ลักษณะสมบัติของยีน *PmSERPIN3* จากกุ้งกุลาดำ *Penaeus monodon*

นางสาวณัฐิยา เวศพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต
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CHARACTERIZATION OF *PmSERPIN3* GENE FROM BLACK TIGER SHRIMP *Penaeus monodon*

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ณัฐิยา เวศพันธ์: ลักษณะสมบัติของยีน *PmSERPIN3* จากกุ้งกุลาดำ *Penaeus monodon* (CHARACTERIZATION OF *PmSERPIN3* GENE FROM BLACK TIGER SHRIMP, *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. กุลยา สมบูรณ์วิวัฒน์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. วิเชียร ริมพณิชยกิจ, 114 หน้า

เซอร์ปินหรือตัวยับยั้งเซอรีนโปรติเนสเป็นกลุ่มของตัวยับยั้งโปรติเนสที่ควบคุมกระบวนการทางชีวภาพในสิ่งมีชีวิตหลากหลายชนิด ในปัจจุบันมีการค้นพบเซอร์ปินหลายตัวในกุ้ง ในงานวิจัยนี้ได้ทำการศึกษาลักษณะสมบัติของยีน *PmSERPIN3* ของกุ้งกุลาดำ โดยหาข้อมูลนิวคลีโอไทด์ทั้งหมดด้วยเทคนิค 5'- และ 3'- Rapid Amplification of cDNA Ends (RACE) พบว่าลำดับนิวคลีโอไทด์ของ cDNA ของยีน *PmSERPIN3* มีความยาวทั้งสิ้น 1,456 คู่เบส โดยมีส่วนของ Open Reading Frame เท่ากับ 1,233 คู่เบส สามารถถอดรหัสเป็นกรดอะมิโนได้ทั้งสิ้น 410 กรดอะมิโน และมีส่วนของ signal peptide ยาว 23 กรดอะมิโน จากการศึกษาการจัดเรียงตัวของยีน *PmSERPIN3* บนจีโนม พบว่ายีน *PmSERPIN3* ไม่มี intron ในการศึกษาการแสดงออกของยีน *PmSERPIN3* ด้วยเทคนิค RT-PCR พบว่ายีน *PmSERPIN3* มีการแสดงออกในทุกเนื้อเยื่อที่ทดสอบและมีการแสดงออกในระยะต่างๆของการเจริญ ได้แก่ นอเพลียส ซูเอี๋ย ไมซิส และตัวเต็มวัย แต่การแสดงออกของยีนนี้ ไม่ตอบสนองต่อการติดเชื้อแบคทีเรีย *Vibrio harveyi* เชื้อไวรัสจุดขาว (white spot syndrome virus) และ ไวรัสหัวเหลือง (yellow head virus) จากการใช้เทคนิค immunohistochemistry พบโปรตีน *Pm*SERPIN3 แสดงออกในเซลล์เม็ดเลือดทั้ง 3 ชนิด ได้แก่ hyaline semigranular และ granular โดยมีการแสดงออกมากขึ้นเมื่อติดเชื้อ *Vibrio harveyi* ได้ทำการผลิตโปรตีนรีคอมบิแนนท์ *Pm*SERPIN3 ในเชื้อแบคทีเรีย *Escherichia coli* และทดสอบแอคทิวิตี้ในการยับยั้งเอนไซม์ พบว่าโปรตีน *Pm*SERPIN3 ที่บริสุทธิ์สามารถยับยั้งการทำงานของเอนไซม์ subtilisin ได้ นอกจากนั้นยังสามารถยับยั้งการกระตุ้นระบบโพรฟีนอลออกซิเดส (Prophenoloxidase system) ได้อีกด้วย ศึกษาผลของโปรตีน *Pm*SERPIN3 ต่อการกำจัดเชื้อแบคทีเรียในตัวกุ้งที่ติดเชื้อแบคทีเรีย *V. harveyi* โดยเมื่อฉีดโปรตีน r*Pm*SERPIN3 พร้อมเชื้อ *V. harveyi* เข้าไปในตัวกุ้งและตรวจสอบจำนวนเชื้อแบคทีเรียทั้งหมดในน้ำเลือดกุ้ง พบว่าที่เวลา 30 นาทีหลังการฉีด จำนวนเชื้อแบคทีเรียทั้งหมดและเชื้อแบคทีเรีย *V. harveyi* ของกุ้งกลุ่มที่ฉีดโปรตีน r*Pm*SERPIN3 ร่วมกับเชื้อแบคทีเรีย *V. harveyi* จะมากกว่ากลุ่มควบคุมที่ฉีดเชื้อแบคทีเรีย *V. harveyi* เพียงอย่างเดียวคิดเป็น 3.5 และ 2.9 เท่า และยังพบว่าโปรตีน r*Pm*SERPIN3 ไม่สามารถยับยั้งการเกิดลิ่มเลือดของเลือดกุ้งได้ จากผลการทดลองข้างต้น จึงคาดว่าโปรตีน *Pm*SERPIN3 มีหน้าที่ในการเป็นตัวยับยั้งการทำงานของระบบโปรฟีนอลออกซิเดส โดยความสามารถในการกำจัดเชื้อแบคทีเรียในกุ้ง

