most abundant serpins in the *P. monodon* EST database.

The sequences of all *Pm*SERPINs were analyzed. The ORFs and deduced amino acid sequences of the *Pm*SERPINs were predicted using the Genetyx program. There were only 3 complete serpin genes identified; *Pm*SERPIN6, *Pm*SERPIN7, and *Pm*SERPIN8. The signal sequence of these complete ORF serpins was predicted using the SignalP3.0 server. The results showed that they all contained 19 residues of signal peptides (Table 3.1).

Table 3.1 Identification of *Pm*SERPINs from the *Penaeus monodon* EST database.

Serpin	Contig or singleton	Number of clones	Frequency in EST libraries[a]		Representative clone (ORF) and GenBank accession number	ORF (amino acids)	Predicted signal sequence
***Pm*SERPIN1**	**CT2488**	**2**	**GlEp-N-N** **HPa-N-N**	**1** **1**	**GlEp-N-N01-1536-LF (incomplete ORF) GT968764**	**-**	**-**
***Pm*SERPIN2**	**CT1501**	**3**	**GlEp-N-N** **HC-N-N**	**1** **2**	**GlEp-N-N01-2770-LF (incomplete ORF) GT968765**	**-**	**-**
***Pm*SERPIN3**	**SG5480**	**1**	**GL-H-S**	**1**	**GL-H-S01-0037-LF (incomplete ORF) GT968763**	**-**	**-**
***Pm*SERPIN4**	**SG7094**	**1**	**HC-N-N**	**1**	**HC-N-N01-5643-LF (incomplete ORF) GT968767**	**-**	**-**
***Pm*SERPIN5**	**CT1116**	**5**	**GlEp-N-N** **HC-N-N** **HPO-N-S** **HT-N-S**	**2** **1** **1** **1**	**GlEp-N-N01-1465-LF (incomplete ORF) GT968766**	**-**	**-**

Table 3.1 (cont.) Identification of *Pm*SERPINs from the *Penaeus monodon* EST database.

Serpin	Contig or singleton	Number of clones	Frequency in EST libraries[a]		Representative clone (ORF) and GenBank accession number	ORF (amino acids)	Predicted signal sequence
***Pm*SERPIN6**	**CT1604 (CT2832)**	**5**	**GL-N-STC HC-N-N HT-N-S**	**2 2 1**	**HC-N-N01-2773-LF (complete ORF) GQ260129**	**415**	**MRLLVAMAVTAAVLGLVRP (19 amino acids)**
***Pm*SERPIN7**	**CT1087**	**5**	**HC-N-N HC-W-S**	**4 1**	**HC-N-N01-12906-LF (complete ORF) GU358487**	**411**	**MKFAVVGAVAAALVGVVQP (19 amino acids)**
***Pm*SERPIN8**	**SG5654**	**1**	**GL-H-S**	**1**	**GL-H-S01-0891-LF (complete ORF) GU358488**	**417**	**MKCLVALAAAAAVLGLGRP (19 amino acids)**

[a] GlEp-N-N, HPa-N-N, and HC-N-N are normalized (3rd N) normal (2nd N) gill-epipodite (GlEp), hepatopancreas (HPa) and hemocyte (HC) cDNA libraries. HPO-N-S and HT-N-S are standard (3rd S) normal (2nd N) hematopoietic tissue (HPO) and heart (HT) cDNA libraries. GL-H-S and GL-N-STC are standard heat-treated (2nd H) and subtractive (3rd STC) gill (GL) cDNA libraries, respectively. HC-W-S is standard white spot virus-infected (2nd W) hemocyte cDNA library.

3.2 Amino acid sequence analysis of *Pm*SERPINs

To reveal the conserved serpin structure of *Pm*SERPIN6 -8 and to compare the homology of all complete ORF serpins obtained, ClustalW program was used. Two serpins such as serpin6 from the tobacco hornworm, *Manduca sexta* (*Ms*SPN6) (AAV91026), an inhibitor in proPO system, and *Fc*-serpin from the chinese shrimp, *Fenneropenaeus chinensis* (ABC33916) were selected for the alignment with *Pm*SERPIN6 – 8. The amino acid sequence alignment revealed the serpin conserved sequences of *Pm*SERPIN6 – 8 which are the reactive center loop (RCL) at the C-terminal region of protein and the highly conserved hinge region (P_{17} to P_8; EEGTEAAAAT) at the N-terminal portion of RCL as well as the serpin signature (FHCNRPFVFLI) (Figure 3.1). In general, serpin with proteinase inhibitory activity contains the scissile bond between P_1-P_1' residues which is cleaved by its target proteinase and the P_1 residue determines the target specificity of the serpin to proteinase (Gettins, 2002). Based on the sequence alignment, we found in this study that the predicted P_1 residue of *Pm*SERPIN6 - 7 and 8 were Arg and Lys, respectively (Figure 3.1). However, the biochemical assay is required for characterization of the inhibitory activity and the true P_1 residues of *Pm*SERPINs. According to pairwise alignment, *Pm*SERPIN6, 7 and 8 showed 63%, 94% and 55% amino acid sequence identity to *Fc*-serpin. This indicated that *Pm*SERPIN7 is the *Fc*-serpin variant. Moreover, the amino acid sequence alignment of *Pm*SERPIN6 – 8 with those of *Ms*SPN6, revealed that the *Pm*SERPIN6 – 8 had 36 %, 34 % and 33 % identity, respectively. Due to the highest similarity to *Ms*SPN6, the *Pm*SERPIN6 was then selected for further characterization.

Figure 3.1 Alignment of amino acid sequences of *Pm*SERPIN6 - 8, *Fc*-serpin and *M. sexta* serpin-6 (*Ms*_serpin-6). The conserved hinge region, the putative signal sequence and the serpin signature of each gene are shown in grey, black and open boxes, respectively. The predicted P_1 and P_1´ positions are underlined. The asterisks (*) indicate identical amino acid residues. Semicolons (:) indicate conservative amino acid substitutions. Dots (.) show semi-conservative amino acid substitutions.

3.3 Phylogenetic analysis

The amino acid sequences of the mature protein of *Pm*SERPIN6 – 8 and *Pm*SERPINB3, the putative serpin from *P. leniusculus* (Pl), *F. chinensis* (Fc), *D. melanogaster* (Dm), *M. sexta* (Ms), *B. mori* (Bm) and *T. tridentatus* (Tt) were used to create phylogenetic tree using the Phylip program. Figure 3.2 showed that *Pm*SERPIN6 was clustered in the same group with *Fc*-serpin, putative serpin from *P. leniusculus*, serpin2 and serpin6 from *M. sexta*, serpin4A and serpin6 from *D. melanogaster*. According to the tree, *Pm*SERPIN6 was closely related to serpin from *F. chinensis*.

3.4 Cloning of the full-length sequence of *Pm*SERPIN6

According to sequence analysis, the contig nos. CT1604 and CT2832 were identified as *Pm*SERPIN6. To obtain the full-length sequence of *Pm*SERPIN6 gene, gene specific primers were designed and then used for PCR amplification as described in materials and methods section 2.8. The expected PCR product of 1,248 bp was obtained (Figure 3.3). It was then cloned into T&A cloning vector, subsequently checked for positive clones by colony PCR (Figure 3.4) and sequenced to confirm the result. The *Pm*SERPIN6 ORF encoded for 415 amino acid residues protein with 19 residues of signal sequence (Figure 3.5). The calculated molecular mass and isoelectric point of the mature protein were 44.5 kDa and 7.33, respectively. Four potential *N*-linked glycosylation sites were predicted. The deduced amino acid sequences of complete ORF *Pm*SERPIN6 showed 63% identities to *Fc*-serpin from *F. chinensis*, 41% to serpin from *P. leniusculus*, and 36% to serpin6 from *M. sexta* (Table 3.2).

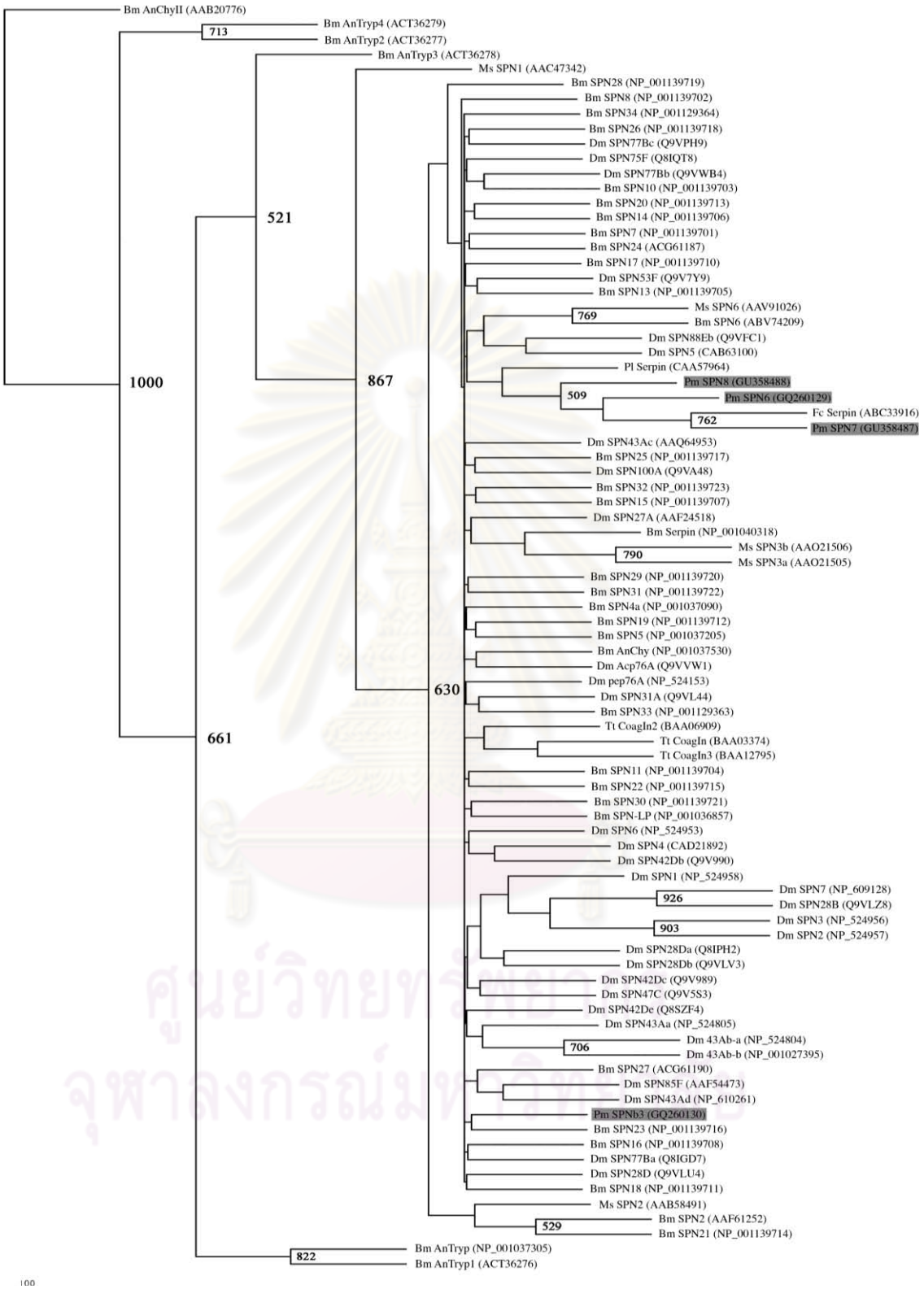

Bm AnChyII (AAB20776)
713
Bm AnTryp4 (ACT36279)
Bm AnTryp2 (ACT36277)
Bm AnTryp3 (ACT36278)
Ms SPN1 (AAC47342)
Bm SPN28 (NP_001139719)
Bm SPN8 (NP_001139702)
Bm SPN34 (NP_001129364)
Bm SPN26 (NP_001139718)
Dm SPN77Bc (Q9VPH9)
Dm SPN75F (Q8IQT8)
Dm SPN77Bb (Q9VWB4)
Bm SPN10 (NP_001139703)
Bm SPN20 (NP_001139713)
Bm SPN14 (NP_001139706)
Bm SPN7 (NP_001139701)
Bm SPN24 (ACG61187)
Bm SPN17 (NP_001139710)
Dm SPN53F (Q9V7Y9)
Bm SPN13 (NP_001139705)
Ms SPN6 (AAV91026)
769
Bm SPN6 (ABV74209)
Dm SPN88Eb (Q9VFC1)
Dm SPN5 (CAB63100)
Pl Serpin (CAA57964)
Pm SPN8 (GU358488)
509
Pm SPN6 (GQ260129)
Fc Serpin (ABC33916)
762
Pm SPN7 (GU358487)
Dm SPN43Ac (AAQ64953)
Bm SPN25 (NP_001139717)
Dm SPN100A (Q9VA48)
Bm SPN32 (NP_001139723)
Bm SPN15 (NP_001139707)
Dm SPN27A (AAF24518)
Bm Serpin (NP_001040318)
Ms SPN3b (AAO21506)
790
Ms SPN3a (AAO21505)
Bm SPN29 (NP_001139720)
Bm SPN31 (NP_001139722)
Bm SPN4a (NP_001037090)
Bm SPN19 (NP_001139712)
Bm SPN5 (NP_001037205)
Bm AnChy (NP_001037530)
Dm Acp76A (Q9VVW1)
Dm pep76A (NP_524153)
Dm SPN31A (Q9VL44)
Bm SPN33 (NP_001129363)
Tt CoagIn2 (BAA06909)
Tt CoagIn (BAA03374)
Tt CoagIn3 (BAA12795)
Bm SPN11 (NP_001139704)
Bm SPN22 (NP_001139715)
Bm SPN30 (NP_001139721)
Bm SPN-LP (NP_001036857)
Dm SPN6 (NP_524953)
Dm SPN4 (CAD21892)
Dm SPN42Db (Q9V990)
Dm SPN1 (NP_524958)
Dm SPN7 (NP_609128)
926
Dm SPN28B (Q9VLZ8)
Dm SPN3 (NP_524956)
903
Dm SPN2 (NP_524957)
Dm SPN28Da (Q8IPH2)
Dm SPN28Db (Q9VLV3)
Dm SPN42Dc (Q9V989)
Dm SPN47C (Q9V5S3)
Dm SPN42De (Q8SZF4)
Dm SPN43Aa (NP_524805)
Dm 43Ab-a (NP_524804)
706
Dm 43Ab-b (NP_001027395)
Bm SPN27 (ACG61190)
Dm SPN85F (AAF54473)
Dm SPN43Ad (NP_610261)
Pm SPNb3 (GQ260130)
Bm SPN23 (NP_001139716)
Bm SPN16 (NP_001139708)
Dm SPN77Ba (Q8IGD7)
Dm SPN28D (Q9VLU4)
Bm SPN18 (NP_001139711)
Ms SPN2 (AAB58491)
Bm SPN2 (AAF61252)
529
Bm SPN21 (NP_001139714)
822
Bm AnTryp (NP_001037305)
Bm AnTryp1 (ACT36276)
1000
521
867
661
630
100

Figure 3.2

Figure 3.2 (cont.) Phylogenetic analysis of the *Pm*SERPIN6. A neighbor-joining distance based phylogenetic tree of *Pm*SERPINs (highlighted in grey) and serpins from related organisms, including the serpins from *P. leniusculus* (Pl), *F. chinensis* (Fc), *D. melanogaster* (Dm), *M. sexta* (Ms), *B. mori* (Bm) and *T. tridentatus* (Tt). Values at the node of a bootstrapped neighbor-joining tree indicate the number of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences. The bootstrap support values higher than 500 are shown at the nodes. The GenBank accession number of the serpin genes used for the analysis is shown in the parenthesis.

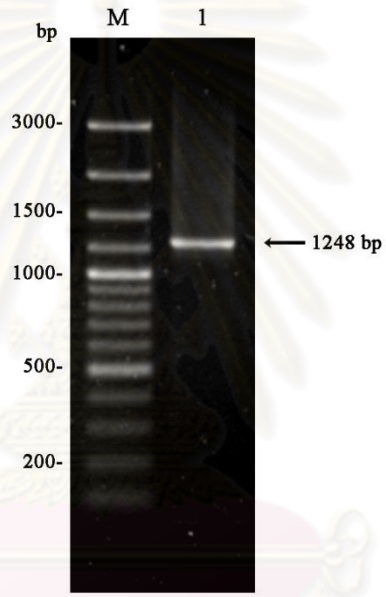


Figure 3.3 Amplification of the full-length *Pm*SERPIN6 gene. Lane M and lane 1 represent 100 bp DNA ladder marker (GeneRuler™ 100 bp DNA ladder, Fermentas) and a purified PCR product of the full-length *Pm*SERPIN6 with 1,248 bp in length, respectively.

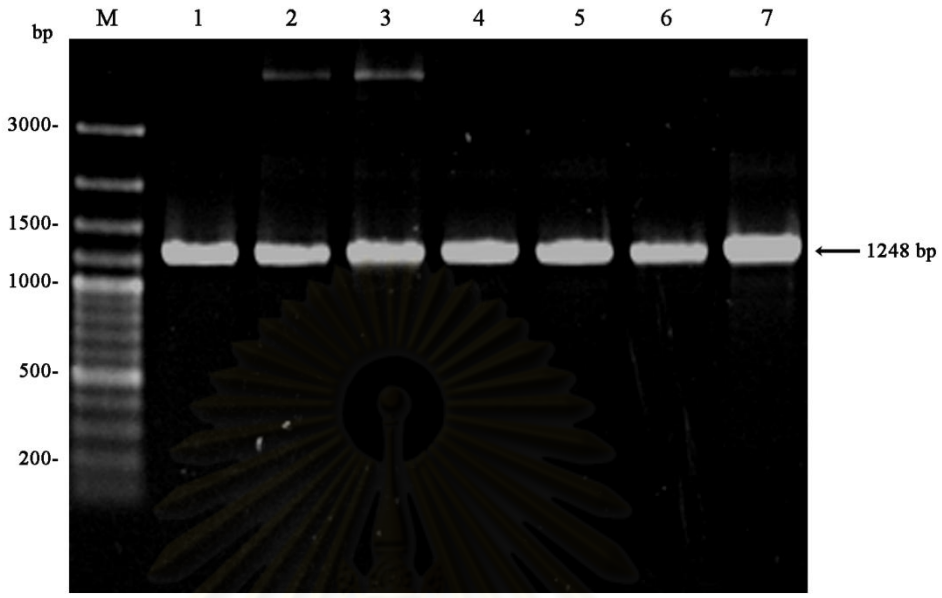


Figure 3.4 Screening of recombinant clones of *Pm*SERPIN6 gene in T&A vector by colony PCR. The colony PCR was performed using *Pm*SERPIN6 specific primers. The positive band of 1,248 bp is shown by the arrow. Lane M: 100 bp DNA ladder marker; Lanes 1-7: PCR product of colony PCR of clones 1-7.

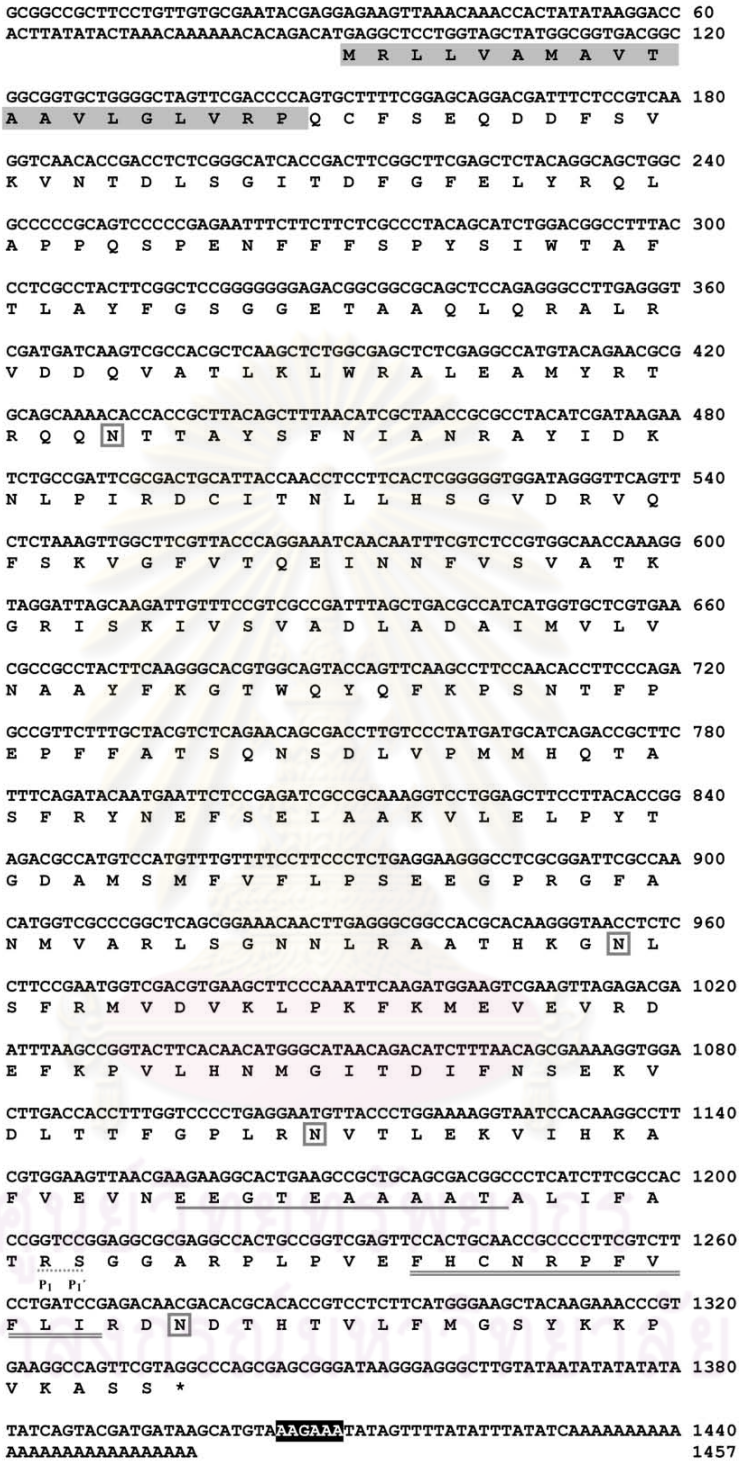


Figure 3.5 The deduced amino acid sequence of the *Pm*SERPIN6 gene. The signal peptide predicted by SignalP3.0 server is highlighted in gray. The predicted *N*-linked glycosylation sites are shown in the gray boxes. The serpin conserved sequence is underlined, and the serpin signature identified by Prosite is shown with double underlining. The predicted P_1-P_1´ residues are also marked under the amino acid residues.

Table 3.2 Homology proteins hit to *Pm*SERPIN6 from the BlastX software.

Sequence homology (Accession no.)	Species	% Identity to *Pm*SERPIN6	Expect values	Score (bits)
Serpin serine proteinase inhibitor (ABC33916)	*Fenneropenaeus chinensis*	251/398 (63%)	9e-148	527
*Pm*SERPIN7 (ADC42878)	*Penaeus monodon*	249/398 (62%)	4e-146	522
*Pm*SERPIN8 (ADC42879)	*Penaeus monodon*	249/395 (63%)	3e-145	519
Putative serine proteinase inhibitor (CAA57964)	*Pacifastin. Leniusculus*	178/434 (41%)	9e-89	331
Serpin-6 (AAV91026)	*Manduca sexta*	152/420 (36%)	4e-65	253
Serpin-6 (ABV74209)	*Bombyx mori*	144/420 (34%)	3e-60	236

3.5 Expression analysis of the *Pm*SERPIN6 transcript

3.5.1 Tissue distribution analysis

The distribution of *Pm*SERPIN6 gene in shrimp tissues such as lymphoid organ, epipodite, hemocytes, stomach, antennal gland, intestine, eye stalk, heart, gills, and hepatopancreas, was examined by RT-PCR. After the quality of total RNA from shrimp tissues was checked (Figure 3.6), the cDNA was synthesized and used as template for RT-PCR. From semi-quantitative RT-PCR analysis, it was found that *Pm*SERPIN6 transcript was expressed in almost tissues tested except in hepatopancreas as shown in Figure 3.7. Among those, it was highly expressed in lymphoid organ, epipodite, hemocytes, heart, and gills. A weak band was observed in stomach, antennal gland, intestine, and eye stalk.

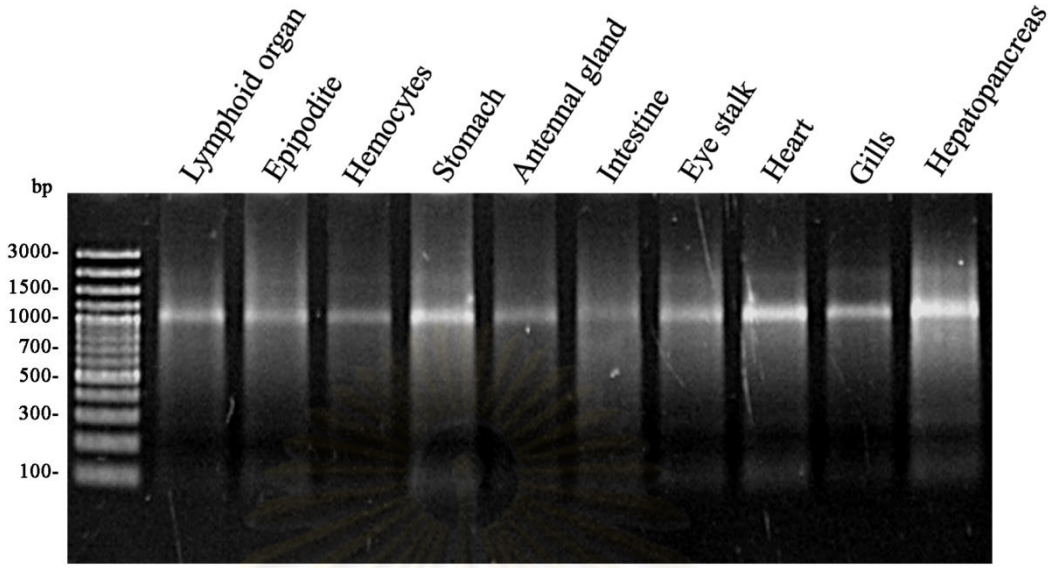


Figure 3.6 Total RNA extracted from ten tissues of unchallenged shrimp with 100 bp DNA ladder marker. Total RNAs were run on 1.5% agarose gel and stained with ethidium bromide.

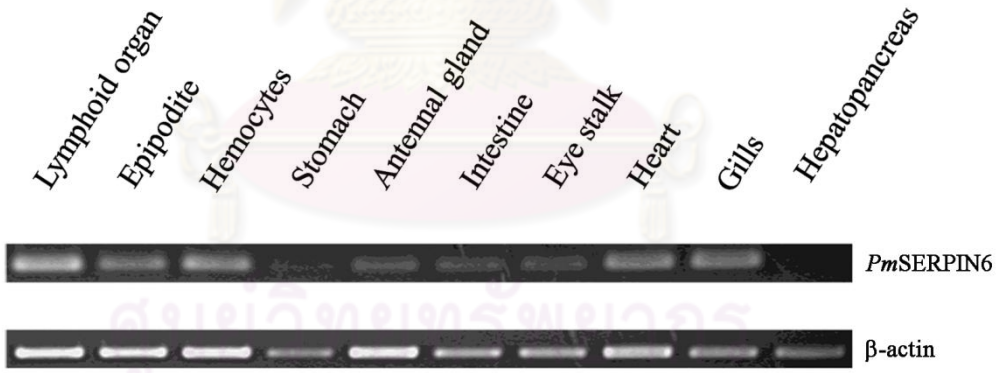


Figure 3.7 Tissue distribution analysis of *Pm*SERPIN6 transcript in ten shrimp tissues. The *Pm*SERPIN6 gene was amplified from various shrimp tissues by RT-PCR using β-actin as an internal control.

3.5.2 Temporal expression of *Pm*SERPIN6 mRNA during WSSV and *V. harveyi* infection

To understand the involvement of *Pm*SERPIN6 in shrimp immunity, we determined the gene expression in shrimp hemocyte in response to viral or bacterial infection at various time points. Before challenging shrimp with pathogen, the presence of WSSV or *V. harveyi* was randomly checked to make sure that the shrimp are free from both pathogens (Figures 3.8 and 3.9). After challenge with WSSV or *V. harveyi*, the total RNA from hemocyte at various time points were extracted and analyzed for their quality on 1.5% agarose gel with ethidium bromide staining (Figure 3.10).

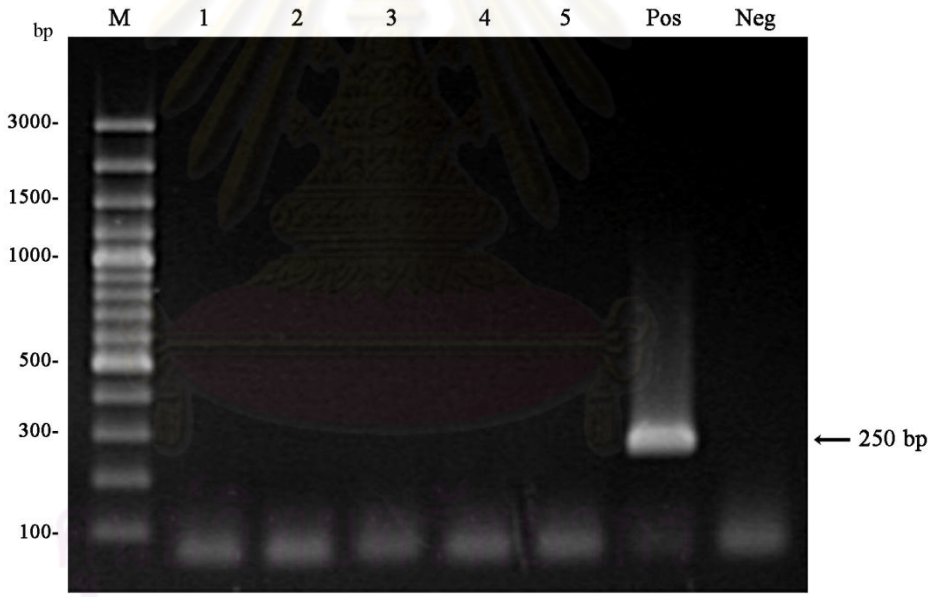


Figure 3.8 Detection of WSSV infection in five individual shrimps. Lane M: 100 bp DNA ladder marker; Lanes 1-5: PCR product of five individual shrimps; Lane Pos: positive control using WSSV as a PCR template; Lane Neg: negative control for PCR reaction. A positive band of 250 bp is shown by the arrow.

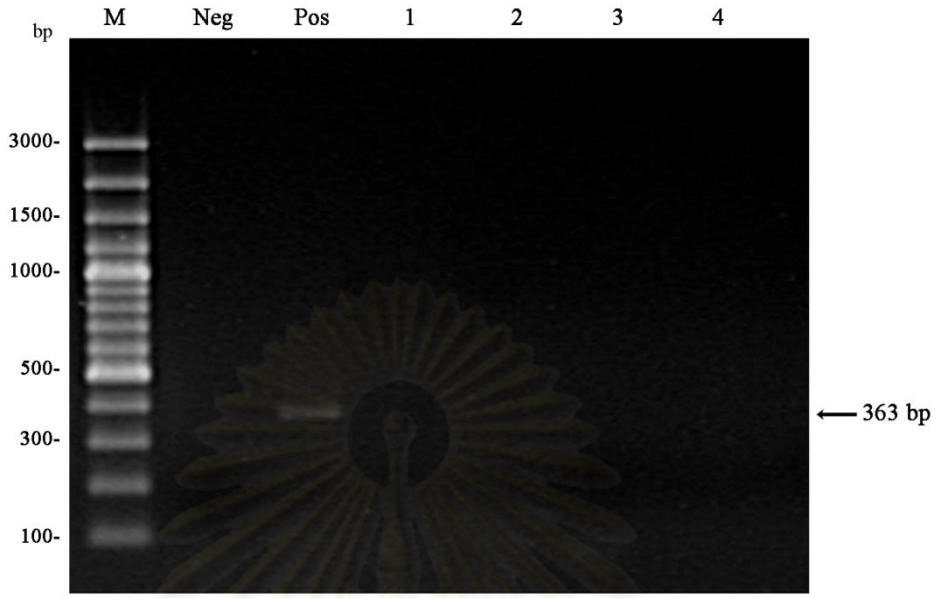


Figure 3.9 Detection of *V. harveyi* infection in four individual shrimps. Lane M: 100 bp DNA ladder marker; Lane Pos: positive control using gill-DNA extraction from the *V. harveyi* infected shrimp as a PCR template; Lane Neg: negative control for PCR reaction; Lanes 1-4: PCR product from four individual shrimps. A positive band of 363 bp is shown by the arrow.

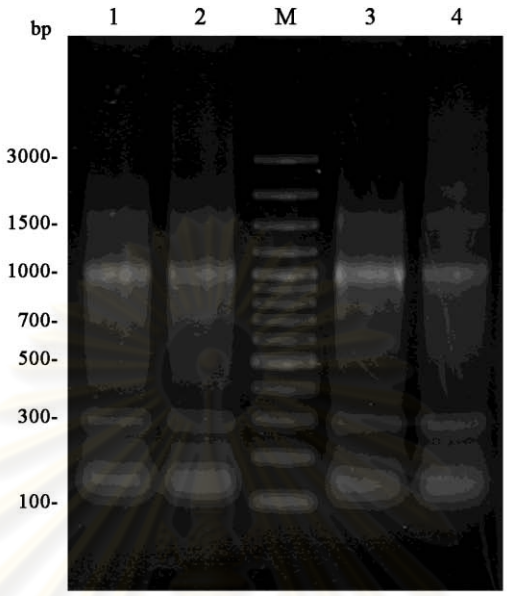


Figure 3.10 Quatitative analysis of total RNA extracted from the hemocyte. Lane M: 100 bp DNA ladder marker; Lanes 1-4 are the total RNA sample used in semi-quantitative RT-PCR experiment.

In WSSV-challenged shrimp, we observed the changes in *Pm*SERPIN6 gene expression at 0, 12, 24, and 48 hpi, and found that the relative expression ratio of *Pm*SERPIN6 gene was significantly decreased at 24 hpi compared to that of 0 hpi (Figure 3.11).

The observation on the alteration of the *Pm*SERPIN6 gene expression at 0, 6, 12, 24, and 48 h post *V. harveyi* infection was performed. The expression profile of *Pm*SERPIN6 transcript upon *V. harveyi* challenge as shown in Figure 3.12 remained unchanged during 0 – 48 hpi. The results clearly showed that alteration of the *Pm*SERPIN6 gene expression was observed only in shrimp infected with WSSV.

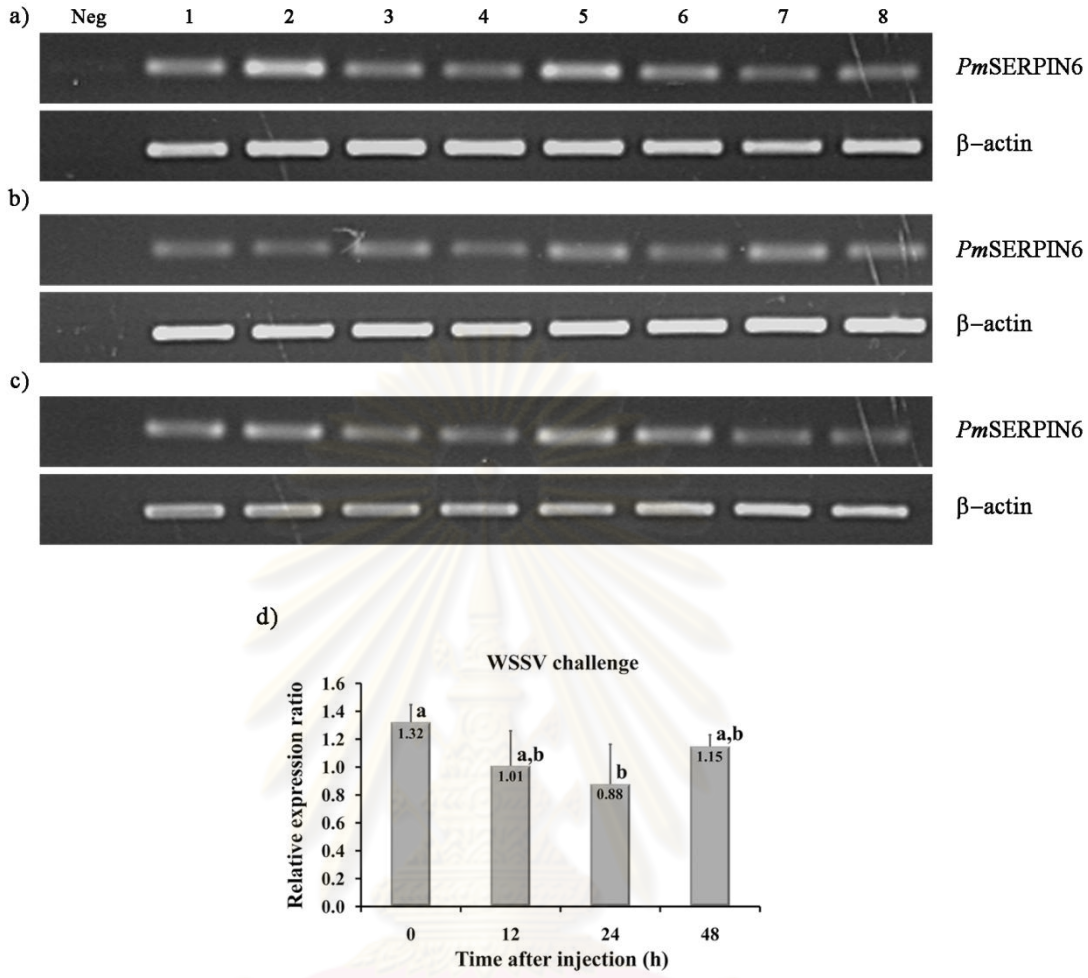


Figure 3.11 Semi-quantitative RT-PCR analysis of *Pm*SERPIN6 gene expression in *P. monodon* hemocyte in response to WSSV infection. Analysis of *Pm*SERPIN6 gene transcript expression levels was performed in shrimp systemically infected with WSSV at 0, 12, 24, and 48 hpi (n=3). The control shrimp was injected with LHM media. β-actin was used as an internal control. Three independent experiments were performed (a-c). The *Pm*SERPIN6 gene expression level of infected group was normalized to that of the control group and shown as the mean ±SD (d). Mean with different letters are significantly different (P<0.05).

Lane Neg: negative control for PCR reaction;

Lane 1: 0 h post LHM infection; Lane 2: 0 h post WSSV infection;

Lane 3: 12 h post LHM infection; Lane 4: 12 h post WSSV infection;

Lane 5: 24 h post LHM infection; Lane 6: 24 h post WSSV infection;

Lane 7: 48 h post LHM infection; Lane 8: 48 h post WSSV infection.

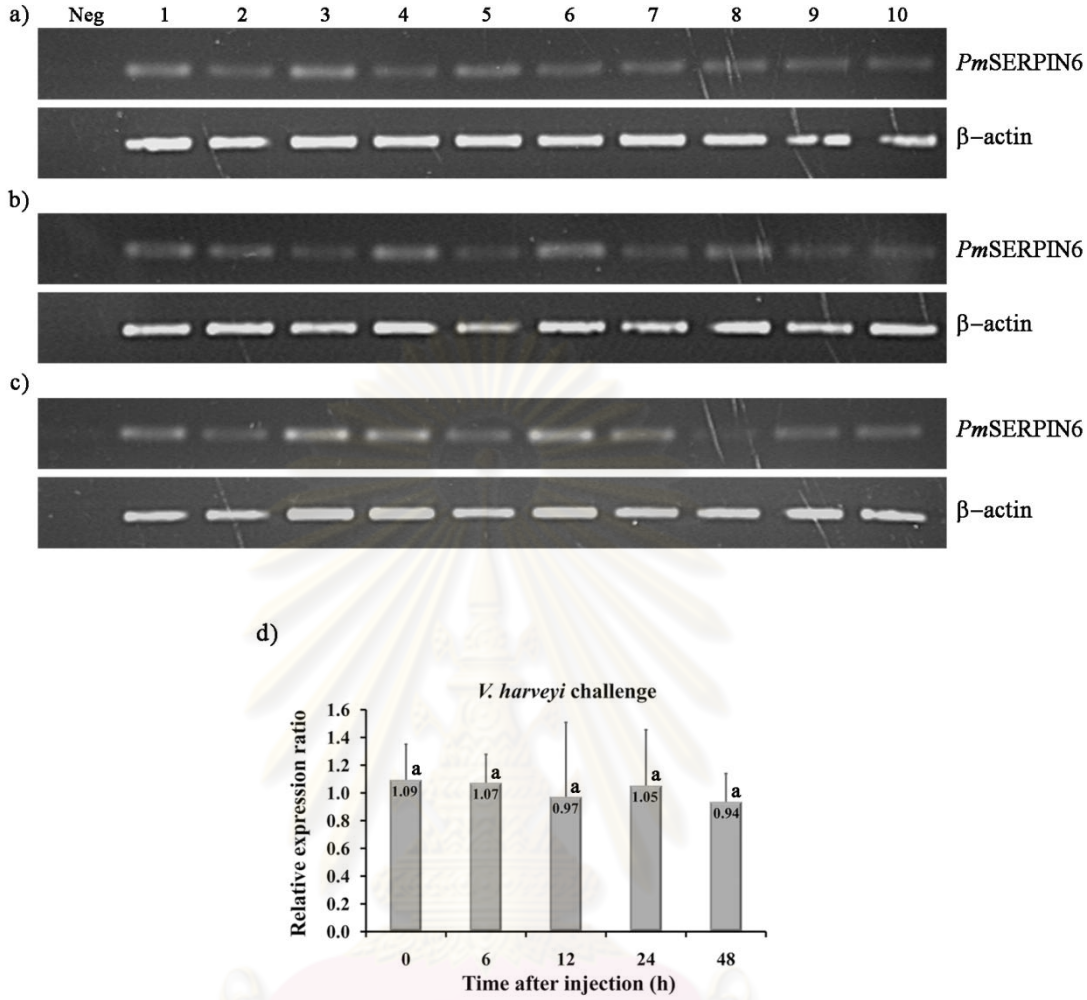


Figure 3.12 Semi-quantitative RT-PCR analysis of *Pm*SERPIN6 gene expression in *P. monodon* hemocyte in response to *V. harveyi* infection. Analysis of *Pm*SERPIN6 gene transcript expression levels was performed in shrimp systemically infected with *V. harveyi* at 0, 6, 12, 24, and 48 hpi (n=3). The control shrimp was injected with 0.85% (w/v) NaCl. β-actin was used as an internal control. Three independent experiments were performed (a-c). The *Pm*SERPIN6 gene expression level of infected group was normalized to that of the control group and shown as the mean ±SD (d). Mean with different letters are significantly different (P<0.05).