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....
สาขาวิชา.....ชีวเคมีและชีววิทยาโมเลกุล..... ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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NATTHIYA WETSAPHAN: CHARACTERIZATION OF *PmSERPIN3* GENE FROM BLACK TIGER SHRIMP *Penaeus monodon* ADVISOR: ASST. PROF. KUNLAYA SOMBOONWIWAT, Ph.D., CO-ADVISOR: ASSOC. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D., 114 pp.

Serpin or serine proteinase inhibitor is a family of a proteinase inhibitor that involves in controlling the proteolytic cascade in various biological processes and has been identified in most of organisms. In shrimp, several serpins have been identified so far. In this study, *PmSERPIN3* gene was characterized. From the 5'- and 3'- Rapid Amplification of cDNA Ends (RACE) techniques, the full-length of *PmSERPIN3* cDNA is about 1,456 bp containing an open reading frame of 1,233 bp encoding for 410 amino acid residues with 23 amino acid residues signal peptide was obtained. Genome sequence analysis revealed that the *PmSERPIN3* was an intronless gene. RT-PCR analysis revealed that it expressed in all shrimp tissues tested. Moreover, the expression of *PmSERPIN3* was also found in all developmental stages including nauplius, zoea, mysis and adult. The expression level of *PmSERPIN3* gene did not respond to *Vibrio harveyi*, white spot syndrome virus and yellow head virus challenges. Using the immunofluorescent staining observed under confocal laser scanning microscope, the result revealed that *Pm*SERPIN3 appeared in 3 main types of hemocytes such as hyaline, semigranular, and granular hemocytes and was up-regulated upon *V. harveyi* infection. The recombinant *Pm*SERPIN3 (r*Pm*SERPIN3) was successfully produced in *Escherichia coli*. The proteinase inhibitory activity assay revealed that the purified r*Pm*SERPIN3 could inhibit subtilisin. Interestingly, r*Pm*SERPIN3 also inhibited the shrimp prophenoloxidase system activation *in vitro*. The effect of *Pm*SERPIN3 on bacterial clearance of shrimp after *V. harveyi* infection was tested. The results revealed that the number of total bacteria and *V. harveyi* (CFU/ml) after *V. harveyi* and r*Pm*SERPIN3 injection at 30 min was higher than the control shrimp for 3.5 and 2.9 fold, respectively. However, r*Pm*SERPIN3 could not inhibit the clot formation of shrimp hemolymph. Taken together, it can be implied that *Pm*SERPIN3 functions as an inhibitor of proPO system activation. Its inhibitory activity takes part in the bacterial clearance efficacy of shrimp.

Department:….....…Biochemistry.....Student's Signature…….....……...
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LIST OF ABBREVIATIONS

°C	degree Celcius
µg	microgram
µl	microlitre
µM	micromolar
A	absorbance
Arg	arginine
bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxyadecytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EMS	early mortality syndrome
FBS	fetal bovine serum
h	hour, hours
His	histidine
hpi	hours post infection
Kb	kilobase
LPS	lipopolysaccharide
M	molar
mA	milliampere
Met	methionine
mg	milligram
min	minute
ml	millilitre
mM	millimolar
ng	nanogram
nm	nanomolar
O.D.	optical density
ORF	open reading frame
Pm	*Penaeus monodon*
PO	phenoloxidase
proPO	prophenoloxidase
RNA	ribonucleic acid
RT	reverse transcription
sec	second
TCBS	thiosulfate-citrate-bile-sucrose

TSA	tryptic soy agar
TSB	tryptic soy broth
TSV	taura syndrome virus
WSSV	white spot syndrome virus
YHD	yellow head disease
YHV	yellow head virus

CHAPTER I

INTRODUCTION

1.1 Taxonomy of *Penaeus monodon*

(Source: http://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=1209)

Figure 1.1 The black tiger shrimp (*Penaeus monodon*)

According to taxonomic definition, *P. monodon*, the black tiger shrimp is classified into the largest kingdom, Animalia (Linnaeus, 1758), Arthopoda phylum (Latreille, 1829), Crustacea subphylum (Brünnich, 1772), Malacostraca class (Latreille, 1802), Decapoda order (Latreille, 1802), Dendrobranchiata suborder (Bate, 1888), Penaidae family (Rafinesque, 1815), and *Penaeus* genus (Fabricius, 1798) by the general characteristics such as pair appendages and protective cuticle or exoskeleton.