Lane Neg: negative control for PCR reaction;

Lane 1: 0 h post saline infection;
Lane 2: 6 h post saline infection;
Lane 3: 12 h post saline infection;
Lane 4: 24 h post saline infection;
Lane 5: 48 h post saline infection;
Lane 6: 0 h post *V. harveyi* infection;
Lane 7: 6 h post *V. harveyi* infection;
Lane 8: 12 h post *V. harveyi* infection;
Lane 9: 24 h post *V. harveyi* infection;
Lane 10: 48 h post *V. harveyi* infection.

3.6 Recombinant *Pm*SERPIN6 protein expression

In order to obtain the *Pm*SERPIN6 protein for further characterization, the recombinant *Pm*SERPIN6 (r*Pm*SERPIN6) was produced in *E. coli* system using two different constructs of expression plasmid.

3.6.1 Construction of the recombinant plasmid pET22b/*Pm*SERPIN6 and pVR500/*Pm*SERPIN6

First, the nucleotide sequences corresponding to the mature peptide of *Pm*SERPIN6 was amplified with forward primer and reverse primer containing His_6-tag coding sequence at their 5´ end. Then, the purified PCR product (Figure 3.13) was digested with *Nco*I and *Bam*HI and cloned into pET-22b(+) cut with the same enzymes. The ligation mixtures were transformed into *E. coli* strain XL-1-Blue. The positive cells containing recombinant plasmid were screened by colony PCR using T7 promoter and T7 terminator primers (Figure 3.14), and then the plasmid was extracted and sequenced.

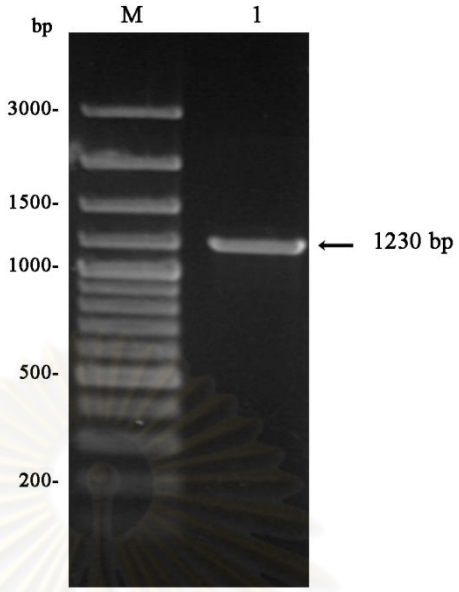


Figure 3.13 Amplified PCR product of the mature *Pm*SERPIN6 gene to be expressed in *E. coli* expression system. Lane M: 100 bp DNA ladder marker; Lane 1: PCR product of the mature *Pm*SERPIN6 gene, about 1,230 bp in size, indicated by the arrow.

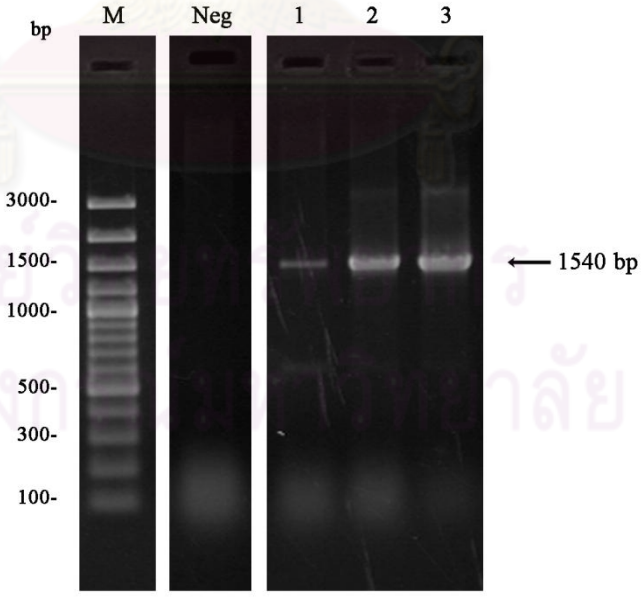


Figure 3.14 Colony PCR screening for positive clones of *E. coli* XL-1-Blue containing pET22b/*Pm*SERPIN6 vector. Lane M: 100 bp DNA ladder marker; Lane Neg: negative control for PCR reaction; Lanes 1-3: product from colony PCR of 3 clones. The positive band of about 1540 bp is shown by the arrow.

The inserted *Pm*SERPIN6 gene from pET22b-*Pm*SERPIN6 vector was sub-cloned into pVR500 at the *Nco*I / *Bam*HI site. The transformants containing desired insert were checked by digestion the recombinant vector with *Nco*I and *Bam*HI (Figure 3.15). The obtained plasmids were then checked for the correctness of sequence.

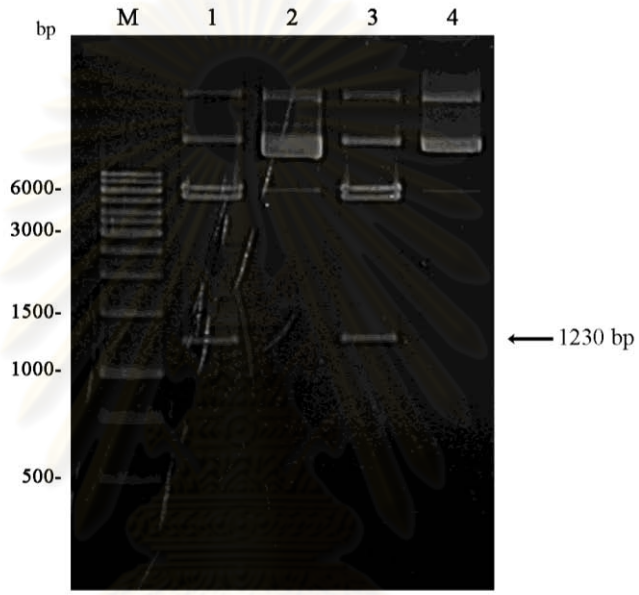


Figure 3.15 Digestion of the recombinant pVR500-*Pm*SERPIN6 plasmid with *Nco*I and *Bam*HI. Lane M: 1kb DNA ladder marker; Lanes 1 and 3 are digested product of pVR500-*Pm*SERPIN6 plasmid; Lanes 2 and 4 are the uncut plasmid. An expected band of about 1,230 bp is indicated by the arrow.

The pET22b-*Pm*SERPIN6 and pVR500-*Pm*SERPIN6 with correct sequence of inserted gene were transformed into expression hosts, *E. coli* strains BL21(DE3) and Rosetta(DE3)pLysS, respectively. The transformants obtained from transformation of pET22b-*Pm*SERPIN6 into *E. coli* BL21(DE3) and pVR500-*Pm*SERPIN6 into Rosetta(DE3)pLysS were finally checked for the presence of the expression plasmid.

3.6.2 r*Pm*SERPIN6 expression in *E. coli* BL21(DE3) using pET-22b(+) expression plasmid

The r*Pm*SERPIN6 was produced after induction the *E. coli* BL21(DE3) containing pET22b-*Pm*SERPIN6 expression plasmid with 1 mM IPTG. The whole cell was collected at various times (0-6 h) after induction and analyzed for the expressed protein by 12.5% SDS-PAGE and coomassie staining. The result showed an increase in expression of the expected r*Pm*SERPIN6 band with the molecular weight of about 45 kDa with maximum intensity at 6 h after induction as shown in Figure 3.16. To determine whether r*Pm*SERPIN6 was expressed in the soluble or inclusion bodies forms, an aliquot of cells was resuspended in 50 mM Tris-Cl, pH 8.4, 5% Glycerol, and 50 mM NaCl and frozen-thawed to break the bacterial cells. The pellet and soluble fractions were then analyzed by 12.5% SDS-PAGE. The result showed that r*Pm*SERPIN6 was expressed in the inclusion bodies form (Figure 3.17).

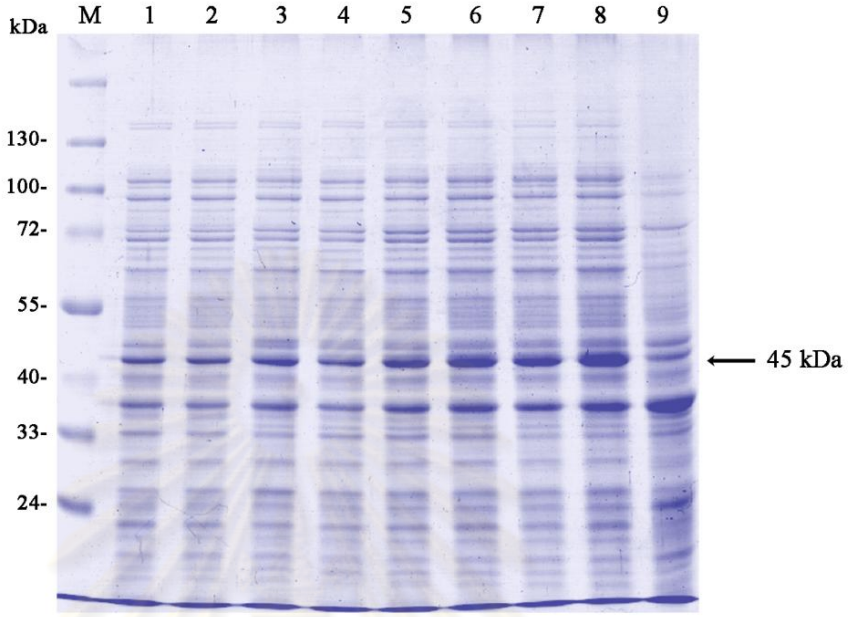


Figure 3.16 SDS-PAGE analysis of small-scale expression of pET22b-*Pm*SERPIN6 in *E. coli* strain BL21(DE3). Lane M: prestained protein marker (PageRuler™ Prestained protein ladder, Fermentas); Lane 1: whole cell at 0 h with no IPTG induction (control); Lanes 2-8: whole cell at 0-6 h after 1 mM IPTG induction; Lane 9: whole cell at 6 h with no IPTG induction (control). An arrow reveals the expected 45 kDa recombinant protein.

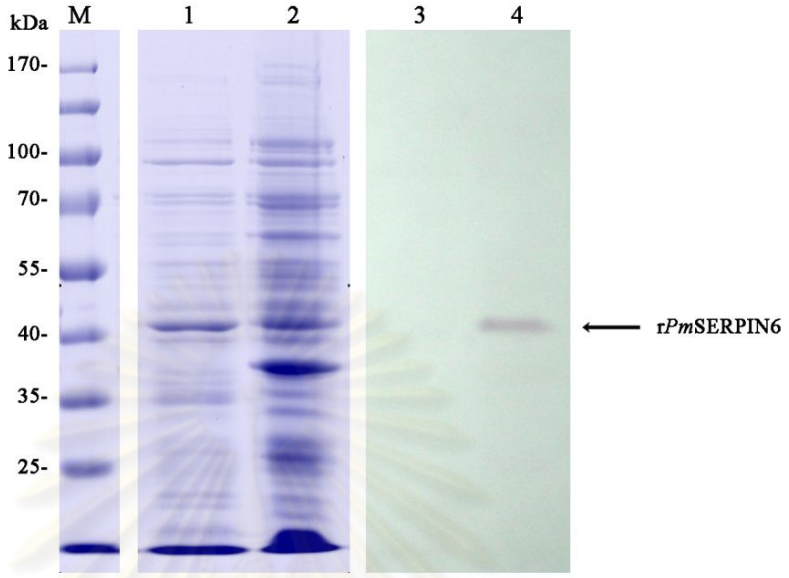


Figure 3.17 SDS-PAGE analysis of r*Pm*SERPIN6 expressed in *E. coli* BL21(DE3) containing pET22b-*Pm*SERPIN6 plasmid at 6 h after IPTG induction. Lane M: prestained protein marker; Lanes 1 and 2: coomassie staining of soluble and inclusion fractions, respectively; Lanes 3 and 4: Western blotting of soluble and inclusion fractions using anti-His_6 antibody as a primary antibody. The expected band of r*Pm*SERPIN6 is shown by the arrow.

As shown in Figure 3.17, the r*Pm*SERPIN6 was expressed as the inclusion bodies form. To obtain the soluble protein from inclusion bodies, the appropriate buffers for solubilization of protein were tested. The r*Pm*SERPIN6 inclusion pellet was aliquoted into two tubes and solubilized for overnight in 50 mM sodium carbonate buffer, pH 10.0 and 50 mM sodium phosphate buffer, pH 12.0, respectively. After that the soluble fractions were analyzed by SDS-PAGE and Western blotting. From Figure 3.18, it was shown that r*Pm*SERPIN6 could not solubilize in both buffers tested. Therefore, the denaturant, 8 M urea, was chosen to solubilize the recombinant protein from the inclusion bodies in the next experiment.

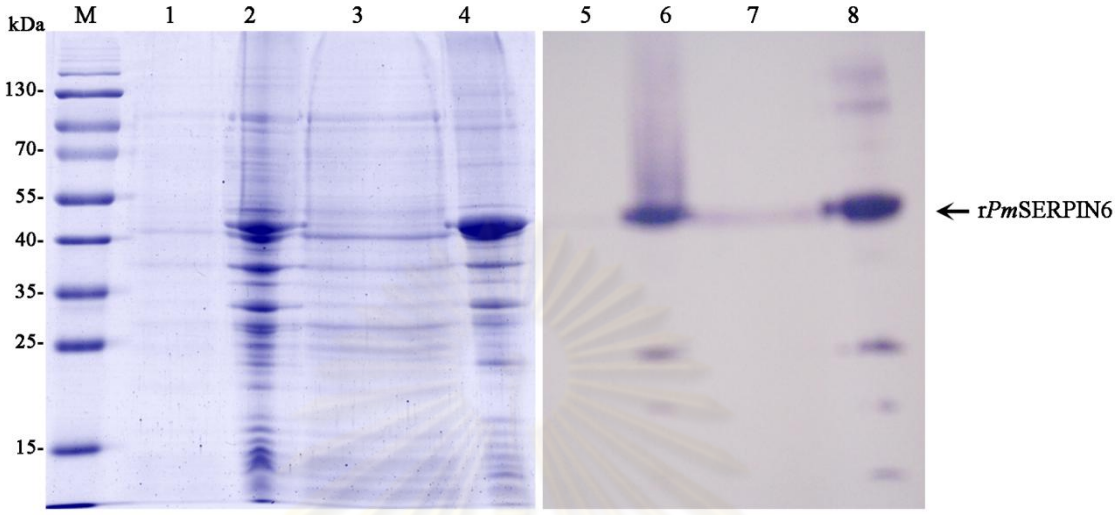


Figure 3.18 Solubilization of r*Pm*SERPIN6 from inclusion bodies with two buffer systems. Lanes M and 1-4 represented coomassie staining gel and lanes 5-8 represented nitrocellulose membrane of Western blot analysis.

Lane M is prestained protein marker;

Lanes 1 and 5 are soluble fraction of inclusion bodies in 50 mM sodium carbonate buffer, pH 10.0;

Lanes 2 and 6 are insoluble fraction of inclusion bodies in 50 mM sodium carbonate buffer, pH 10.0;

Lanes 3 and 7 are soluble fraction of inclusion bodies in 50 mM sodium phosphate buffer, pH 12.0;

Lanes 4 and 8 are insoluble fraction of inclusion bodies in 50 mM sodium phosphate buffer, pH 12.0.

To obtain an adequate amount of protein, the large scale over-expression was carried out. After 6 hours of IPTG induction, cells were harvested and resuspended in 1x PBS buffer, pH 7.4, and subjected to repeat frozen and thawed for 3 times. The cell suspension was completely lysed under high pressure using a French Press. The pellet was then collected. The inclusion bodies was solubilized by 8M urea and purified by HiTrap chelating HP column under denaturing condition and eluted with 200 mM imidazole in binding buffer (Figure 3.19 and 3.20). The purified protein was dialyzed

against 25 mM Tris-Cl, pH 8.0. Unsurprisingly, the r*Pm*SERPIN6 appeared as precipitate after the dialysis. However, the precipitate of r*Pm*SERPIN6 was used as antigen for rabbit immunization in order to produce the specific anti-*Pm*SERPIN6 polyclonal antibody.

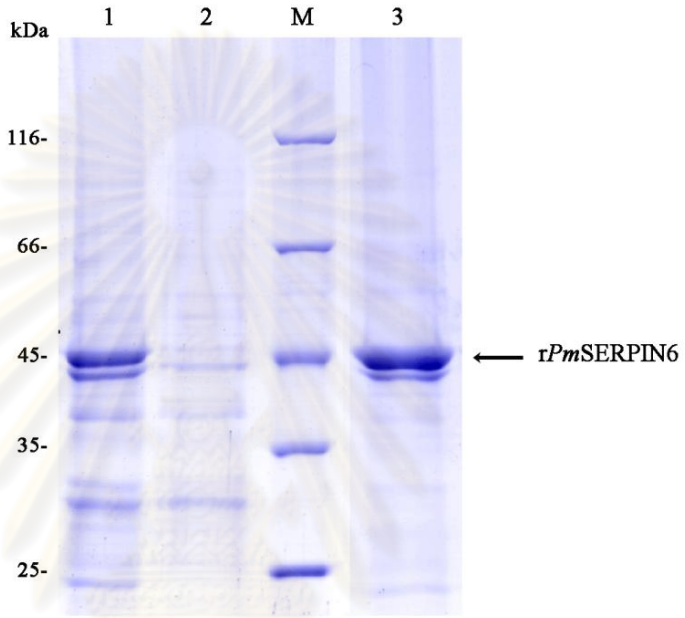


Figure 3.19 SDS-PAGE analysis of the purified r*Pm*SERPIN6. The crude r*Pm*SERPIN6 was purified through Hi-Trap chelating HP column under denaturing condition. Lane M: unstained protein marker (PageRuler™ unstained protein ladder, Fermentas); Lane 1: crude r*Pm*SERPIN6 in 8 M urea buffer; Lane 2: flowthrough fraction; Lane 3: purified r*Pm*SERPIN6 fraction.

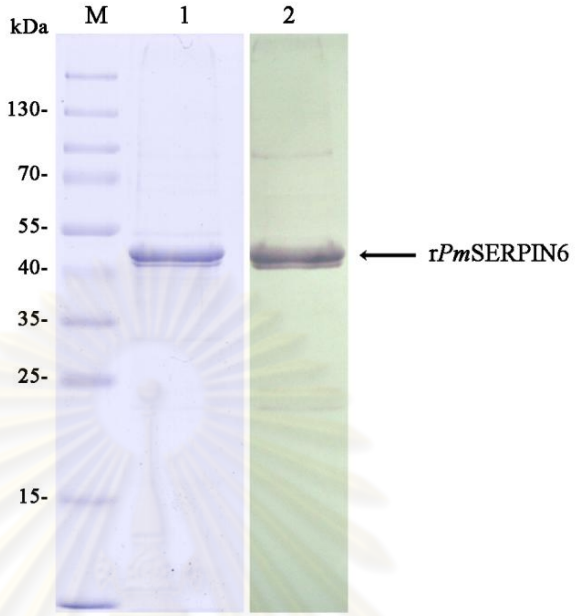


Figure 3.20 Western blotting of the purified r*Pm*SERPIN6 using anti-His_6 antibody. Lane M: prestained protein marker; Lane 1: coomassie stained 12.5% SDS-PAGE gel; Lane 2: Western blot analysis.

3.6.3 r*Pm*SERPIN6 expression in *E. coli* Rosetta(DE3)pLysS using pVR500 expression plasmid

The transformant of *E. coli* Rosetta(DE3)pLysS containing pVR500-*Pm*SERPIN6 plasmid was checked for the protein expression by induction the cells with 1 mM IPTG. The whole cell was collected at 0, 2, 4, and 6 h after induction and analyzed for the expressed protein by 12.5% SDS-PAGE and coomassie staining. The result showed an increase in the expression of the expected r*Pm*SERPIN6-thioredoxin tag fusion protein band with the molecular weight of about 58 kDa as shown in Figure 3.21. The highest expression was observed at 4 h after induction. Analysis of the expressed protein revealed that r*Pm*SERPIN6 was expressed in the inclusion bodies form (Figure 3.22).

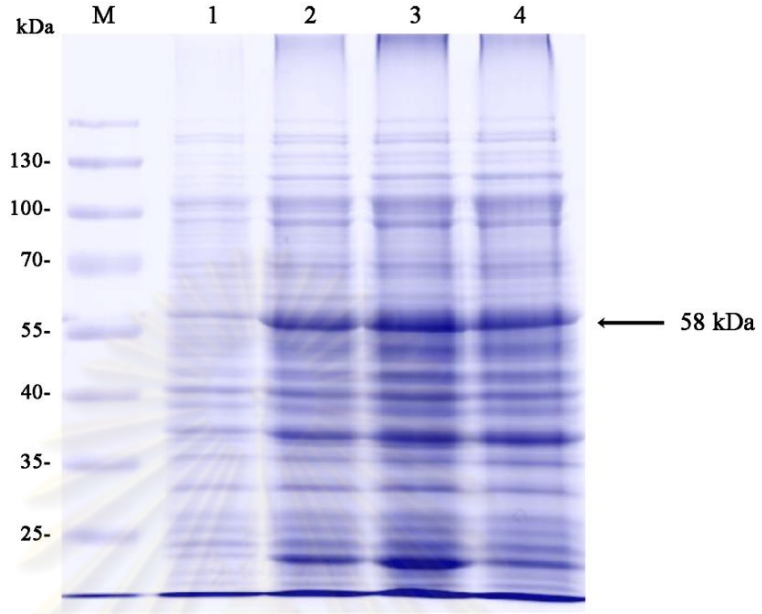


Figure 3.21 SDS-PAGE analysis of small-scale expression of pVR500-*Pm*SERPIN6 in *E. coli* strain Rosetta(De3)pLysS. The protein was run onto 12.5% SDS-PAGE and stained with coomassie brilliant blue. Lane M: prestained protein marker; Lanes 1-4: whole cell at 0, 2, 4, and 6 h after 1 mM IPTG induction. An arrow indicates the expected 58 kDa recombinant protein.