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

General name: Bangkear (Cambodia)**,** Black tiger prawn, Blue tiger prawn, Giant tiger prawn Jar-Pazun (Burma), Jinga (India, Bombay region), Jumbo tiger prawn, Kalri

(Pakistan), Kamba ndogo (Kenya), Kung kula-dum (Thailand), Leader prawn, Panda prawn, and Sugpo (Phillipines)

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

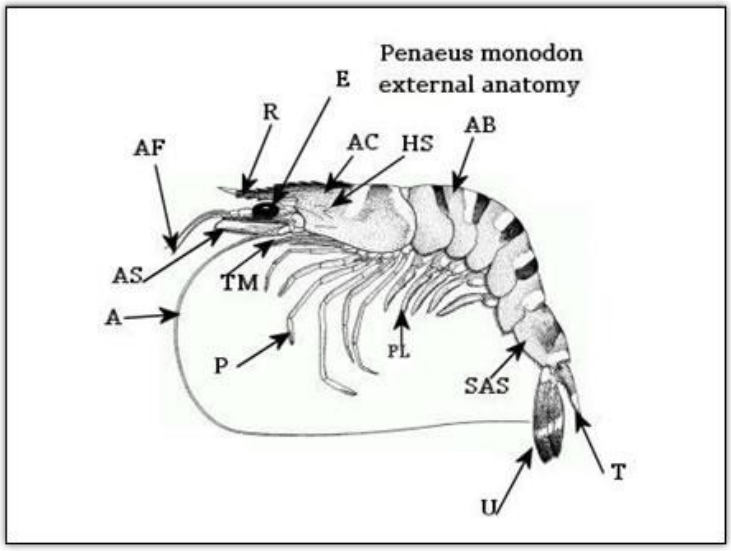
Table 1.1 The taxonomic definition of the black tiger shrimp, *P. monodon.*

Scientific classification	
Kingdom:	Animalia
Phylum:	Arthropoda
Subphylum:	Crustacea
Class:	Malacostraca
Order:	Decapoda
Suborder:	Dendrobranchiata
Family:	Penaeidae
Genus:	*Penaeus*
Species:	*Penaeus monodon*

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

1.2 The biological features of shrimp

The general color of *Penaeus monodon* is dark, with the black and white banded on carapace and abdomen (Figure 1.1). The rest of the body is alterable, ranging from light brown to blue or red, while some smaller specimens show a dull red dorsal strip from the rostrum to the sixth abdominal segment (Grey, Dall and Baker, 1983). The external morphology of *P. monodon* contains three parts, cephalon (head), tholax, and abdomen (tail) (Figure 1.2).



(Source: http://affris.org/giant_tiger_prawn/morphological.ph)

Figure 1.2 Extenal anatomy of *Penaeus monodon*

A = antenna, AB = abdominal segment, AC = adrostral carina, AF = antennular flagellum, AS = antennal scale, E = eyestalk, HS = hepatic spine, P = pereiopods, Pl = pleopods, R = rostrum, SAS = sixth abdominal segment, T = telson, TM = third maxilliped, U = uropod.

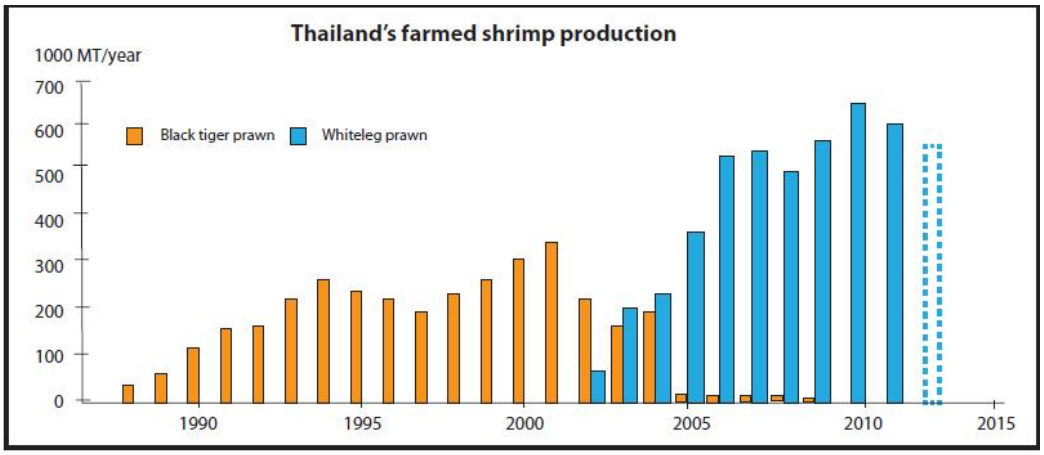
1.3 The shrimp farming industry in Thailand