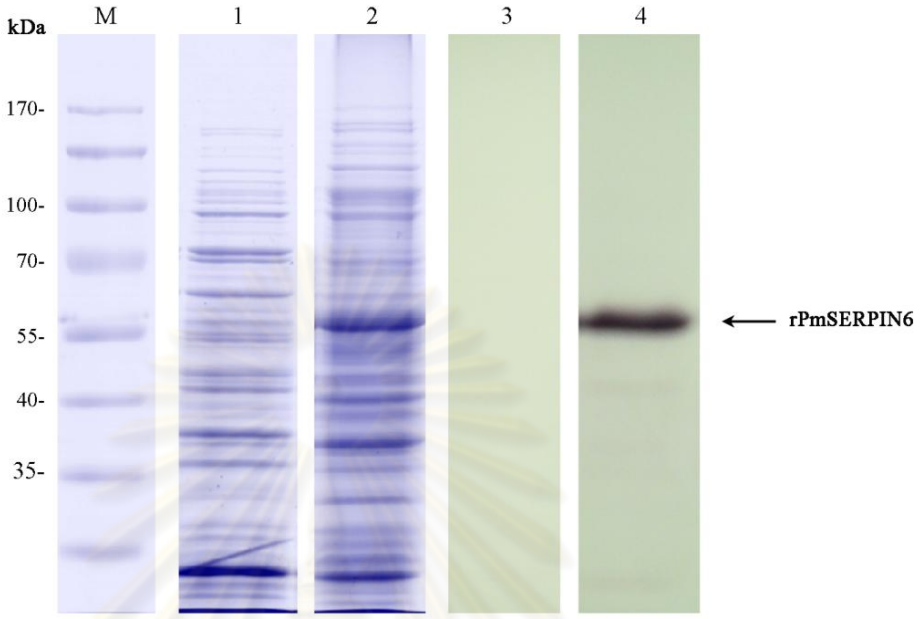


Figure 3.22 SDS-PAGE analysis of r*Pm*SERPIN6 protein expressed in *E. coli* Rosetta(DE3)pLysS containing pVR500-*Pm*SERPIN6 plasmid at 2 h after IPTG induction. Lane M: prestained protein marker; Lanes 1 and 2: coomassie stained 12.5% SDS-PAGE of soluble and inclusion fractions, respectively; Lanes 3 and 4: Western blotting of soluble and inclusion fraction using anti-His_6 antibody as a primary antibody. The expected band of r*Pm*SERPIN6 is shown by the arrow.

Because the r*Pm*SERPIN6 was expressed in inclusion bodies form, the suitable condition for solubilizing the r*Pm*SERPIN6 was then determined. Various buffer solutions including sodium phosphate buffer, pH 8.0, sodium carbonate buffer, pH 10.0, and sodium phosphate buffer, pH 12.0 were used to resuspend the inclusion bodies. After analysis by SDS-PAGE and Western blotting, it was revealed that r*Pm*SERPIN6-thioredoxin tag fusion protein could be partially solubilized from the inclusion bodies with 50 mM sodium phosphate buffer, pH 12.0 (Figure 3.23).

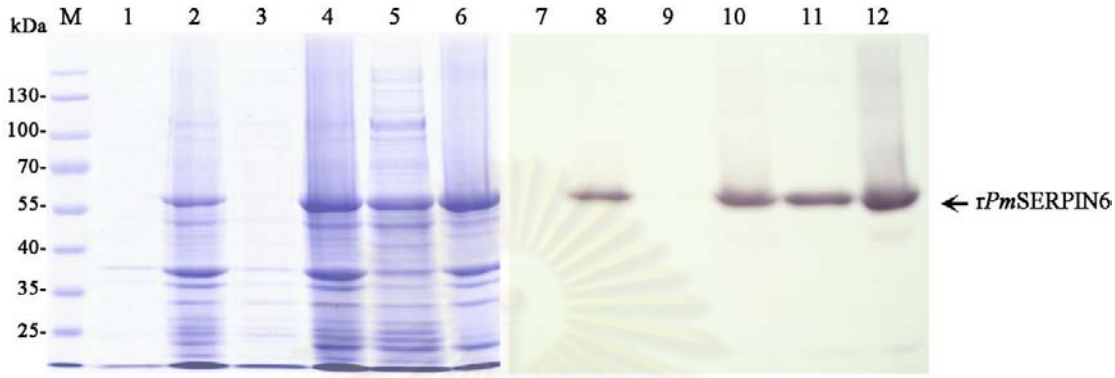


Figure 3.23 Solubilization of inclusion bodies containing r*Pm*SERPIN6-thioredoxin tag fusion protein. The inclusion body pellet was resuspend in various buffer solutions and incubated at 4°C for overnight. The soluble and pellet fractions were separated by centrifugation and subjected to SDS-PAGE and Western blot analysis. Lanes M and 1-6 represent coomassie staining gel and lanes 7-12 represent Western blot analysis.

Lane M: prestained protein marker;

Lanes 1 and 7 are soluble fraction of inclusion bodies in 50 mM sodium phosphate buffer, pH 8.0;

Lanes 2 and 8 are insoluble fraction of inclusion bodies in 50 mM sodium phosphate buffer, pH 8.0;

Lanes 3 and 9 are soluble fraction of inclusion bodies in 50 mM sodium carbonate buffer, pH 10.0;

Lanes 4 and 10 are insoluble fraction of inclusion bodies in 50 mM sodium carbonate buffer, pH 10.0;

Lanes 5 and 11 are soluble fraction of inclusion bodies in 50 mM sodium phosphate buffer, pH 12.0;

Lanes 6 and 12 are insoluble fraction of inclusion bodies in 50 mM sodium phosphate buffer, pH 12.0.

The r*Pm*SERPIN6 protein expression was scaled-up to produce more protein by increasing the volume of the culture medium. After 6 hours of IPTG induction, cells were harvested and resuspended in 1x PBS buffer, pH 7.4, and then repeat frozen and thawed for 3 times. The cell suspension was completely lysed by sonication. The protein in inclusion bodies form was solubilized in 50 mM sodium phosphate buffer, pH 12.0. The supernatant was then dialyzed against 20 mM sodium phosphate buffer, pH 8.0 and then subjected to purification using Ni Sepharose 6 Fast Flow bead (Figures 3.24 and 3.25). The r*Pm*SERPIN6 bound to the column was eluted with 20 mM sodium phosphate buffer containing 200 mM imidazole, pH 8.0. After purification, the protein was dialyzed against 25 mM Tris-Cl, pH 8.0 for further use.

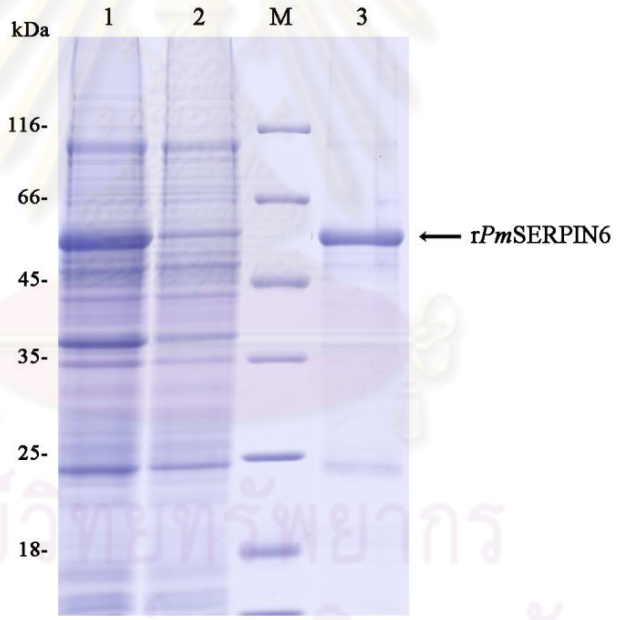


Figure 3.24 r*Pm*SERPIN6-thioredoxin tag fusion protein purification using Ni-NTA column. Lane M: unstained protein marker; Lane 1: crude r*Pm*SERPIN6 in sodium phosphate buffer, pH 12.0; Lane 2: flowthrough fraction; Lane 3: purified r*Pm*SERPIN6 fraction eluted with 20 mM sodium phosphate buffer containing 200 mM imidazole, pH 8.0.

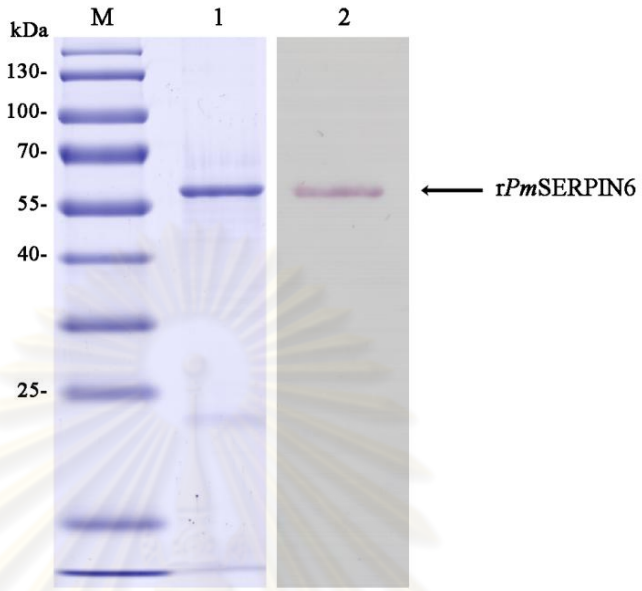


Figure 3.25 Western blotting of purified r*Pm*SERPIN6-thioredoxin tag fusion protein using anti-His_6 antibody. Lane M: prestained protein marker; Lane 1: coomassie staining; Lane 2: Western blot analysis.

3.6.4 Expression of thioredoxin from pET-32a(+) vector

Thioredoxin protein was expressed and used as control in the activity assay. The pET-32a(+) vector was transformed into *E. coli* Rosetta(DE3)pLysS. The transformant was cultured and induced with 1mM IPTG to produce thioredoxin protein.

After 6 h of induction, the cell was collected and resuspended in 20 mM sodium phosphate buffer, pH 8.0. After cell lysis, the supernatant crude thioredoxin protein was collected and subjected to the Ni Sepharose column. The purified protein was digested with thrombin to remove the thioredoxin tag and then re-purified as above. The purified protein was finally checked on SDS-PAGE (Figure 3.26).

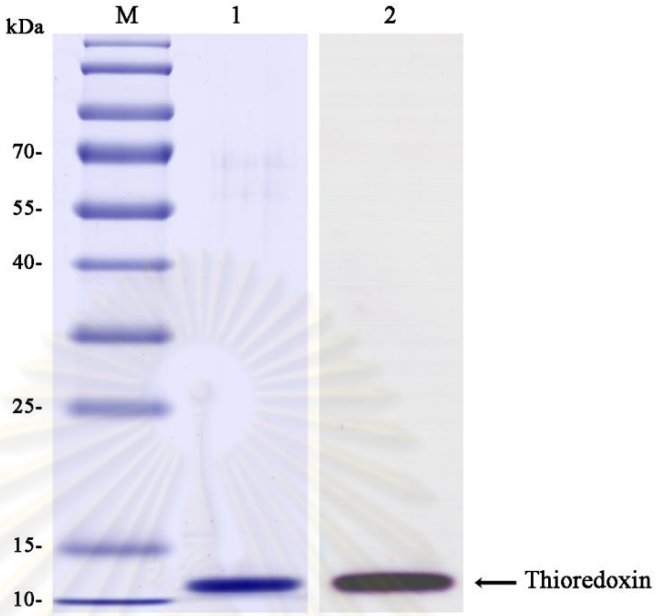


Figure 3.26 The purified thioredoxin protein digested with thrombin. Lane M: prestained protein marker; Lane 1: coomassie staining gel; Lane 2: Western blot analysis using anti-His_6 antibody.

3.7 Detection of native *Pm*SERPIN6 protein in shrimp hemolymph

To detect native *Pm*SERPIN6 protein in hemocyte lysate and cell-free hemolymph of unchallenged shrimp, the anti-*Pm*SERPIN6 polyclonal antiserum was used. Following Western blot analysis, the expected band of about 45 kDa was observed in hemocyte lysate but not in cell-free hemolymph sample (Figure 3.27). This indicated that *Pm*SERPIN6 protein was presented in the hemocyte of unchallenged shrimp as the native protein. The rabbit preimmune serum was used instead of the anti-*Pm*SERPIN6 polyclonal antiserum as a control. No positive band about of 45 kDa was observed in both hemocyte lysate and cell-free hemolymph (data not shown).

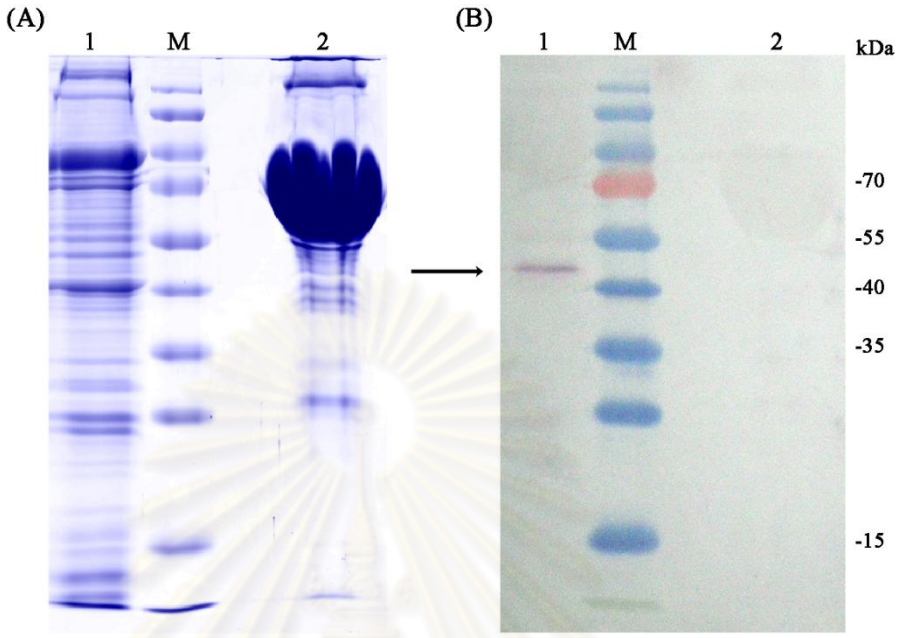


Figure 3.27 Detection of native *Pm*SERPIN6 in hemocyte and cell-free hemolymph of unchallenged shrimp. Fifty micrograms of hemocyte lysate (lane 1) and 200 µg of cell-free hemolymph (lane 2) were separated on 12.5% SDS-PAGE (A) coomassie staining and (B) analyzed by Western blotting using rabbit anti-*Pm*SERPIN6 antiserum. The arrow indicates the expected immune reactive band of about 45 kDa.

3.8 Localization of *Pm*SERPIN6 protein in hemocytes of *V. harveyi* and WSSV infected shrimp

Beside the expression analysis of *Pm*SERPIN6 transcripts under the viral and bacterial infection conditions, we also determined the *Pm*SERPIN6 protein expression using immunocytochemistry technique. The polyclonal anti-*Pm*SERPIN6 antiserum was used to detect the *Pm*SERPIN6 producing hemocytes in both WSSV and *V. harveyi*-infected shrimp (Figure 3.28 (A-E)). The antibody was found to be specific to *Pm*SERPIN6 because no positive cells could be detected in the control where rabbit pre-immune serum was used (Figure 3.28 (a-e)). The positive cells were counted and then calculated as a percentage. The percentages of positive cells of WSSV-challenged shrimp at 0, 6, 24, 48, and 72 hpi were 4.53, 3.92, 3.87, 3.50, and 10.86,

respectively. These indicated that the expression of the *Pm*SERPIN6 protein in hemocyte of WSSV infected shrimp was unchanged during 0 to 48 hpi but significantly increased at 72 hpi by about 2.7 fold (Table 3.3). The expression of *Pm*SERPIN6 protein in response to bacterial challenge was also determined at the same time point as the WSSV infection. It was shown by the percentages of positive cells at 0 to 72 hpi were 4.15, 6.14, 3.25, 4.57, and 7.62, respectively. After *V. harveyi* infection, the *Pm*SERPIN6 positive cells were significantly decreased at 24 hpi as compared to at 6 hpi and then significantly increased again at 72 hpi (Table 3.3). It should be noted that the highest number of positive *Pm*SERPIN6 producing hemocytes upon *V. harveyi* challenge was again at 72 hpi. This implicated that *Pm*SERPIN6 might play role in shrimp anti-viral and -bacterial immunity at the late phase of infection.

Table 3.3 Time-course analysis of *Pm*SERPIN6 protein expression in hemocytes after pathogen infection by immunocytochemistry.

Time after injection (h)	WSSV challenge (Mean of %Positive cells ±SD)	*V. harveyi* challenge (Mean of %Positive cells ±SD)
0	4.5 ±2.8	4.2 ±1.9
6	3.9 ±1.4	6.1 ±0.9
24	3.9 ±1.8	3.3 ±0.8
48	3.5 ±1.2	4.6 ±0.4
72	10.9 ±2.5	7.6 ±2.3

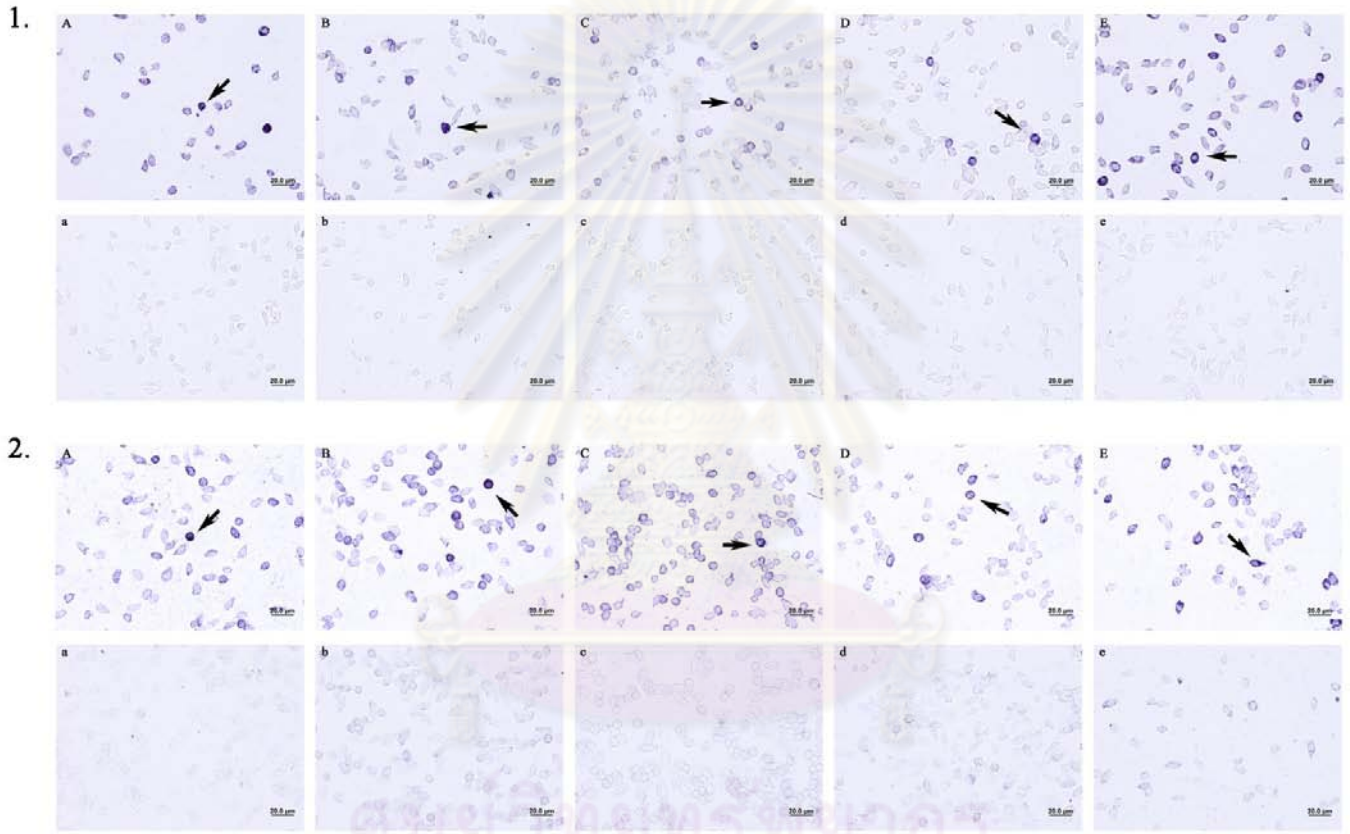


Figure 3.28 Localization of *Pm*SERPIN6 protein in hemocytes of bacterial and viral challenged shrimp by Immunocytochemistry. Infected shrimp hemocytes at 0 (A), 6 (B), 24 (C), 48 (D) and 72 (E) hpi probed with anti-*Pm*SERPIN6 antibody. Rabbit pre-immune serum was used as a control at each time point (a-e). Numbers 1 and 2 are hemocytes from WSSV- and *V. harveyi*-infected shrimp, respectively. The arrows show the positive cells.