Shrimp farming is an aquaculture trade that is in either a marine or freshwater habitat. The marine shrimp farming began in the 1970s and became the market demands of the United States, Japan and Western Europe. The total global production of farmed shrimp reached more than 1.6 million tonnes in 2003, representing a value of nearly 9 billion U.S. dollars. The production of farmed shrimp in Asia, in particular in China and Thailand are about 75%. The other 25% is produced mainly in Latin America, where Brazil, Ecuador, and Mexico are the largest producers (Source: FAO databases,

2007). Interestingly, Thailand is the top exporting country since 1990s, and became the biggest exporter of the black tiger shrimp, *P. monodon* in marketing of shrimp (Wyban, 2007).

A worldwide industry of shrimp farming has begun in Southeast Asia. Technological advances have led to growing shrimp at ever higher densities, and broodstock is shipped worldwide. All farmed shrimp are of the family Penaeidae, and just two species *Litopenaeus vannamei* (Pacific white shrimp) and *P. monodon* (black tiger shrimp) account for roughly 80% of all farmed shrimp. These industrial monocultures are very sensitive to diseases, which have caused several local decimate of farmed shrimp populations.

According to the graph (Figure 1.2) the black tiger shrimp farming started in Thailand in the late 1980s. Its production sharply increased in the first 10 years and reached a peak of about 250,000 MT in 1994. Then, the white spot disease hit Thailand and the production went down to 180,000 MT in 1997. Fortunately, the diseases were finally addressed and the production bounced back to a new record of 340,000 MT in 2001. But only 4 years later, another disease attacked the country. Thai black tiger output dropped dramatically to 10,000 MT per year and never gets up again.



Thai shrimp production developments

(Source: http://vietfish.org/2012121401447480p49c64/goal-2012-making-a-different-through-responsible-aquaculture.htm)

Figure 1.3 The Thailand's farmed shrimp production in the period from 1988 to 2012. The yellow and the blue bars showed the number of black tiger shrimp and whiteleg shrimp, respectively.

1.4 Disease outbreaks in shrimp production

1.4.1 Bacterial diseases

1.4.1.1 Vibriosis

Vibriosis is a shrimp disease caused by bacteria in the genus *Vibrio*, that contribute to morbidity and is a causative of shrimp mortality. *Vibrio* is a gram-negative bacterium. All members have polar flagella for motility by rod shape in the saltwater. The typical feature is that it is luminous bacteria that can emit a blue-green color light through the luciferase catalysis reaction. The species that can affect to commercially farmed penaeid shrimp is *Vibrio harveyi* (Austin and Zhang, 2006) (Figure 1.2). The mortalities from vibriosis expressed when shrimps are under stresses caused by poor water quality,

high water temperature, low oxygen exchange, and crowding (Lewis, 1973; Lightner and Lewis, 1975; Brock and Lightner, 1990). According to Phuoc et al (2008), it have been found that shrimp that are co-infected with *Vibrio* and white spot syndrome virus, have faster and higher mortality rates than the shrimp infected with *Vibrio* only.

(Source: http://portlandfoodanddrink.com/excuse-me-waiter-my-shrimp-is-glowing/)

Figure 1.4 The luminescent shrimp from *Vibrio harveyi*

(Source: http://mail-cenaim.espol.edu.ec/noti/cursos_material/curso19/ligthner/Photo4_2.htm)

Figure 1.5 The *P. monodon* death which caused by a *Vibrio* sp., probably *V. harveyi* compared to the near normal appearing shrimp on the far left, the other three shrimp with a pale reddish discoloration (bacterial "red disease") of the cuticle and an atrophied, pale white hepatopancreas.