3.9 Activity assay

The r*Pm*SERPIN6 protein was tested for its proteinase inhibitory activities against commercial proteinases and on the prophenoloxidase activating system.

3.9.1 Proteinase inhibitory activity assay

To investigate the ability of r*Pm*SERPIN6 to inhibit the proteinase activity, four commercial proteinases; trypsin, subtilisin A, chymotrypsin, and elastase were used. The r*Pm*SERPIN6 at the mole ratio of about 200 exhibited the inhibitory activity against all the tested proteinases. Considering at the highest mole ratio (about 400) of the inhibitor to proteinase tested, the remaining activities of trypsin, subtilisin A, chymotrypsin, and elastase were 13, 13, 39, and 66%, respectively (Figure 3.29).

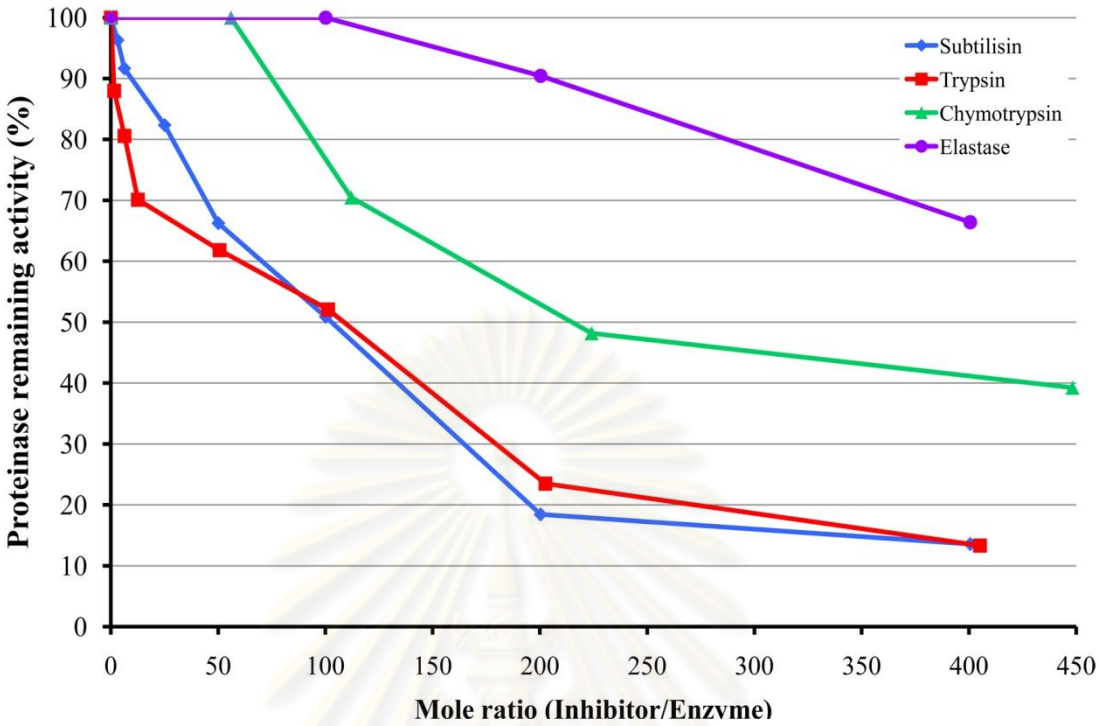


Figure 3.29 Proteinase inhibitory activities of r*Pm*SERPIN6 against commercial proteinases. The inhibitors was incubated with each proteinase at various mole ratio with subtilisin (◆), trypsin (■), chymotrypsin (▲), and elastase (●) in the reaction containing chromogenic substrates. After 20 min of incubation, the remaining activities of proteinases were determined.

The thioredoxin protein was used as a control at the same mole ratio as used in r*Pm*SERPIN6 assay. The results showed that thioredoxin protein did not affect the proteinase activities (data not shown).

3.9.2 Phenoloxidase inhibitory assay

To determine the involvement of the *Pm*SERPIN6 protein in prophenoloxidase (proPO) system, the shrimp hemocyte lysate (HLS) was prepared to assay for the PO activity. The r*Pm*SERPIN6 protein was pre-incubated with HLS followed by PO activation using larminarin. The residual activity of PO activation was monitored by measuring the A_{492} after adding L-DOPA for 30 min. The control

reactions using BSA or thioredoxin instead of r*Pm*SERPIN6 were performed. Blank was the reaction without any protein. Proteinase inhibitor mix was used as a positive control. It was found that the r*Pm*SERPIN6 did not affect the PO activity as compared to the negative control. This indicated that r*Pm*SERPIN6 might not be the inhibitor of the proPO system. The results showed that none of those proteins could inhibit PO activity (Table 3.4).

Table 3.4 Percentage of remaining activity of phenoloxidase in PO system in the presence of r*Pm*SERPIN6.

Protein concentration (μM)	Phenoloxidase remaining activity (%)		
	BSA (Mean)	Thioredoxin (Mean)	r*Pm*SERPIN6 (Mean ± SD)
0.4	-	-	110 ± 15.6
0.8	88.8	77.1	118 ± 15.6
1.6	85.7	107	126 ± 13.3
3.1	81.7	127	154 ± 16.1
Positive control (6X Inhibitor mix (GE Healthcare))	28.3 ± 8.83		

CHAPTER IV

DISCUSSIONS

Shrimp innate immunity composes of several immune reactions providing immediate defense against infection. Of those, the prophenoloxidase activating system and apoptosis involve proteinase cascades that catalyzed change of inactive serine proteinases into their active form. Activation of proteinase cascades benefit to the animal; however, excessive activation is also deleterious. To maintain the optimal level of proteinase cascade activation, serine proteinase inhibitors are synthesized and functioned as regulators of such cascade. In crustaceans, many families of proteinase inhibitors consisting of Kazal, Kunitz, Serpin, α-macroglobulin, and pacifastin have been identified (Liang and Söderhäll, 1995; Liang et al., 1997; Kanost, 1999). In shrimp, only Kazal type serine proteinase inhibitor has been well characterized (Somprasong et al., 2006; Visetnan et al., 2009; Donpudsa et al., 2010).

In invertebrate such as *D. melanogaster*, *M. sexta*, and *B. mori*, several serpins were identified as one of the important serine proteinase inhibitors that play role in the immune responses (Garrett et al., 2009; Zou et al., 2009). Previously, only two shrimp serpins have been identified. Firstly, *P. monodon* serpin called *Pm*SERPINB3 was identified by differential display technique and found to be responded to bacterial infection (Somboonwiwat et al., 2006). In 2009, Liu et al. found the other serpin gene from *F. chinensis*, named *Fc*-serpin, expressed fluctuantly in response to WSSV and bacterial challenge. The availability of the *Penaeus monodon* EST database allows us to search for all shrimp serpin corresponding genes. Unsurprisingly, eight more serpin genes were found in several libraries but at high frequency in hemocyte, gill and gill-epipodite cDNA libraries, designated as *Pm*SERPIN1 - 8. Therefore, at least 9 serpins existed in *P. monodon*. Still, the function of each serpin has to be determined further. Currently, only 3 *Pm*SERPINs from EST database, *Pm*SERPIN6 - 8, had complete ORF. In comparison with previously reported serpins from related organisms, we found that *Pm*SERPIN7 is the same as the *Fc*-serpin with some variations in amino acid sequence. Like *Fc*-serpin, all 3 *Pm*SERPINs showed homology with *Ms*-serpin6

which can negatively regulate prophenoloxidase system in *M. sexta* by inhibiting prophenoloxidase activating proteinase-3 (PAP-3) (Zou and Jiang, 2005).

The phylogenetic tree of serpin amino acid sequences from the related organisms of invertebrate and crustacean revealed that *Pm*SERPIN6 - 8 were clustered in the same group and with *Fc*-serpin, putative serpin from *P. leniusculus*, serpin2 and serpin6 from *M. sexta*, serpin4A and serpin6 from *D. melanogaster*, but *Pm*SERPINB3 was separated into a different group.

Several serpins have been clearly characterized for their functions and target proteinases. Serpins normally act as inhibitor of proteinases in various biological processes. However, non-inhibitory serpins also exist (Gettins, 2002). The conserved three dimensional structures reveal the presence of a scissile bond at the P_1-P_1' site within an exposed loop called reactive center loop (RCL). This specific peptide bond is cleaved by the target proteinase. The P_1 residue normally determines the inhibitory specificity. Primary amino acid sequences of RCL at P_{17}-P_{14} and P_{12}-P_8 are conserved among the inhibitory serpins (P_{17}:E, P_{16}:E/K/R, P_{15}:G, P_{14}:T/S, P_{12}-P_9:A/G/S, and P_8:T) which is rarely found in the non-inhibitory sepins. After proteinase digests the peptide bond between P_1 and P_1' on serpin, the remaining RCL will be inserted into beta-sheetA of core protein leading to conformational change and complex formation. Although the basis of conservation pattern of hinge region is still unknown, it is possible that this sequence facilitates the insertion process. Therefore, non-inhibitory molecules which represent other amino acids instead of the conservation and lack their inhibitory activity might because the side chain is incompatible for sequence insertion. Comparison to the previously identified inhibitory serpin from the tobacco hornworm, *M. sexta*, the shrimp serpins, *Pm*SERPIN6 - 8, and *Fc*-serpin showed the identical of conserved hinge region (P_{17}-$_{14}$; EEGT, and P_{12}-P_8; AAAAT) suggesting that *Pm*SERPIN6 - 8 might be classified as the inhibitory serpin.

In human, there were several reports concerning the mediation of the cellular internalization and the clearance of several serpin-proteinase complexes by serpin-enzyme complex (SEC) receptor but there is no common mechanism and no structural determinant in the serpin-proteinase complex clearance (Joslin et al., 1991). However, the exposure of a binding site, such as the highly conserved-pentapeptide domain

(FVFLM) of human α1-antitrypsin and the loop structure consist of seven underlined amino acid residues (PHDNIVISP) located in the region of the N-terminus of mature protease nexin I, in the complex form is considered to be involved in the clearance mechanism (Knauer et al., 1997). In *P. monodon*, we found the conserved pentapeptide F(L/V)FLI in all *Pm*SERPIN6 - 8. The possible amino acid residues that corresponded to the loop structure located at the amino terminal region of the mature *Pm*SERPIN6 - 8 was ^{37}P(R/E)NFFFSP^{44}. The mechanism of *Pm*SERPIN-proteinase complex clearance mediated by these sequence motifs should be furthere determined.

Mutagenesis of several serpin at the P_1 residue has been carried out to reveal the nature of serpin-proteinase specificity. They found that changing of amino acid at the P_1 position can alter the rate of inhibition with different target proteinases (Kanost, 1999). The predicted P_1 residue of *Pm*SERPIN6 - 7 and 8 were arginine and lysine, respectively. Previously, it was found that serpins containing Arg at their P_1 site can specifically inhibit trypsin, thrombin, or plasmin, suggesting that *Pm*SERPIN6 and 7 might be inhibitors of trypsin, thrombin, or plasmin. Lysine was found at P_1 position of *Pm*SERPIN8 sugesting that its specific proteinase was also might be trypsin-like (Gettins, 2002).

Because we are interested in a serpin which participate in shrimp immunity especially in the PO system, *Pm*SERPIN6 which showed the highest amino acid sequence identity to serpin6 from *M. sexta*, a regulator of pro-phenoloxidase system, was chosen for further characterization in this study. The expression of *Pm*SERPIN6 gene in various shrimp tissues was examined and found that, like *Fc*-serpin (Liu et al., 2009) and a serpin from the crayfish, *P. leniusculus* (Liang and Söderhäll, 1995), *Pm*SERPIN6 was not expressed in hepatopancreas. The presence of *Pm*SERPIN6 as a mature protein in hemocyte but not in cell-free hemolymph of unchallenged shrimp was also demonstrated in this study.

In addition to tissue distribution analysis, we also studied the *Pm*SERPIN6 expression at transcriptional and translational levels in response to microbial injection in order to elucidate its involvement in shrimp immunity. Previously, serpin-6 of *M. sexta* (*Ms*-serpin6) showed significantly increased in the mRNA expression in hemocyte and fat body at 24 h post-*E. coli* injection. Moreover, its protein level was

also significantly induced at 24 h post *E. coli* or *Micrococcus luteus* infection (Wang and Jiang, 2004). *Fc*-serpin was reported as a pathogen responsive gene which is down-regulated at 6 and 12 h post-mixed bacterial challenge. However, it was found to be up-regulated at 8 h post-WSSV challenge and gradually decreased from 14, 23 and 37 h post-infection (Liu et al., 2009). The significant down-regulation of *Pm*SERPIN6 mRNA at the late phase of WSSV infection was also observed. Unlikely, *Pm*SERPIN6 mRNA was not responded to bacterial challenge. Interestingly, the change in expression level upon bacterial and viral challenges of *Pm*SERPIN6 was obviously evidenced at the translational level. Infection shrimp with *V. harveyi* resulted in significant decrease of *Pm*SERPIN6 protein at 24 h post-challenge. The remarkable increment in the expression of the *Pm*SERPIN6 protein was detected at 72 h post-*V. harveyi* and -WSSV infection. Our data suggested that *Pm*SERPIN6 might play roles in shrimp immune response upon both bacteria and virus invasion especially at the late phase of infection.

To assay for *Pm*SERPIN6 inhibitory activities, the recombinant protein was produced in *E. coli* expression system. In the first trial, the mature *Pm*SERPIN6 gene was cloned into the pET-22b(+) vector and transformed to the expression host *E. coli* strain BL21(DE3). The r*Pm*SERPIN6 with a molecular mass of about 45 kDa was produced and purified. Unfortunately, during the refolding step, the soluble r*Pm*SERPIN6 became precipitate. Therefore, the collected precipitated recombinant protein was only used for immunization in the rabbit to generate the antibody against *Pm*SERPIN6 protein but not for activity assay. In the second trial, to overcome the problem of protein precipitation, we constructed a new expression vector in which the r*Pm*SERPIN6 was expressed as a fusion protein. The pVR500 vector, a derivative of pET-32a(+) containing the thioredoxin sequence tag which is able to increase the solubility of the obtained recombinant protein was chosen. The protein production was performed using *E. coli* Rosetta(DE3)pLysS as an expression host. The predicted 58 kDa r*Pm*SERPIN6-thioredoxin fusion protein was purified through His-tag affinity column at pH 8.0. The obtained purified protein was used to assay for the inhibitory activities against commercial proteinases and on phenoloxidase system.

Although, a shrimp serpin, *Fc*-serpin, has been reported, its inhibitory activity has not been revealed (Liu et al., 2009). In this study, the recombinant protein of

*Pm*SERPIN6 was successfully produced. Therefore, we examined the inhibitory activities of r*Pm*SERPIN6. We found that r*Pm*SERPIN6 was able to inhibit all tested proteinases including trypsin, subtilisin A, chymotrypsin, and elastase at the different strength. The r*Pm*SERPIN6 exhibited the strongest inhibitory activity on trypsin followed by subtilisin, chymotrypsin, and elastase, respectively. According to the primary protein sequence analysis, the predicted P_1 residue of *Pm*SERPIN6 was arginine that made it was specific against trypsin. Surprisingly, the results showed that the remaining proteinase activity of all tested proteinases in this assay did not decline to the baseline level. It is possible that exposure of r*Pm*SERPIN6 at the high pH for a long period during inclusion bodies solubilization resulted in loss of their scaffold or their function. Another reason is that the thioredoxin fusion tag within the r*Pm*SERPIN6 protein might cause improper folding of the protein which leads to the reduction of its proteinase inhibitory activity.

Prophenoloxidase system is an important proteinase cascade mainly involve in defense response of insect and crustacean. Several proteinase inhibitors such as melanisation inhibition protein (MIP), pacifastin, and serpins act as a negative regulator of the proPO system. So far, MIP has been identified from *P. leniusculus* (*Pl*MIP) and *P. monodon* (*Pm*MIP) (Söderhäll et al., 2009; Angthong et al., 2010). However, only *Pl*MIP has been proPO reported for its ability to regulate activation. In the freshwater crayfish, *P. leniusculus*, the pacifastin has been identified. It exhibits the inhibitory activity on the crayfish hemolymph proteinase involving in the proPO cascade activation (Hergenhahn et al., 1987; Liang et al., 1997). Furthermore, many proteins in serpin family were characterized as the inhibitor of proPO system at the different step. In *Drosophila*, serpin-27A regulates the melanisation cascade through specific inhibition at the terminal proPO activating enzyme, whereas serpin-28D controls PO availability at its initial release (Nappi et al., 2005; Scherfer et al., 2008). In tobacco hornworm, *M. sexta*, serpin-3 and -6 were reported as the inhibitor of prophenoloxidase activating proteinase (PAP) (Zhu et al., 2003; Zou and Jiang, 2005), while serpin-4 and -5 regulate at the upstream of PAP (Tong and Kanost, 2005). Until now, researches on regulation of proPO system by shrimp serpin have not been reported. In this study, the inhibitory activity assay on prophenoloxidase system of the r*Pm*SERPIN6 was investigated. Unfortunately, no significant difference in

phenoloxidase activity was found after r*Pm*SERPIN6, thioredoxin, or BSA incubation. The obtained results implied that *Pm*SERPIN6 could not inhibit the phenoloxidase activity and might not involve in regulation of PO system. As shown in Appendix A that the *Pm*SERPIN6 protein contained four predicted *N*-linked glycosylation sites, therefore, the protein glycosylation might be important to protein functions. In this study, the r*Pm*SERPIN6 was produced in the *E. coli* system which has no the post-translational modification. This might be the cause of lack of *Pm*SERPIN6 ability to inhibit the phenoloxidase activity. The involvement of r*Pm*SERPIN6 in proPO system should be further investigated using the recombinant protein produced in the eukaryotic system.

CHAPTER V

CONCLUSIONS

1 From the *Penaeus monodon* EST database, at least eight different types of serpins were identified, named *Pm*SERPIN1 – 8.

2 Only three serpins from the *P. monodon* EST database, *Pm*SERPIN6 - 8, were identified with the complete open reading frame encoding for protein with 415, 411, and 417 amino acid residues in length, respectively. The 19 residues of the signal peptide of all three *Pm*SERPINs were predicted.

3 The full-length cDNA of a *Pm*SERPIN6 gene was successfully amplified from cDNA of unchallenged shrimp. The calculateed molecular weight and pI of the *Pm*SERPIN6 protein were 44.5 kDa and 7.33, respectively. Four positions of the *N*- linked glycosylation site along the sequence of the *Pm*SERPIN6 protein were predicted.

4 The phylogenetic tree showed that the *Pm*SERPIN6 was clustered in the same group with *Fc*-serpin, putative serpin from *P. leniusculus*, serpin2 and serpin6 from *M. sexta*, serpin4A and serpin6 from *D. melanogaster*.

5 *Pm*SERPIN6 transcript was expressed in most of the tissues tested especially in lymphoid organ, hemocyte, heart, and gill but not in hepatopancreas.

6 In response to WSSV challenge, the significant decrease in the relative expression ratio of the *Pm*SERPIN6 gene in shrimp hemocyte was observed at 24 hpi compared to that of 0 hpi.

7 No significant difference in the relative expression ratio of the *Pm*SERPIN6 gene in shrimp hemocyte was observed upon *V. harveyi* challenge,

8 The native *Pm*SERPIN6 protein was found in hemocyte but not in cell-free hemolymph of unchallenged shrimp.

9 Immunocytochemistry using a specific anti-*Pm*SERPIN6 polyclonal antibody revealed that the *Pm*SERPIN6 producing hemocyte was increased in both WSSV- and *V. harveyi*-challenged shrimp at 72 hpi implying that the *Pm*SERPIN6 protein responded in the late phase of infection with virus or bacteria and might be implicated in shrimp immunity.

10 The r*Pm*SERPIN6 exhibited proteinase inhibitory activity against trypsin, subtilisin A, chymotrypsin, and elastase. The remaining activities of those proteinases after incubation with the r*Pm*SERPIN6 at mole ratio of inhibitor/proteinase of about 400 were 13, 13, 39, and 66%, respectively.

11 The residual activity of PO after pre-incubation of the r*Pm*SERPIN6 with hemocyte lysate was unchanged suggesting that the *Pm*SERPIN6 protein did not inhibit the PO activity. The result implied that the *Pm*SERPIN6 might not involve in PO system regulation.

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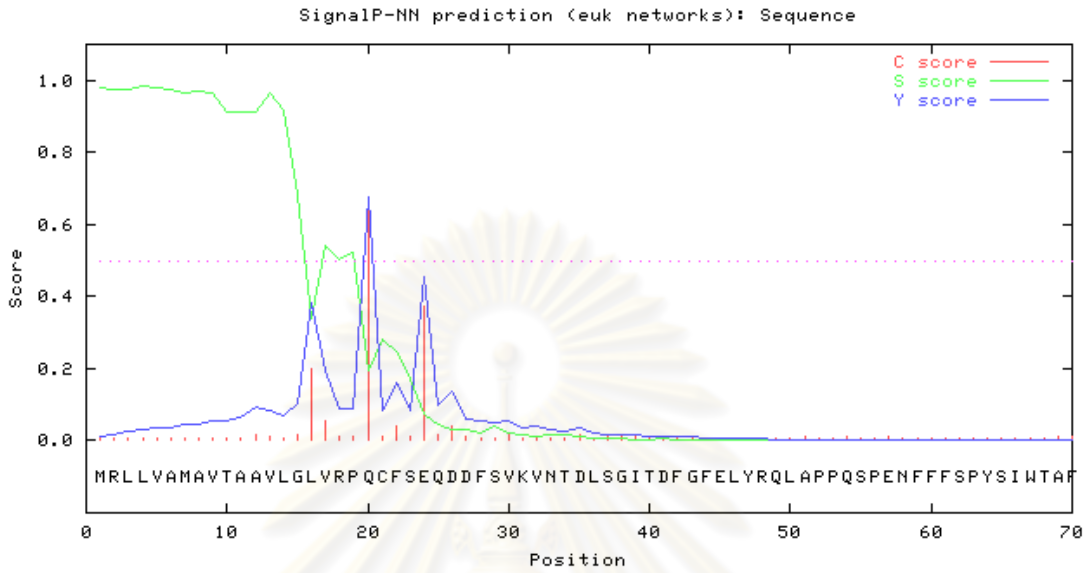
Appendices

Appendix A

Signal peptide prediction by SignalP Server



>*Pm*SERPIN6 length = 70

#	Measure	Position	Value	Cutoff	signal peptide?
	max. C	20	0.645	0.32	YES
	max. Y	20	0.675	0.33	YES
	max. S	4	0.984	0.87	YES
	mean S	1-19	0.841	0.48	YES
	D	1-19	0.758	0.43	YES

Most likely cleavage site between pos. 19 and 20: VRP-QC

The N-Glycosylation sites prediction with NetNglyc server

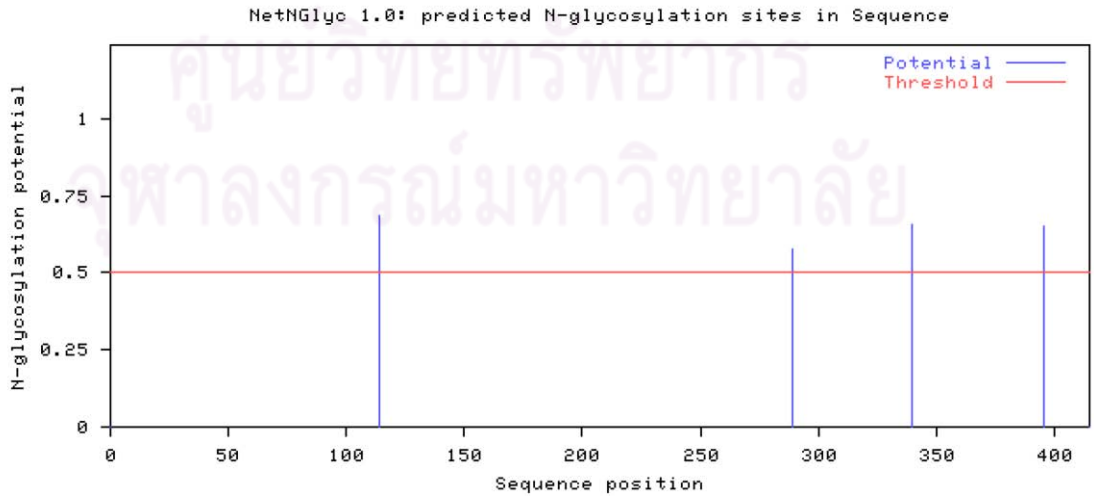
Name: *Pm*SERPIN6 Length: 415

```
MRLLVAMAVTAAVLGLVRPQCFSEQDDFSVKVNTDLSGITDFGFELYRQLAPPQSPENFFFSPYSIWTAFTLAYFGSGGE 80
TAAQLQRALRVDDQVATLKLWRALEAMYRTRQQNTTAYSFNIANRAYIDKNLPIRDCITNLLHSGVDRVQFSKVGFVTQE 160
INNFVSVATKGRISKIVSVADLADAIMVLVNAAYFKGTWQYQFKPSNTFPEPFFATSQNSDLVPMMHQTASFRYNEFSEI 240
AAKVLELPYTGDAMSMFVFLPSEEGPRGFANMVARLSGNNLRAATHKGNLSFRMVDVKLPKFKMEVEVRDEFKPVLHNMG 320
ITDIFNSEKVDLTTFGPLRNVTLEKVIHKAFVEVNEEGTEAAAATALIFATRSGGARPLPVEFHCNRPFVFLIRDNDTHT 400
VLFMGSYKKPVKASS
```

```
.............................................................................. 80
.............................N................................................ 160
.............................................................................. 240
.............................................N................................ 320
..................N.....................................................N.... 400
...............                                                              480
```

(Threshold=0.5)

SeqName	Position	Potential	Jury	N-Glyc agreement result	
Sequence	114	NTTA	0.6846	(9/9)	++
Sequence	289	NLSF	0.5797	(7/9)	+
Sequence	340	NVTL	0.6562	(9/9)	++
Sequence	396	NDTH	0.6516	(8/9)	+



Protein molecular mass prediction by Genetyx program

*Pm*SERPIN6 mature protein

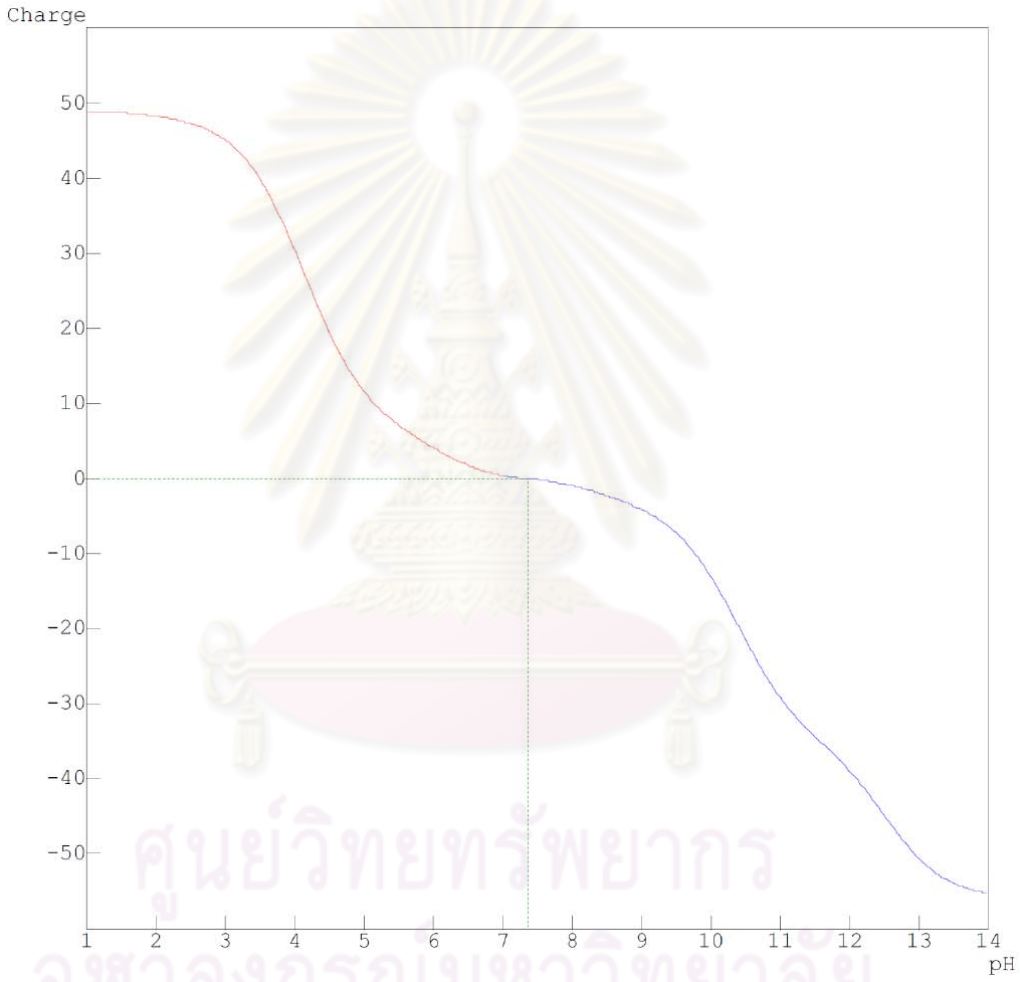
```
Sequence Size    : 396
Sequence Position: 1 - 396

hydrophobic:   201( 50.76)
neutral    :    95( 23.99)
hydrophilic:   100( 25.25)
other      :     0(  0.00)

[ hydrophobic residues ]
Gly(G) 20( 5.05%)  Ala(A) 36( 9.09%)  Val(V) 32( 8.08%)  Leu(L) 30( 7.58%)
Ile(I) 16( 4.04%)  Met(M) 11( 2.78%)  Phe(F) 34( 8.59%)  Trp(W)  3( 0.76%)
Pro(P) 19( 4.80%)

[ neutral residues ]
Ser(S) 27( 6.82%)  Thr(T) 27( 6.82%)  Asn(N) 23( 5.81%)  Gln(Q) 15( 3.79%)
Cys(C)  3( 0.76%)

[ hydrophilic residues ]
Asp(D) 19( 4.80%)  Glu(E) 22( 5.56%)  Lys(K) 20( 5.05%)  His(H)  7( 1.77%)
Arg(R) 21( 5.30%)  Tyr(Y) 11( 2.78%)

[ other residues ]
Asx(B)  0( 0.00%)  Glx(Z)  0( 0.00%)  Xaa(X)  0( 0.00%)  ???(?)  0( 0.00%)

Average Molecular Weight = 44507.99
Monoisotopic Molecular Weight = 44480.4660
```

The prediction of pI of mature protein by Genetyx program

_Pm_SERPIN6 mature protein

Sequence Size : 396

Sequence Position: 1 - 396



Amino Acid	Number	pKa
Arg (R)	21	12.5
His (H)	7	6.0
Lys (K)	20	10.5
Asp (D)	19	3.9
Cys (C)	3	8.3
Glu (E)	22	4.3
Tyr (Y)	11	10.1
N-terminal Gln (Q)		9.1
C-terminal Ser (S)		2.2

Isoelectric point: 7.33

Appendix B

GenBank accession numbers for phylogenetic analysis

Table B1 Species, abbreviations, GenBank accession numbers and gene names of serpins used for the phylogenetic analysis.

Species, Abbreviation	GenBank Accession number	Gene names
Penaeus monodon		
Pm SPN6	GQ260129	*Pm*SERPIN6
Pm SPN7	GU358487	*Pm*SERPIN7
Pm SPN8	GU358488	*Pm*SERPIN8
Pm SPNb3	GQ260130	*Pm*SERPINB3
Fenneropenaeuse chinensis		
Fc Serpin	ABC33916	Serpin serine protease inhibitor
Manduca sexta		
Ms SPN1	AAC47342	Serpin-1
Ms SPN2	AAB58491	Serpin-2
Ms SPN3a	AAO21505	Serpin-3a
Ms SPN3b	AAO21506	Serpin-3b
Ms SPN6	AAV91026	Serpin-6
Pacifastacus leniusculus		
Pl Serpin	CAA57964	Putative serine proteinase inhibitor
Tachypleus tridentatus		
Tt CoagIn	BAA03374	Intracellular coagulation inhibitor precursor
Tt CoagIn2	BAA06909	*Limulus* intracellular coagulation inhibitor type 2 precursor
Tt CoagIn3	BAA12795	Intracellular coagulation inhibitor type3

Table B1 (cont.) Species, abbreviations, GenBank accession numbers and gene names of serpins used for the phylogenetic analysis.

Species, Abbreviation	GenBank Accession number	Gene names
Bombyx mori		
Bm AnTryp	NP_001037305	Antitrypsin precursor
Bm AnTryp1	ACT36276	Antitrypsin isoform 1
Bm AnTryp2	ACT36277	Antitrypsin isoform 2
Bm AnTryp3	ACT36278	Antitrypsin isoform 3
Bm AnTryp4	ACT36279	Antitrypsin isoform 4
Bm AnChy	NP_001037530	Antichymotrypsin precursor precursor
Bm AnChyII	AAB20776	Antichymotrypsin II
Bm Serpin	NP_001040318	Serine protease inhibitor serpin
Bm SPN LP	NP_001036857	Serpin like protein (SEP LP)
Bm SPN2	AAF61252	Serpin-2
Bm SPN4a	NP_001037090	Serpin-4A
Bm SPN5	NP_001037205	Serpin-5
Bm SPN6	ABV74209	Serpin-6
Bm SPN7	NP_001139701	Serpin-7
Bm SPN8	NP_001139702	Serpin-8
Bm SPN10	NP_001139703	Serpin-10
Bm SPN11	NP_001139704	Serpin-11
Bm SPN13	NP_001139705	Serpin-13
Bm SPN14	NP_001139706	Serpin-14
Bm SPN15	NP_001139707	Serpin-15
Bm SPN16	NP_001139708	Serpin-16
Bm SPN17	NP_001139710	Serpin-17
Bm SPN18	NP_001139711	Serpin-18
Bm SPN19	NP_001139712	Serpin-19
Bm SPN20	NP_001139713	Serpin-20
Bm SPN21	NP_001139714	Serpin-21
Bm SPN22	NP_001139715	Serpin-22
Bm SPN23	NP_001139716	Serpin-23
Bm SPN24	ACG61187	Serpin-24
Bm SPN25	NP_001139717	Serpin-25
Bm SPN26	NP_001139718	Serpin-26
Bm SPN27	ACG61190	Serpin-27
Bm SPN28	NP_001139719	Serpin-28
Bm SPN29	NP_001139720	Serpin-29
Bm SPN30	NP_001139721	Serpin-30
Bm SPN31	NP_001139722	Serpin-31
Bm SPN32	NP_001139723	Serpin-32
Bm SPN33	NP_001129363	Serpin-33
Bm SPN34	NP_001129364	Serpin-34

Table B1 (cont.) Species, abbreviations, GenBank accession numbers and gene names of serpins used for the phylogenetic analysis.

Species, Abbreviation	GenBank Accession number	Gene names
Drosophila melamogaster		
Dm SPN1	NP_524958	Serine protease inhibitor 1
Dm SPN2	NP_524957	Serine protease inhibitor 2
Dm SPN3	NP_524956	Serine protease inhibitor 3
Dm SPN4	CAD21892	Serpin 4
Dm SPN5	CAB63100	Serine protease inhibitor (serpin-5)
Dm SPN6	NP_524953	Serine protease inhibitor 6
Dm SPN7	NP_609128	Serine protease inhibitor 7
Dm SPN27A	AAF24518	Serpin-27A
Dm SPN28B	Q9VLZ8	Spn28B*
Dm SPN28D	Q9VLU4	Spn28D*
Dm SPN28Da	Q8IPH2	Spn28Da*
Dm SPN28Db	Q9VLV3	Spn28Db*
Dm SPN31A	Q9VL44	Spn31A*
Dm SPN42Db	Q9V990	Spn42Db*
Dm SPN42Dc	Q9V989	Spn42Dc*
Dm SPN42De	Q8SZF4	Spn42De*
Dm SPN43Aa	NP_524805	Serine protease inhibitor 43Aa
Dm 43Ab b	NP_001027395	Serine protease inhibitor 43Ab, isoform B
Dm 43Ab a	NP_524804	Serine protease inhibitor 43Ab, isoform A
Dm SPN43Ac	AAQ64953	Spn43Ac
Dm SPN43Ad	NP_610261	Spn43Ad
Dm SPN47C	Q9V5S3	Spn47C*
Dm SPN53F	Q9V7Y9	Spn53F*
Dm SPN75F	Q8IQT8	Spn75F*
Dm Acp76A	Q9VVW1	Spn76A*
Dm SPN77Ba	Q8IGD7	Spn77Ba*
Dm SPN77Bb	Q9VWB4	Spn77Bb*
Dm SPN77Bc	Q9VPH9	Spn77Bc*
Dm SPN85F	AAF54473	Spn85F*
Dm SPN88Eb	Q9VFC1	Spn88Eb*
Dm SPN100A	Q9VA48	Spn100A*
Dm pep76A	NP_524153	Accessory gland specific peptide 76A

*Reference
Reichhart J M. (2005). "Tip of another iceberg: *Drosophila* serpins". *Trends Cell Biol* 15(12):659-665.

Appendix C

Statistic analysis by ANOVA and DUNCAN test

Expression analysis in response to pathogen infection by RT-PCR

1. **Expression profiling of *Pm*SERPIN6 mRNA upon WSSV infection**.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.326	3	0.109	2.603	0.124
Within Groups	0.334	8	0.042		
Total	0.661	11			

DUNCAN

Hours	N	Subset for alpha = .05	
		1	2
24.00	3	0.8773	
12.00	3	1.0087	1.0087
48.00	3	1.1470	1.1470
0.00	3		1.3217
Sig.		0.1597	0.1097

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

2. Expression profiling of *Pm*SERPIN6 mRNA upon *V. harveyi* infection.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.056	4	0.014	0.117	0.974
Within Groups	1.196	10	0.120		
Total	1.252	14			

DUNCAN

Hours	N	Subset for alpha = .05
		1
48.00	3	0.9357
12.00	3	0.9717
24.00	3	1.0527
6.00	3	1.0727
0.00	3	1.0940
Sig.		0.6147

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

Appendix D

Presentations

1. The 35th Congress on Science and Technology of Thailand (STT35). Science and Technology for a Better Future. October 15-17, 2009. The Tide Resort (Bangsaen Beach), Chonburi, Thailand. "Expression Analysis of SERPIN*Pm*6 of the Black Tiger Shrimp *Penaeus monodon* in Response to Pathogen Infection". (Oral presentation)

2 The 18th Science Forum 2010. March 11-12, 2010. Faculty of Science, Chulalongkorn University, Bangkok, Thailand. "The Involvement of *Pm*SERPIN6 from the Black Tiger Shrimp *Penaeus monodon* in the Immune System". (Oral presentation)

Award: The runner-up oral presentation award in the biological science session of the Science Forum 2010 at Chulalongkorn University, Thailand

Publication

1. Homvises T, Tassanakajon A, Somboonwiwat K. (2010). "*Penaeus monodon* SERPIN, *Pm*SERPIN6, is implicated in the shrimp innate immunity". *Fish Shellfish Immunol* 29(5):890-898.

Fish & Shellfish Immunology 29 (2010) 890–898

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Short communication

Penaeus monodon SERPIN, *Pm*SERPIN6, is implicated in the shrimp innate immunity

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ABSTRACT

Serine proteinase inhibitors (SERPINs or serpins) have been found in a diverse range of organisms. Herein, eight serpin genes, namely *Pm*SERPIN1 – 8, were identified from the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/home.jsp). Among those, *Pm*SERPIN6 was selected for further characterization. Tissue distribution analysis revealed that *Pm*SERPIN6 transcripts were expressed in the lymphoid organ, hemocyte, heart and gill, but not in the hepatopancreas. Semi-quantitative RT-PCR analysis at 0–48 h after pathogen challenge demonstrated that the *Pm*SERPIN6 gene transcript expression levels in hemocytes was slightly decreased after systemic white spot syndrome virus (WSSV) injection but remained unchanged upon *Vibrio harveyi* injection. Interestingly, immunocytochemistry using anti-*Pm*SERPIN6 polyclonal antiserum showed an increase in the number of *Pm*SERPIN6 producing hemocytes at 72 h after both WSSV and *V. harveyi* injections indicating that the expression of *Pm*SERPIN6 responded to pathogen in the late phase of infection. Our results suggest a likely important function of *Pm*SERPIN6 in the shrimp's defense against invading pathogens.

1. Introduction

In all multicellular organisms, the innate immunity is the key system to defend against foreign invaders and can be divided into the cellular and humoral responses. Some immune responses, such as hemolymph coagulation, complement activation, melanization, phagocytosis and encapsulation, are mediated by proteinase cascades. These defense mechanisms are shown to be regulated by serine proteinase inhibitors [1]. Different serine proteinase inhibitors, such as Kazal, Kunitz, α-macroglobulin, serpin etc., have been found [2].

Serpin is a class of inhibitors that act as suicide-like substrates [3]. They irreversibly inhibit their specific target proteinases. They are found in most organisms, except fungi, and have a relatively large molecular weight of 40–60 kDa and about 400 amino acid residues in length. Serpins function as a regulator of various biological processes [4]. However, whilst many serpins have a proteinase inhibitory activity, some serpins cannot inhibit any proteinases and are called non-inhibitory serpins [5].

A typical feature of serpins is the reactive center loop (RCL), which is an exposed protein motif composed of about 20 amino acids located near its C-terminus. This motif contains a scissile bond between two residues, called P_1 and P_1', which is cleaved by the target proteinase [6]. The inhibitory serpin forms a complex with the specific target proteinase and is cleaved by the proteinase leading to a large conformational change. A very stable serpin-proteinase complex is subsequently formed resulting in the inactivation of the proteinase activity [7]. The key amino acid residue that determines the target specificity is the amino acid at the P_1 residue [4].

Within insects a diverse number of serpin genes have been identified by genomic approaches in *Bombyx mori* [8] and *Drosophila melanogaster* [9]. Some of them have been characterized for their functions. In *D. melanogaster*, some serpins were identified as a regulator of different immune reactions. Serpin-28D regulates hemolymph phenoloxidase (PO), a key enzyme in the proPO system and serpin-28D deficient flies show an extensive melanization in tissues that are exposed to air [10]. Serpin-27A functions as a negative inhibitor of proPO activation by inhibiting the proPO activating enzyme (PPAE) [11].

In the tobacco hornworm, *Manduca sexta*, several serpin members have been characterized. The *M. sexta* serpin-3, which regulates the proPO system by inhibiting the proPO activating proteinases (PAPs), is up-regulated in the hemolymph upon microbial challenge [12]. Also, as regulators of proPO system, serpin-4 and serpin-5 inhibit proteinases upstream of the PAP

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reactions but not directly to the PAPs [13]. Serpin-6 was identified in the complexes of serpins with PAP-3 and hemolymph proteinase 8 [14].