1.4.1.2 Early Mortality Syndrome (EMS)

The novel emerging bacterial infectious disease found in commercial shrimp farm is called early mortality syndrome (EMS). It has been firstly reported in China in 2009 (Panakorn, 2012). Later, the disease outbreak have been reported in many countries such as, Vietnam in 2010 (Mooney, 2012), Malaysia in mid-2010 and Thailand recently in 2012 (Flegel, 2012). EMS also termed acute hepatopancreatic necrosis syndrome or AHPNS. The primary pathogen (considering the disease is infectious) has not been identified, while the presence of some microbes including *Vibrio*, microsporidians and nematode has been observed in some samples. (Lightner et al., 2012) described the pathological and etiological details of this disease. This disease contributes to both of *P. monodon* and *L. vannamei* in characteristic of mass mortalities, arrival to 100 percent in 20-30 day. The symptom of the disease is slow growth, corkscrew swimming, pale coloration, loose shells, abnormal shrunken, small, swollen, and discolored hepatopancreas (http://naturalshrimp.com/university-of-arizona-dr-donald-lightner/).

(Source:http://www.fis.com/fis/worldnews/worldnews.asp?monthyear=&day=18&id=58172&l=e&special=0&ndb=0)

Figure 1.6 The bacterial infected shrimp were death with Early Mortality Syndrome (EMS).

1.4.2 Viral diseases

The major viral pathogens in shrimp are caused by Taura Syndrome Virus, White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV).

1.4.2.1 Taura syndrome virus (TSV)

The Taura syndrome virus, TSV was first reviewed by Jimenes in 1992 as a shrimp disease that caused by Taura virus. The Taura virus is a cytoplasmic, non-enveloped icosahedral virus containing a single-stranded positive sense RNA genome of 10,205 nucleotides of 32 nm diameter (Bonami et al., 1997; Mari et al., 2002). It was classified as a possible member of the family Picornaviridae based on biological and physical characteristics. It was later reclassified in the Dicistroviridae family, genus Cripavirus (Mayo, 2002 and 2005). It has since been reassigned to a second genus in the same family - the Aparavirus. Seriously, this disease is a causative of shrimp mortality in *P. monodon* and *L. vannamei*. In 2004, Taura syndrome virus was hit to

Thailand shrimp farm, it made a lot of damages and the farmers have to change from cultivation of *P. monodon* to *L. vannamei*. The symptoms of Taura syndrome virus in shrimp are tail fan and pleopods particularly were red (Figure 1.6), shell soft, darkening of body from infection.

(Source: http://www.dld.go.th/niah/AnimalDisease/aquatic_TauraShrimp.htm)

Figure 1.7 The presenting symptom of the Taura syndrome in black tiger shrimp. The tail fan and pleopods partially present in red.

1.4.2.2 White spot syndrome virus (WSSV)

White spot syndrome virus (WSSV) is a virus that can infect crustacean species such as penaeid shrimps and crabs (Lo et al., 1996; Peng et al., 1998; Sumattaya et al., 1998; Chen et al., 2000). Firstly, it cause of severe death of shrimp in Taiwan (Chou, 1995) and further reported in Thailand (Lo et al., 1996). The disease is highly lethal and spreads and kills shrimps quickly. The outbreaks of this disease have cleared within a few days the whole populations of many shrimp farms throughout the world. The disease is caused by a family of related viruses subsumed as the White spot Syndrome Baculovirus complex (WSSV). The clinical signs of this disease are lethargy,

a pink to reddish-brown discoloration. The typical symptom of this disease is presenting the white spot of 0.5-2 nm on shrimp's carapace of the cephalothorax (Chou et al., 1995; Lightner, 1996). WSSV is an envelope with double stranded DNA. This virus was classified in Whispovirus genus, Nimaviridae family (Mayo, 2002; Vlak et al., 2005).

(Source: http://business.mega.mu/2012/05/22/new-virus-hits-aquaculture-indian-ocean/)

Figure 1.8 White spot presenting on shrimp carapace after WSSV infection.

1.4.2.3 Yellow-head disease (YHD)

Yellow-head disease (YHD) is a viral infection always found in shrimp such as the black tiger shrimp (*P. monodon*) and the white leg shrimp (*L. vannamei*). This disease caused the economic losses in Thailand shrimp aquiculture since 1992 (Limsuwan, 1991; Boonyaratpalin et al., 1993; Chantanachookin et al., 1993). Moreover, this shrimp disease was found in shrimp farm in the Asia (Walker et al., 2001). It is caused by a positive-sense single-stranded RNA virus namely yellow head virus (YHV) with rod-shaped and enveloped viral particle of about 40-60 nm × 150-200 nm. This virus was classified in Okavirus genus, Roniviridae family and Nidovirales order

(Wongteerasupaya, 1995 and Walker, 2005). The symptoms of this disease can be observed as abnormally high rate feeding, yellow light at cephalothorax and hepatopancreas and dramatrically increase shrimp mortality to 100% in 3-5 days (Chantanachookin, 1993).

(Source: http://library.enaca.org/Health/FieldGuide/html/cv010yhd.htm#)

Figure 1.9 Yellow head disease in the black tiger shrimp (*Penaeus monodon*). The yellow heads of infected shrimp was shown on the left-hand side and normal shrimp was shown on the right-hand side.

1.5 The crustacean immune response

All living organisms have the defense system for fighting against all pathogenic organisms. Invertebrates rely on the innate immunity. This immune system composes of two responses that are able to build up a forcible non adaptive response against microorganisms upon microbial challenge, such as cellular and humoral responses. The cellular response dose not implicates antibodies but implicates the activation directly by hemocytes, for examples, phagocytosis, blood coagulation, nodule formation and encapsulation. The humoral response is the defense mechanism mediated by immune

components which are synthesized from those cells, such as various enzymes and proteins in blood coagulation system, prophenoloxidase activating system, agglutination, proteinase inhibitors and antimicrobial peptides (Hoffmann et al., 1999; Iwanaga and Lee, 2005; Söderhäll, 1999).

The immune response in crustaceans is classified as innate immunity, which is activated to protect the cells against pathogen invading by both of humoral and cell mediated immune responses. In crustaceans, the hemocytes are the major immune related cells that play a crucial role in immunity. The hemocytes were classified into three types, such as hyaline cell (agranular), semigranular cell (small granular), and granular cell (large granular) (Bauchau, 1980; Tsing et al., 1989). As discussed most recently by Lin and Söderhäll (2011), the biological functions of each hemocyte type was reported, hyaline cell hemocyte have potential roles in phagocytosis and cytolysis for blood clotting (Smith and Söderhäll, 1983; Vargas-Albores F. et al., 1998).

1.6 Shrimp immunity

The immunity of shrimp immediately acts against pathogen invading. Their immune system mainly involves in the 3 types of hemocytes, which are able to carry out encapsulation, nodule formation, and phagocytosis in removing microorganisms (Kobayashi et al., 1990; Pech and Strand, 2000; Sung et al., 1998). Moreover, several plasma components such as antimicrobial peptides, histones, lysosomal enzymes, recognition molecules (Lee and Söderhäll, 2002), lipopolysaccharide- and β-1, 3-glucan-binding proteins (Amparyup, 2012) are importants in penaied shrimp defense reactions.

Among immune reactions in shrimp innate immunity, prophenoloxidase (proPO) activating system is one of major immune reaction in shrimp.

1.6.1 Prophenoloxidase system

The proPO cascade in arthopods is set off in a stepwise process with the recognition of bacterial cell wall components by pattern recognition proteins. The proPO system is activated by bacterial and fungal components. First, the pattern recognition proteins recognize the bacterial and viral invasion, then, the process of melanization is activated. The granulocytes, which are storing and secreting the proPO reaction are induced resulting in the oxidation of toxic quinone intermediate and finally producing melanin. This process, in turn, initiates the activation of a serine protease cascade that leads to the conversion of the proPO-activating enzyme (PPAE) to an active proteinase that converts the inactive enzyme precursor, proPO, into phenoloxidase (PO). The melanin produced helps removing microorganisms by nodule formation (Söderhäll and Cerenius, 1998; Amparyup et al, 2012) (Figure 1.10). The activation of proPO system should be tightly regulated to prevent host cell damage. The presence of proPO system has been reported in arthropod immune system, such as crayfish *Pacifastacua leniusculus*, *Manduca sexta*, *Bombyx mori*, and *Drosophila melanogater* (Aspánvet al,1995; Fujimoto et al, 1995; Hall et al., 1995; Kawabata et al., 1995).

In penaeid shrimp, the cells that stored the enzymes involving in the proPO system are semigranular and granular cells (Perazzolo and Barracco, 1997). In *P. monodon*, *Pm*PPAE1 and *Pm*PPAE2 have been identified as protinases participating in shrimp proPO system. Moreover, *Pm*proPO1 and *Pm*proPO2 were identified as important

enzymes for shrimp in fighting against bacterial infection. From gene silencing results, the significant reduction of the PO activity, the higher mortality rate and bacterial counted was found in the *V. harveyi*-challenged *Pm*proPO1 and *Pm*proPO2 knocked-down shrimp (Amparyup et al., 2009; Charoensapsri et al., 2009; Charoensapsri et al., 2011).