In crustaceans, only a few serpins have been reported which seems an omission given their otherwise fairly well conserved immune systems at a broad level. A serpin cDNA has been identified from the hemocyte of crayfish, *Pacifastacus leniusculus*, but the functions have not been investigated [15]. Three serpins from the Japanese horseshoe crab, *Tachypleus tridentatus*, were reported to be intracellular coagulation inhibitors, designated as TICI types 1–3 [16–18]. Recently, some serpin genes were found to be up-regulated in *Scylla paramamosain* in response to lipopolysaccharide challenge [19]. Two serpin genes such as serine protease inhibitor Serpin homolog and serpin-6 have been identified from hemocyte of *Eriocheir sinensis* infected with the mixture of *Listonella anguillarum* and *Staphylococcus aureus* [20]. So far, only few serpin genes have been reported in shrimp. A shrimp serpin was first reported in *P. monodon*, called *Pm*SERPINB3, and its transcript was found to be up-regulated upon bacterial infection [21]. A serpin from *Fenneropenaeus chinensis* (*Fc*-serpin) was identified and expression profiling showed differences in its response to bacterial and viral stimulation [22].

In light of the important functions of serpins especially in the immune system, we therefore took an advantage of the data available in the *P. monodon* EST database to search for all serpins from the database and then analyze their sequences. An interesting serpin, named *Pm*SERPIN6, was chosen for further analyses to reveal the importance of this gene in shrimp immunity. In addition to the analysis of the expression of *Pm*SERPIN6 transcripts in various shrimp tissues, the expression of *Pm*SERPIN6 both in the transcriptional and in the translational levels upon viral or bacterial infection was determined using semi-quantitative RT-PCR and immunocytochemistry, respectively.

2. Materials and methods

2.1. Data mining of serpin sequences from the P. monodon EST database

The *P. monodon* EST database (http://pmonodon.biotec.or.th/home.jsp) was searched by homology for nucleotide sequence clusters corresponding to the serpin genes. The appropriate representative clones from the contigs or singletons were re-sequenced to confirm the correctness of sequence information. The BLASTx program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the sequences against the GenBank database [23]. The open reading frames (ORFs) and the deduced amino acid sequences were predicted using the Genetyx program. The online ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to compare the amino acid sequences among the serpins [24]. The signal sequences were predicted using the online SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/) [25].

2.2. Experimental shrimp and tissue collection

The black tiger shrimp, *P. monodon*, each weighing about 15–20 g were purchased from a local shrimp farm at Surat thani Province, Thailand. Shrimps were acclimatized in the laboratory aquaria at a temperature of 28 ± 4 °C and at the salinity of 15 ppt for at least 1 week before use.

For tissue distribution analysis, the hemocyte, gill, hepatopancreas, lymphoid organ, heart, epipodite, eye stalk, antennal gland, intestine and stomach were collected from unchallenged shrimp ($n = 3$) and immediately frozen in liquid N_2.

For the challenge experiments, shrimps were divided into four groups, each of thirty shrimps, such as the challenge groups; the white spot syndrome virus (WSSV)- and *Vibrio harveyi*-challenged groups, and the control groups for each challenged group. Hemocyte from five individual shrimps per time point was collected for each experiment group. Three independent experiments were performed. The WSSV challenge experiment was performed by injection into the last abdominal segment of each shrimp with 100 μl of a 1: 8000 diluted WSSV in lobster hemolymph medium (LHM: 486 mM NaCl, 15 mM $CaCl_2$, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 8.1 mM $MgSO_4$, 36 mM $NaHCO_3$, 0.05% (w/v) dextrose in Minimum Essential Medium (Invitrogen)). This dosage of WSSV used in this experiment was empirically determined to be sufficient to kill the shrimp in about 4 days (data not shown). The control shrimp were treated in the same manner except they were injected without any virus (100 μl of LHM). At 0, 12, 24 and 48 h post injection (hpi), the hemolymph was collected individually using 10% (w/v) trisodium citrate as an anticoagulant. Hemocytes were separated by centrifugation at 800 × g for 10 min at 4 °C, and then immediately frozen in liquid N_2 and stored as such until used.

For the bacterial infection experiment, 10^5 CFU of *V. harveyi* strain 639 in 0.85% (w/v) NaCl was injected into the shrimp. Hemolymph was collected at 0, 6, 12, 24, and 48 h after injection. Hemocytes were then separated as described above. Control shrimps were treated in the same manner except they were injected with the equivalent volume of 0.85% (w/v) NaCl solution without *V. harveyi*.

2.3. Total RNA extraction and cDNA synthesis

Hemocytes and tissue samples were homogenized in 1 ml of TriReagent® (Molecular Research Center). Total RNA from shrimp tissues was extracted according to the manufacturer's instructions and treated with RQ1 RNase-free DNase (Promega) to remove any contaminating DNA.

For the transcript expression analysis in response to pathogen infection, equal amounts of total RNA from three individual shrimps of the same group were pooled. Then, 1 μg of DNase-treated total RNA was used as the template for cDNA synthesis using the RevertAID™ first strand cDNA synthesis kit (Fermentas).

2.4. Cloning of the full-length PmSERPIN6 cDNA

To obtain a full-length of *Pm*SERPIN6 cDNA, the specific primer pair (forward: 5′ ATGAGGCTCCTGGTAGCTAT 3′ and reverse: 5′ CTACGAACTGGCCTTCAC 3′), were designed from the nucleotide sequences of the EST clone. The complete ORF of *Pm*SERPIN6 was amplified by PCR from the cDNA sample of unchallenged shrimp hemocytes. The PCR product was cloned into the T & A vector using the T & A cloning vector kit (RBC Bioscience). The clones were then screened and sequenced in both directions using M13 forward and reverse primers with an automated sequencer by a commercial service (Macrogen Inc., Korea).

2.5. Phylogenetic analysis

The deduced amino acid sequences of the mature proteins of *Pm*SERPIN6 – 8, *Pm*SERPINB3 and selected serpins from other invertebrate organisms reported in the GenBank database were aligned using the ClustalX program [26]. Phylogenetic analysis was then performed on these aligned amino acid sequences using the neighbor-joining distance algorithm implemented the Phylip program with default settings [27].

2.6. Tissue distribution analysis

RT-PCR was carried out to investigate the expression profile of *Pm*SERPIN6 transcript in different tissues of *P. monodon*, including the hemocyte, gill, hepatopancreas, lymphoid organ, heart, epipodite, eye stalk, antennal gland, intestine and stomach. Two specific primers were designed from the full-length cDNA sequence of the *Pm*SERPIN6, the forward primer: 5′ GTCGATGATCAAGTCGCC ACGCTCAA 3′ and the reverse primer: 5′ TATCGATGTAGGCGCGGTT AGCGATG 3′ to amplify a 118 bp fragment of the gene. PCR reactions were carried out using 5 μl of the 10-fold diluted cDNA as the template in a total reaction volume of 25 μl. The PCR profile was 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. PCR amplification of a fragment of the β-actin gene was used as a template concentration and quality control gene, where the PCR primers were forward: 5′ GCTTGCTGATCCACATCTGCT 3′ and reverse: 5′ ATCACCATCGGCAACGAGA 3′, which amplify a 337 bp fragment of the gene. The PCR condition was the same as of *Pm*SERPIN6 gene except that the reaction cycle was optimized to 25 cycles. The amplification product was analyzed on a TBE-1.5% (w/v) agarose gel. The experiment was performed in triplicate.

2.7. Expression analysis by semi-quantitative RT-PCR

To investigate the expression of *Pm*SERPIN6 transcripts in response to pathogen infection, PCR was performed using hemocyte cDNAs of the control, WSSV- and *V. harveyi*-infected shrimps as templates. The primers and PCR conditions were the same as above (Section 2.6). The PCR products were analyzed by TBE-1.5% (w/v) agarose gel electrophoresis. The expression level of *Pm*SERPIN6 gene was quantified and normalized to that of the β-actin gene using the GeneTools program (Syngene). The *Pm*SERPIN6 gene expression level of infected groups was subsequently normalized to that of the control groups. The resulting relative expression ratios of *Pm*SERPIN6 gene were calculated from three independent experimental groups. Statistical analysis was carried out using a One-Way ANOVA and Post Hoc test, with significance accepted at $P < 0.05$.

2.8. Recombinant PmSERPIN6 expression, purification and antibody production

The recombinant *Pm*SERPIN6 protein (r*Pm*SERPIN6) was produced in an *Escherichia coli* expression system. Two specific primers were designed to amplify the mature peptide. The NcoI restriction site (underlined) was added to the 5′ end of the forward primer: 5′ TATACCATGGGCCAGTGCTTTTCGGAGCAG 3′. The 6 × His tag (bolded) followed by the BamHI restriction site (underlined) was added to the 5′ end of the reverse primer: 5′ TATAGGATCCCTA **ATGATGATGATGATGATG**CGAACTGGCCTTCAC 3′, so as to allow positional in frame cloning into the pET-22b(+) expression vector MCS and subsequent Ni^{2+} based chromatographic purification of the recombinant protein. The PCR was performed using *Pfu* DNA polymerase (Promega). The purified PCR product was digested with NcoI and BamHI, then cloned into the pET-22b(+) expression vector (Novagen) cut with the same restriction enzymes. The recombinant plasmid was isolated, sequenced and subsequently transformed into the expression host, *E. coli* strain BL21(DE3).

The recombinant *Pm*SERPIN6 production was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 6 h of induction, total cell pellet was collected and then resuspended in PBS pH 7.4. The cell suspension was frozen and thawed three times before the cells were completely lysed under high pressure using a French Press. The inclusion bodies were collected by centrifugation and solubilized in 20 mM sodium phosphate buffer pH 8.0 containing 0.2 M NaCl, 20 mM imidazole and 8 M urea. The r*Pm*SERPIN6 was purified by HiTrap chelating HP column (GE Healthcare) coated with Ni^{2+} ion under denaturing conditions and eluted with 0.3 M imidazole in 20 mM sodium phosphate buffer pH 8.0 containing 0.2 M NaCl and 8 M urea. The eluted protein was then dialyzed against 25 mM Tris–HCl pH 8.0, and subsequentially analyzed by resolving through a 12.5% (w/v) acrylamide SDS-PAGE and western blot using anti-His antibody (GE healthcare). The protein content of the purified r*Pm*SERPIN6 was measured using the 2-D Quant kit (GE healthcare). Two milligrams of protein were used to immunize a rabbit in order to generate anti-*Pm*SERPIN6 polyclonal antibody at the Biomedical Technology Research Unit, Chiangmai University, Thailand.

2.9. Immunocytochemistry

To analyze the expression profile of *Pm*SERPIN6 in response to pathogen infection, immunocytochemistry was performed using polyclonal antiserum specific to *Pm*SERPIN6. The hemocytes from the WSSV- and *V. harveyi*-infected shrimp at 0, 6, 24, 48 and 72 h after challenge were fixed with 4% (w/v) paraformaldehyde in MAS solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose and 9 mM EDTA, pH 7.0). The hemocytes were aliquoted at 10^5 cells and fixed onto the poly-L-lysine coated slide. The hemocytes from three individual shrimps at each time point were used. The fixed hemocytes were permeabilized by soaking the slides in 0.2% (w/v) gelatine in TBS containing 0.5% (v/v) Triton X-100 at room temperature for 10 min. The slides were then incubated with a 1:2000 dilution of the rabbit anti-*Pm*SERPIN6 antiserum in PBS supplemented with 0.1% (v/v) Tween 20, 1% (w/v) BSA and 1% (v/v) normal goat serum at 37 °C for 3 h. Rabbit pre-immune serum was used as a control. After extensively washing with 0.1% Tween 20 in PBS for three times, alkaline phosphatase-conjugated anti-rabbit IgG at the dilution of 1:2000 was added and incubated at room temperature for 1 h. The slides were then washed as above. The positive cells were detected by adding the alkaline phosphatase substrate (NBT/BCIP solution), as previously described [28], counted and reported as the percentage of positive cells. For each sample, a minimum of 300 cells per slide were screened for the positive cells.

2.10. Qualitative analysis of PmSERPIN6 protein in the cell-free hemolymph and hemocytes

The hemolymph of unchallenged shrimp was collected in an equal volume of the MAS solution and centrifuged at 800 × g for 15 min to separate the hemocytes. The hemocyte pellet was washed three times with the MAS solution, and then resuspended in 150 mM NaCl. The hemocyte lysate was prepared by homogenizing the sample and then collecting the supernatant after centrifugation at 14,000 × g for 15 min. The protein contents of the cell-free hemolymph and hemocyte lysate were measured using the Bradford reagent. Fifty micrograms protein of hemocyte lysate and 200 μg protein of cell-free hemolymph were separated on a 12.5% (w/v) acrylamide SDS-PAGE and then subjected to western blot analysis using rabbit anti-*Pm*SERPIN6 antiserum (37 °C for 3 h) and alkaline phosphatase-conjugated anti-rabbit IgG, as described above. The blot was developed using NBT/BCIP solution as described above.

3. Results

3.1. Data mining of serpin sequences from the P. monodon EST database

To identify genes of *P. monodon* belonging to the serpin superfamily, we searched and analyzed the data in the *P. monodon* EST

database. From the 10,536 unique clusters in the database, nine contigs and singletons corresponding to serpin genes were identified (Table 1), based upon sequence homology to known serpins in GenBank using the BLASTn and BLASTx algorithms. The representative clones of each contigs or singletons were re-sequenced to confirm the correctness of the sequence information. The confirmed sequences were then searched against the GenBank database to identify similar proteins using the BLASTx program. Two contigs, CT1604 and CT2832, were found to represent the same protein. Therefore, a total of eight different *P. monodon* serpins (*Pm*SERPIN1 − 8) were identified. Of these, only three (*Pm*SERPIN6 − 8) contained complete ORFs, encoding for a predicted 415, 411 and 417 amino acid residues, respectively. All three serpins contained 19 residues of putative signal peptides with nine identical residues amongst all three serpins (11 and 14 identical amino acids between *Pm*SERPIN8 and either *Pm*SERPIN7 or *Pm*SERPIN6, respectively) (Table 1).

3.2. Amino acid sequence analysis of three P. monodon serpins, PmSERPIN6 − 8

The deduced amino acid sequences of *Pm*SERPIN6 − 8, were compared with those of the *Fc*-serpin from *F. chinensis* and serpin-6 from *M. sexta*, which are the two serpins found to be highly homologous to these *P. monodon* ones from the BLAST results. According to the conserved sequences previously identified in the serpins [29], the *Pm*SERPIN6 − 8 sequences contained the highly conserved hinge region (P_{17} to P_8; EEGTEAAAAT) at the N-terminal portion of the reactive center loop and the serpin signature (FHCNRPF-V/L-FLI) near its C-terminus (Fig. 1a). Typically, serpins that act as proteinase inhibitors are cleaved at the peptide bond between P_1 and P_1' amino acid residues by the target proteinase, and the P_1 residue determines the target specificity of the serpin to proteinase [4]. Based on the amino acid sequence alignment, we predicted that the putative P_1 residues of *Pm*SERPIN6, 7 and 8 were Arg, Arg and Lys, respectively (Fig. 1a).

The pairwise alignment showed that *Pm*SERPIN6, 7 and 8 had 63, 94 and 55% sequence identity to the *Fc*-serpin, respectively, and so *Pm*SERPIN7 was clearly an orthologue of *Fc*-serpin. *Pm*SERPIN6 showed 36% amino acid sequence homology to the *M. sexta* serpin-6, previously characterized as a regulator of the prophenoloxidase system [14]. They also shared the same putative P_1 Arg. Therefore, *Pm*SERPIN6 was chosen for further study.

3.3. Phylogenetic analysis

The four *P. monodon* serpins, *Pm*SERPIN6 − 8 and *Pm*SERPINB3, were compared with serpins from other crustaceans (a putative serpin from crayfish *P. leniusculus* and a serpin from Japanese horseshoe crab *T. tridentatus*) and insects (serpins from the tobacco hornworm *M. sexta*, fruit fly *D. melanogaster* and silkworm *B. mori*). The alignment of the predicted amino acid residue sequences was used to create a phylogenetic tree using the neighbor-joining distance method, implemented in the Phylip program. The distance tree showed that *Pm*SERPIN6 − 8 were all clustered in the same group as the *Fc*-serpin, with a putative serpin from *P. leniusculus* as slightly more distant, and a weakly discriminated sister clade that contained the serpin-6 from *M. sexta*, serpin-6 from *B. mori*, and serpin-5 and serpin88Eb from *D. melanogaster* (Fig. 1b). The other serpin from *P. monodon*, *Pm*SERPINB3 was positioned in a separate but poorly supported clade with *B. mori* serpin-23 amongst others.

Table 1
The serpin genes identified from the *Penaeus monodon* EST database.

Serpin	Contig or singleton	Number of clones	Frequency in EST libraries[a]		Representative clone (ORF) and GenBank accession number	ORF (amino acids)	Predicted signal sequence
*Pm*SERPIN1	CT2488	2	GIEp-N-N HPa-N-N	1 1	GIEp-N-N01-1536-LF (incomplete ORF) GT968764	–	–
*Pm*SERPIN2	CT1501	3	GIEp-N-N HC-N-N	1 2	GIEp-N-N01-2770-LF (incomplete ORF) GT968765	–	–
*Pm*SERPIN3	SG5480	1	GL-H-S	1	GL-H-S01-0037-LF (incomplete ORF) GT968763	–	–
*Pm*SERPIN4	SG7094	1	HC-N-N	1	HC-N-N01-5643-LF (incomplete ORF) GT968767	–	–
*Pm*SERPIN5	CT1116	5	GIEp-N-N HC-N-N HPO-N-S HT-N-S	2 1 1 1	GIEp-N-N01-1465-LF (incomplete ORF) GT968766	–	–
*Pm*SERPIN6	CT1604 (CT2832)	5	GL-N-STC HC-N-N HT-N-S	2 2 1	HC-N-N01-2773-LF (complete ORF) GQ260129	415	MRLLVAMAVTAAVLGLVRP (19 amino acids)
*Pm*SERPIN7	CT1087	5	HC-N-N HC-W-S	4 1	HC-N-N01-12906-LF (complete ORF) GU358487	411	MKFAVVGAVAAALVGVVQP (19 amino acids)
*Pm*SERPIN8	SG5654	1	GL-H-S	1	GL-H-S01-0891-LF (complete ORF) GU358488	417	MKCLVALAAAAAVLGLGRP (19 amino acids)

[a] GIEp-N-N, HPa-N-N, and HC-N-N are normalized (3rd N) normal (2nd N) gill-epipodite (GIEp), hepatopancreas (HPa) and hemocyte (HC) cDNA libraries. HPO-N-S and HT-N-S are standard (3rd S) normal (2nd N) hematopoietic tissue (HPO) and heart (HT) cDNA libraries. GL-H-S and GL-N-STC are standard heat-treated (2nd H) and subtractive (3rd STC) gill (GL) cDNA libraries, respectively. HC-W-S is standard white spot virus-infected (2nd W) hemocyte cDNA library.

a

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PmSERPIN7       MKFAVVGAVAAALVGVVQPQCFTDNDNF----LIKVNTDLSGVTDFGFDLYRRLDSPSSP 56
Fc-serpin       MKFAALGAVAAALVGLAQPQCFTDNDNF----LIKVNTDLSGVTDFGFDLYRRLDSPSSP 56
PmSERPIN6       MRLLVAMAVTAAVLGLVRPQCFSEQDDF----SVKVNTDLSGITDFGFELYRQLAPPQSP 56
PmSERPIN8       MKCLVALAAAAAVLGLGRPQCLPGRGSS----SGRISTDLSGIADFGFELYRQLAPPQSP 56
Ms_serpin-6     M--LKSAALVLLVATCVSSQCFSKDDSSKKLDPGARTSLYSGQLAFTLNLFQTINSAVPD 58

PmSERPIN7       RNFFFSPFSIWSAFILAYLGSAGETEAQLQRALRVDGKVETFKIWRALEALYQT--SNND 114
Fc-serpin       KNFFFSPFSIWSAFILAYLGSAGETEAQLQRALRVDGKVETFKIWRALEAMYQT--SNND 114
PmSERPIN6       ENFFFSPYSIWTAFTLAYFGSGGETAAQLQRALRVDDQVATLKLWRALEAMYRT--RQQN 114
PmSERPIN8       ENFFFSPYSIWTAFTLVYFGTGGETAAQLQRALRVGDQATTLGLWRELEAKYQQ--RQAN 114
Ms_serpin-6     DNIFFSPFSVYQSLLLAYFSTGGRTEESLKKSLEIEDNMDKMNLMTAYKVDKRSRMTNNN 118

PmSERPIN7       ---YTFNIANRAYIDNVLPIRPCIIELLSNEFERINFR-DVFSAVNRINNFASTNTKGKI 170
Fc-serpin       ---YTFNIANRAYIDNVLPIRPCILEMLSNEFERINFR-DVFSAVNRINNFASTNTKGKI 170
PmSERPIN6       TTAYSFNIANRAYIDKNLPIRDCITNLLHSEVDRVQFS-KVGFVTQEINNFVSVATKGRI 173
PmSERPIN8       NKAYTFTVANRAFIHNNLPIRPCISNLLKTEVERVNFL-DTLTLVAHINNFASASTKGRI 173
Ms_serpin-6     SDSYEFTTANKLFVANELQVRQCMFDLFGEEIEALNFRENPEVSREYINNWVERITKNHI 178