1.7 Serine proteinase inhibitor

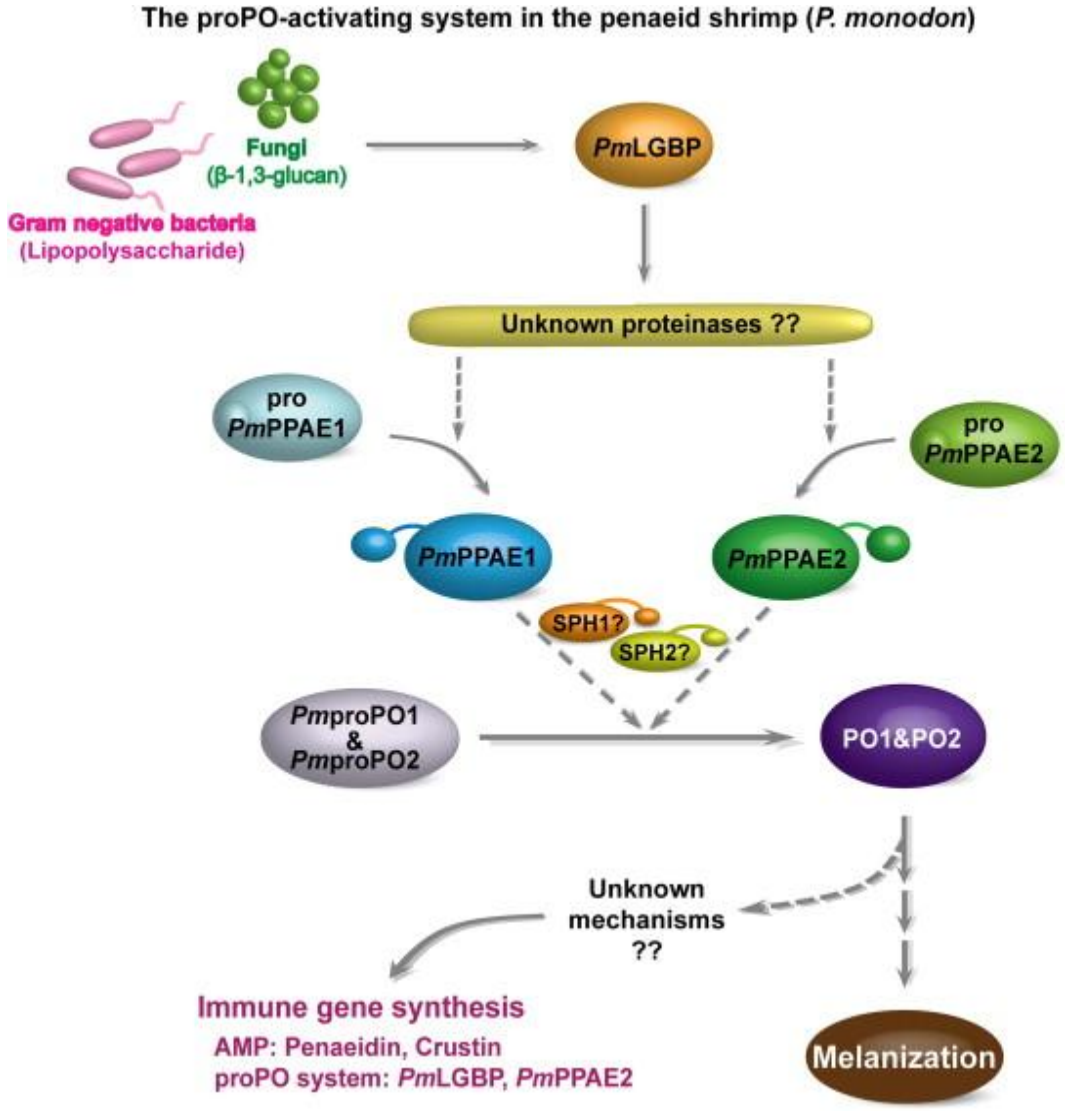
Serine proteinase inhibitors have been found in several organisms, they function as a regulator of the peoteinases involved in many biological processes such as blood coagulation, fibrinolysis, complement activation and hormone transport. They can be divided into subgroups depend on their 3D structure including Kazal, Kunitz, Serpin, α-macroglobulin and pacifastin (Liang et al., 1997).

1.7.1 The low molecular mass proteinase inhibitors

The low molecular mass proteinase inhibitors are classified into three groups including Kazal, Kunitz, and pacifastin.

The first group, Kazal (Kazal-type serine proteinase inhibitors or KPIs), is a single proteins or chains of inhibitor domains. This protein molecules compose of about 40-60 amino acid residues including some spacer amino acids which are domain's linkers. The inhibition mechanism of KPI is accessed by the reactive center loop (RCL), the peptide segment that contains the specific site P1 and P1′ of each domain. KPIs act as a substrate for their specific proteinase and attack to the active site of proteinase by competing inhibition (For review: Rimphanitchayakit and Tassanakajon, 2010).