PmSERPIN7       NDLVTVENIEG-IHMAIVNAAYFKGTWQFQFKPTSTASERFFVTPQSHQMVPMMNQISAF 229
Fc-serpin       NERVTVENIEG-IHMAIVNAAYFKGTWQFQFKPTSTVSERFFVTPQNHQMVPMMNQISAF 229
PmSERPIN6       SKIVSVADLAD-AIMVLVNAAYFKGTWQYQFKPSNTFPEPFFATSQNNDLVPMMHQTASF 232
PmSERPIN8       TEIVSADDLVD-ALMVLVNAAYFKGTWQYFFDAAATTPREFYVTPGDSVMTPMMKQATSL 232
Ms_serpin-6     KKLLPADGVSEFTKLVLANAAYFKGVWASKFSPERTKKEPFFVSETRQTLVPFMKQKGTF 238

PmSERPIN7       NFGEFDQVAASVLELPYTGERVSMFLFLPVQEGPQGFANMVSKLSGNNLRAATHKKNLKK 289
Fc-serpin       RFGEFDQVAASVLELPYTGERVSMFLFLPAQEGPQGFANMVTRLSGNNLRAATHRKNLRK 289
PmSERPIN6       RYNEFSEIAAKVLELPYTGDAMSMFVFLPSEEGPRGFANMVARLSGNNLRAATHKGNLSF 292
PmSERPIN8       RYGEFDHIAARVLELPYAGGAMSMFLLLPMGEGTQGFASMVTKLNENNMQAVTLGNNLVK 292
Ms_serpin-6     HYGVSEELGAQVLELPYKGNDISMFILLPPYSMKEGVTNIIANLNTERLAAVMEESYMS- 297

PmSERPIN7       QDVDLKLPKFRMELKLADEMIPALKDMGIVDIFSSDKVDLTTLGNLRNLTLEKVIHKAFV 349
Fc-serpin       QDVELKLPKFRMELKLADEMIPALKDMGIVDIFNSEKVDLSTLGNLRNLTLEKVIHKAFV 349
PmSERPIN6       RMVDVKLPKFKMEVEVRDELKPALHNMGITDIFNSEKVDLTTFGPLRNVTLEKVIHKAFV 352
PmSERPIN8       KDVDLLLPRFRLEQTVSKTLIPALQNMGIIDIFDSRKVDLTGFGPLRNITVDKAIHKAFV 352
Ms_serpin-6     REVIVEIPKFTIERTLS--LRPILDRLGVGDLFN-VSADFSTLTEDSGIRFDDAVHKAKI 354

                                P17      P8      P1-P1'
PmSERPIN7       EVNEEGTEAAAATVLTFTLRASRR--DPV-VEFHCNRPFLFLIRDNETNNNLFMGVYRAP 406
Fc-serpin       EVNEEGTEAAAATVLTFTLRASRR--DPV-VEFHCNRPFLFLIRDNETNNNLFMGVYRSP 406
PmSERPIN6       EVNEEGTEAAAATALIFATRSGGA--RPLPVEFHCNRPFVFLIRDNDTHTVLFMGSYKKP 410
PmSERPIN8       EVNEEGTEAAAVTAAILVFKSASSSRDDLPIQFHCNRPFVFLIQDNDTQNILFMGAFKNP 412
Ms_serpin-6     QIDEEGTVAAAATAL-FGFRSSRP-AEPT--RFIANFPFVYLIYERPTNSILFFGVYRDP 410

PmSERPIN7       DAARS 411
Fc-serpin       DTARS 411
PmSERPIN6       VKASS 415
PmSERPIN8       RGRAQ 417
Ms_serpin-6     KK--- 412
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Fig. 1. Sequence analysis of *Pm*SERPIN6. (a) Amino acid sequence of *Pm*SERPIN6 - 8, *Fc*-serpin and *M. sexta* serpin-6 (*Ms*_serpin-6) were aligned. The conserved hinge region, the putative signal sequence and the serpin signature of each gene are shown in grey, black and open boxes, respectively. The predicted P_1 and P_1' positions are underlined. (b) Phylogenetic analysis of the *Pm*SERPIN6 is shown. A neighbor-joining distance based phylogenetic tree of *Pm*SERPINs (highlighted in grey) and serpins from related organisms, including the serpins from *P. leniusculus* (Pl), *F. chinensis* (Fc), *D. melanogaster* (Dm), *M. sexta* (Ms), *B. mori* (Bm) and *T. tridentatus* (Tt). Values at the node of a bootstrapped neighbor-joining tree indicate the number of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences. The bootstrap support values higher than 500 are shown at the nodes. The GenBank accession number of the serpin genes used for the analysis is shown in the parenthesis.

3.4. Expression of PmSERPIN6 in shrimp tissues

To analyze the expression of *Pm*SERPIN6 gene transcripts in shrimp tissues, such as the lymphoid organ, epipodite, hemocyte, stomach, antennal gland, intestine, eye stalk, heart, gill and hepatopancreas, RT-PCR was employed. As shown in Fig. 2a, *Pm*SERPIN6 transcripts were expressed in almost all tissues tested, except in the hepatopancreas.

To determine the expression of *Pm*SERPIN6 at the protein level, the rabbit anti-*Pm*SERPIN6 polyclonal antiserum was used to

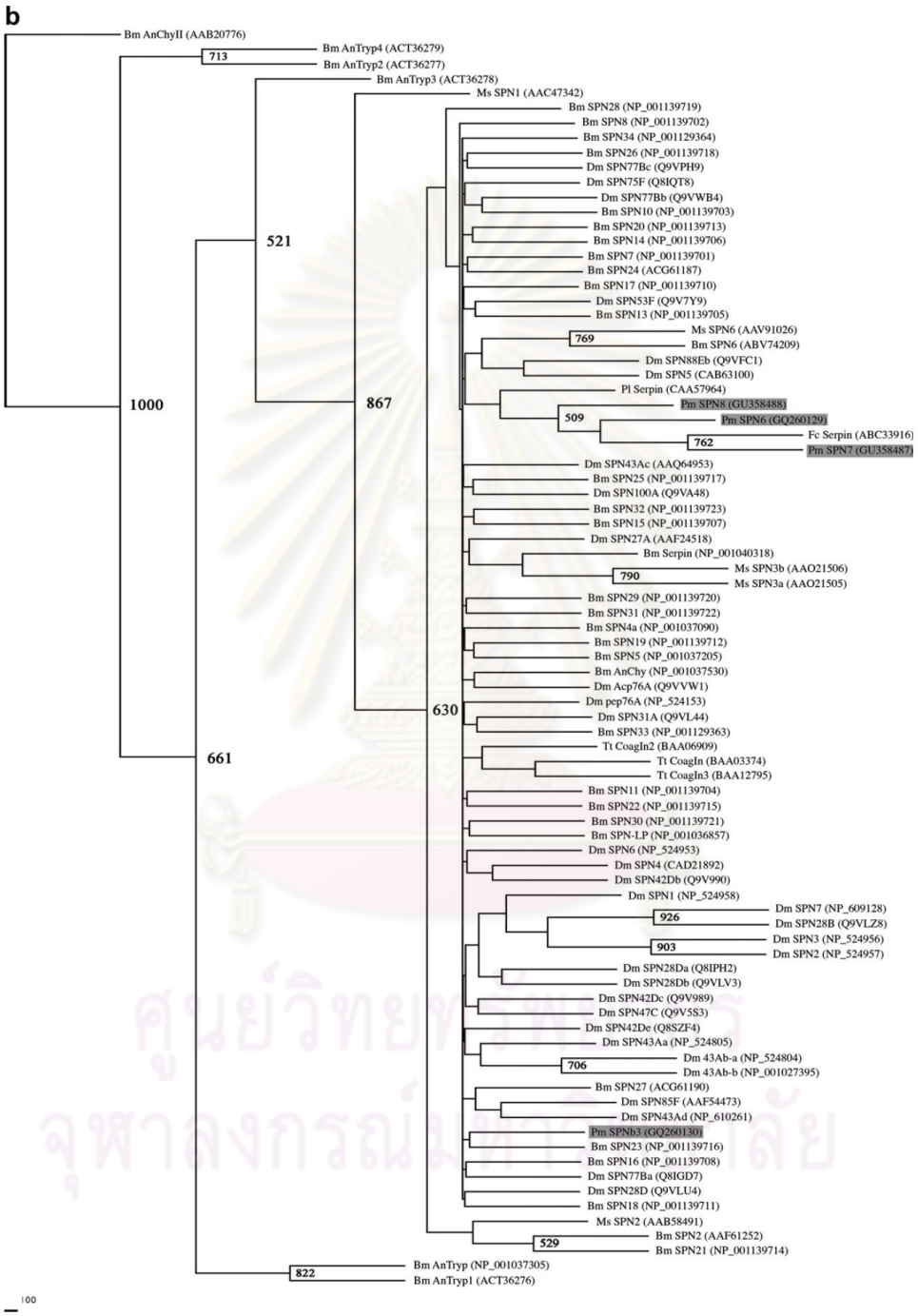

Fig. 1. (*continued*).

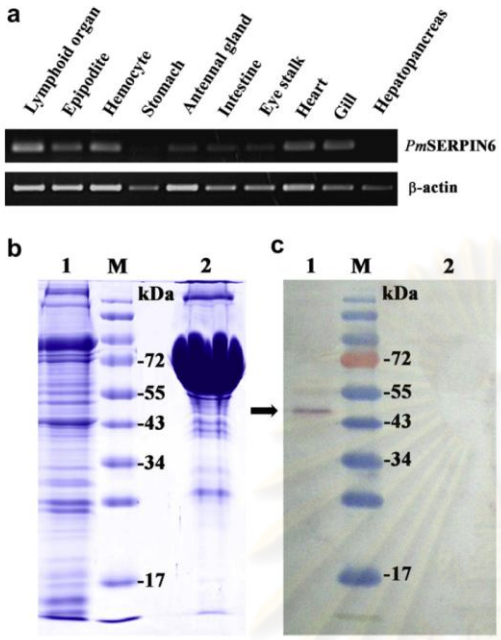

Fig. 2. Expression analysis of *Pm*SERPIN6 in shrimp tissues. (a) The expression of *Pm*SERPIN6 mRNA was determined by RT-PCR in various shrimp tissues (lymphoid organ, epipodite, hemocyte, stomach, antennal gland, intestine, eye stalk, heart, gill and hepatopancreas), whilst the β-actin gene was used as an internal control. Expression of *Pm*SERPIN6 protein in shrimp hemocyte was analyzed by (b) SDS-PAGE and (c) western blot analysis. Fifty micrograms of hemocyte lysate (lane 1) and 200 μg of cell-free hemolymph (lane 2) were separated on (b) 12.5% (w/v) acrylamide SDS-PAGE and (c) analyzed by western blot using rabbit anti-*Pm*SERPIN6 antiserum. The arrow indicates the expected immune reactive band of about 45 kDa.

examine the presence of *Pm*SERPIN6 protein in the hemocyte and hemolymph of unchallenged shrimp by western blot analysis. A positive band with the expected size of about 45 kDa was observed only in the hemocyte lysate sample, but not in the cell-free hemolymph (Fig. 2b and c).

3.5. Expression of PmSERPIN6 transcript after bacterial and viral challenge

The expression pattern of *Pm*SERPIN6 mRNA in the hemocytes of shrimp systemically challenged with either WSSV or with *V. harveyi* was determined by semi-quantitative RT-PCR. In WSSV-challenged shrimp, we observed a slight decrease in the relative expression ratio of *Pm*SERPIN6 mRNA at 12 and 24 h post-viral challenge and these transcript levels then returned to nearly normal levels at 48 hpi (Fig. 3a). However, no significant change in transcript levels was observed upon *V. harveyi* challenge at 0 – 48 hpi (Fig. 3b).

3.6. Expression of PmSERPIN6 protein in the hemocytes

Besides the analysis of expression of *Pm*SERPIN6 transcript under viral and bacterial infection conditions, we also determined the *Pm*SERPIN6 protein expression in hemocytes at 0 – 72 hpi under the same conditions by immunocytochemistry. The polyclonal anti-*Pm*SERPIN6 antiserum was used to detect the *Pm*SERPIN6 producing hemocytes in both WSSV- and *V. harveyi*-infected shrimp. No positive cells could be detected in the controls where the rabbit pre-immune serum was used (data not shown). The cells were counted and the percentage of positive hemocytes was calculated (Table 2). The expression of *Pm*SERPIN6 protein in the hemocytes of WSSV-infected shrimp was relatively unchanged up to 48 hpi (between 3.5% and 4.5% positive hemocytes), but significantly increased at 72 hpi (11% positive hemocytes) (Table 2). In *P. monodon* that were systemically challenged with *V. harveyi*, the percentages of *Pm*SERPIN6-positive cells at 0, 6, 24, 48 and 72 hpi were 4.15, 6.14, 3.25, 4.57 and 7.64 respectively (Table 2). However, the numerical trend was of a decrease in expression levels over the first 24 hpi followed by an increased expression afterwards during the period of 48–72 hpi. In both cases, the highest number of positive hemocytes was detected late (72 hpi) in the systemic infection of either WSSV or *V. harveyi*.

4. Discussion

In invertebrates including shrimp, a fairly diverse array of serpins have been identified as a group of serine proteinase inhibitors that play an important role in the immune responses [10–14,30]. Being interested in the immune modulators in shrimp, we, therefore, searched for other potential shrimp serpins by mining the *P. monodon* EST database. Eight more potential serpin gene homologs were found in cDNA libraries prepared from various shrimp tissues, but at a higher frequency in the hemocyte, gill and gill–epipodite cDNA libraries. However, only three *Pm*SERPINs from the EST database, *Pm*SERPIN6 – 8, possessed complete ORFs. Sequence comparison with serpins from other invertebrates revealed that *Pm*SERPIN7 was highly similar to *Fc*-serpin, with a 94% amino acid sequence identity, suggesting that *Pm*SERPIN7 is an orthologue of *Fc*-serpin. Like *Fc*-serpin, all three *Pm*SERPINs showed homology to the *M. sexta* serpin-6, which was reported to regulate the prophenoloxidase system in *M. sexta* by inhibiting the prophenoloxidase activating proteinase-3 (PAP-3) [14]. However, the biological function of each of these *Pm*SERPINs remains to be determined in further work.

Biological functions and target proteinases of several serpins have been characterized in a variety of species. Serpins normally act as inhibitors of proteinases in various biological processes, such as blood coagulation, complement activation, fibrinolysis and inflammation. However, non-inhibitory serpins are also known to exist [5]. The conserved three-dimensional structures reveal the presence of a scissile bond at the $P_1 - P_1'$ site within the exposed reactive center loop (RCL). This specific peptide bond is cleaved by the target proteinase, and the P_1 residue normally determines the inhibitory specificity. Primary amino acid sequences of RCL at P_{17} - P_{14} and P_{12} - P_8 are conserved among the inhibitory serpins (P_{17}: E, P_{16}: E/K/R, P_{15}: G, P_{14}: T/S, P_{12} - P_9: A/G/S, and P_8: T), and this conserved region is rarely found in the apparently non-inhibitory serpins, in agreement with this notion. After proteinase digestion, the remaining RCL is inserted into the core protein leading to a conformational change and complex formation. Lacking the conserved sequences in the RCL, the non-inhibitory serpins lack their inhibitory activity as well [5,31]. Comparison to the previously identified inhibitory serpins from the tobacco hornworm, *M. sexta* serpin-6 [14], the shrimp serpins, *Pm*SERPIN6 – 8 and *Fc*-serpin [22] should be tentatively classified as inhibitory serpins for they contained the identical conserved hinge region (P_{17} - P_{14}: EEGT, and P_{12} - P_8: AAAAT) (Fig. 1a), although this of course requires confirmation.

Another feature of serpins that is observed in humans is that the serpin–proteinase complex is cleared by cellular internalization, which is mediated by the serpin–enzyme complex (SEC) receptor

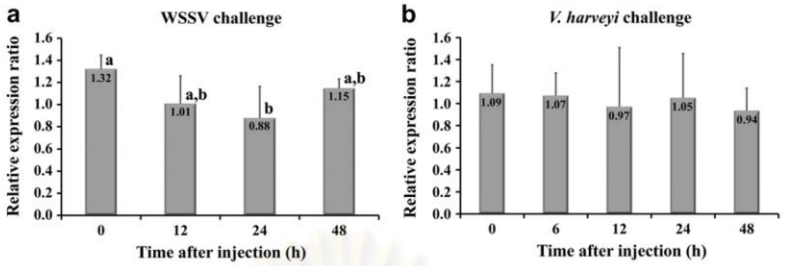

Fig. 3. Expression profiles of *Pm*SERPIN6 gene transcripts upon bacterial and viral infection. Analysis of *Pm*SERPIN6 gene transcript expression levels was performed in shrimp systemically infected with (a) WSSV at 0, 12, 24 and 48 hpi or (b) *V. harveyi* at 0, 6, 12, 24 and 48 hpi. The control shrimp for both groups were injected with LHM media or 0.85% (w/v) NaCl, respectively. The *Pm*SERPIN6 gene expression level of infected groups was normalized to that of the control groups. β-actin gene was used as an internal control. The results are shown as the mean ± SD. Means with different letters are significantly different ($P < 0.05$).

[32,33]. The clearance mechanism is believed to be facilitated by a binding site in the complex, supposedly the highly conserved-pentapeptide domain (FVFLM) of human α1-antitrypsin and/or the loop structure consisting of the seven underlined amino acid residues (PHDNIVISP) at the N-terminal region of the mature protease nexin I [34,35]. We have found the conserved-pentapeptide F(L/V)FLI at the carboxyl terminus of serpin signature in all three *Pm*SERPIN6 − 8 (Fig. 1). The possible amino acid residues corresponding to the loop structure of protease nexin I was also found at the amino terminal region, residues 37 − 44: P(R/E)NFFFSP, of the mature *Pm*SERPIN6 − 8. The potential function of these sequence motifs will be further elucidated.

Mutagenesis of the P_1 residues in several serpins has been carried out to reveal the nature of the serpin–proteinase specificity. Changing the amino acid at the P_1 position can alter the rate of inhibition against different target proteinases [1]. The predicted P_1 residues of *Pm*SERPIN6, 7 and 8 were arginine, arginine and lysine, respectively. It is known that serpins containing Arg at their P_1 site specifically and preferentially inhibit trypsin, thrombin and plasmin [5], therefore suggesting that the *Pm*SERPIN6 and 7 might be inhibitors of trypsin, thrombin and plasmin. In the same way, the *Pm*SERPIN8 with a P_1 Lys residue is expected to specifically inhibit a trypsin-like proteinase [36], although these remain to be formally tested and confirmed.

The expression of *Pm*SERPIN6 gene transcripts in various shrimp tissues was detected in all tissues examined except for the hepatopancreas in accord with the tissue localization observed for the *Fc*-serpin and a serpin from the crayfish, *P. leniusculus* [15,22]. Since hemocytes are one of the main sites of synthesis of immune proteins, along with the fat body, and are a frontline response to infection, it was of interest that the mRNA expression of *M. sexta* serpin-6 was found to be significantly increased in the hemocyte and the fat body at 24 h post-*E. coli* injection, and, in accord, the protein level was also significantly induced at 24 h post-*E. coli* or *Micrococcus luteus* infection [30]. In addition, the pathogen responsive *Fc*-serpin gene in hemocytes was down-regulated at 6 and 12 h post-mixed bacterial challenge. On the contrary, it was up-regulated at 8 h post-WSSV challenge and gradually decreased after 14 h post-infection [22]. Therefore, we studied *Pm*SERPIN6 expression in hemocytes at both the transcriptional and translational levels in response to systemic microbial (*V. harveyi*) and viral (WSSV) injection to elucidate the involvement of *Pm*SERPIN6 in shrimp immunity.

Table 2
Time-course analysis of *Pm*SERPIN6 protein expression in hemocytes after pathogen infection by immunocytochemistry.

Time after injection (h)	WSSV challenge (Mean of % positive cells ± SD)	*V. harveyi* challenge (Mean of % positive cells ± SD)
0	4.53 ± 2.84	4.15 ± 1.86
6	3.92 ± 1.44	6.14 ± 0.92
24	3.87 ± 1.79	3.25 ± 0.84
48	3.50 ± 1.18	4.57 ± 0.44
72	10.86 ± 2.48	7.64 ± 2.34

In *P. monodon*, *Pm*SERPINB3 gene expression levels were gradually increased from 0 to 48 hpi upon *V. harveyi* challenge [21], and only a slight change at the transcription level of *Pm*SERPIN6 was observed upon WSSV infection, whilst no significant response to systemic *V. harveyi* challenge was found. Interestingly, the proportion of circulating *Pm*SERPIN6-positive hemocytes increased for 2.4- and 1.8-fold in response to WSSV- and *V. harveyi* challenge, respectively, at 72 hpi as compared to that observed at 0 hpi. An increase in the expression of *Pm*SERPIN6 protein in the late infection phase might help prevent excessive proteinase actions that are harmful to the cells. In the proPO activation system, serpins are involved in preventing premature and excessive activation of the melanization cascade [37]. Our results then could suggest that *Pm*SERPIN6 might play an important role in the shrimp immune regulatory system. Whether or not its function involves the proPO activation system in shrimps, however, requires further investigation.

In conclusion, as found in other species, several serpin family members were identified in *P. monodon*. Of the three new serpins reported here, they share high amino acid sequence identity with the conserved serpin signature. An increase in the expression of *Pm*SERPIN6 protein in response to microbial challenge implied its role in the shrimp immune system. Further analyses of the inhibitory activity *in vitro* and *in vivo* of *Pm*SERPINs may unveil the function of serpins in the shrimp defense mechanism.

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