In invertebrates, KPIs play the potential roles in immune system as they are up-regulated in response to microbial challenges. Others functions of these KPIs need further study.



(Source: Amparyup et al., 2012)

Figure 1.10 The outline of shrimp prophenoloxidase activating system.

The serine proteinase inhitibitors in the Kunitz group have been found in hemolymph of lepidopteran, dipteran and insect species. It contains a single chain of 60 amino acids and can inhibit trypsin and chymotrypsin (Kanost, 1999). Kunitz domains have been found in multiple tandem repeats such as bovine pancreatic trypsin inhibitor (BPTI). The crystal structure of the trypsin-BPTI complex revealed that the Kunitz domain inserts a protruding reactive site loop into the active clef of its cognate proteinase (Macedo-Ribeiro et al., 2008).

1.7.2 α-Macroglobulin

α-Macroglobulin is a family of serine proteinase inhibitors in which its size is larger than the others and mechanism of inhibition is different. Each α-macroglobulin protein contains an exposed bait region that is susceptible to proteolytic cleavage and an intramolecular β-cysteinyl-γ-glutamyl thioester that is hided in a pocket protected from solvent. Cleavage of the bait region by the specific proteinase leads to a conformational change that traps the proteinase in a cavity formed by the α-macroglobulin tetramer (in vertebrates) or dimer (in invertebrates) (Sottrup-Jensen, 1989 and Kanost, 1999). The change in conformation also leads to formation of covalent crosslinks between the thiol ester region of α-macroglobulin and lysine side-chains of the proteinase, resulting in irreversible inhibition of the proteinase, even though its active site is not affected (Sottrup-Jensen, 1989).

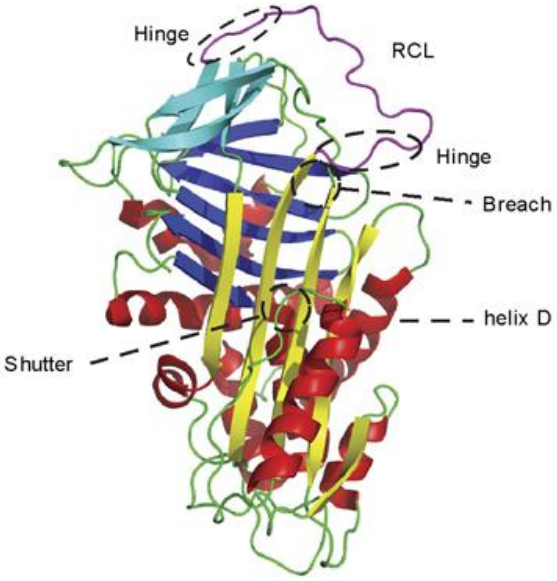
α-Macroglobulins have been identified and characterized in horseshoe crabs and crustaceans, but not yet in insects (Kanost and Jiang, 1996). A cDNA for *Limulus polyphemus* α-macroglobulin was cloned (Iwaki et al., 1996). The amino acid sequence

of the horseshoe crab protein was 28–29% identical to mammalian α-macroglobulins and included a conserved bait region, thiol ester site, and receptor binding domain. Like the mammalian proteins, *L polyphemus* α-macroglobulin is related in sequence to complement proteins C3 and C4. The α-macroglobulin were found in various shrimps such as *Marsupenaeus japonicus*, *Fenneropenaeus chinensis*, *Farfantepenaeus paulensis*, and *P. monodon*, they are expressed in hemocytes and stored in the large granules (Rattanachai et al., 2004; Ma et al., 2010; Perazzolo et al., 2011 and Chaikeeratisak et al., 2012).

1.7.3 Serpin-type serine proteinase inhibitor

Serpins have been found in several organisms and their molecular mass is about 40-50 kDa with approximately 400 amino acid residues in length. Its core domain consists of 3 beta-sheets and 8-9 alpha-helices. A typical feature of serpin is the Reactive Center Loop or 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 residues called P1 and P1′ which is cleaved by the target proteinase (Figure 1.11).

The mechanism of inhibition of serpin has been demonstrated biophysically and structurally as suicide substrate-like inhibitory mechanism (Figure 1.12) where after binding to the target protease it is partitioned between cleaved serpin and serpin-protease complex. Initially serpin binds to protease through a noncovalent Michaelis-like complex by interactions with residues flanking the scissile bond (P1-P′1). Then, the serpin rapidly undergoes conformation change, and finally formed a very stable complex of inactivated enzyme and serpin (Law et al., 2006) (Figure 1.12).



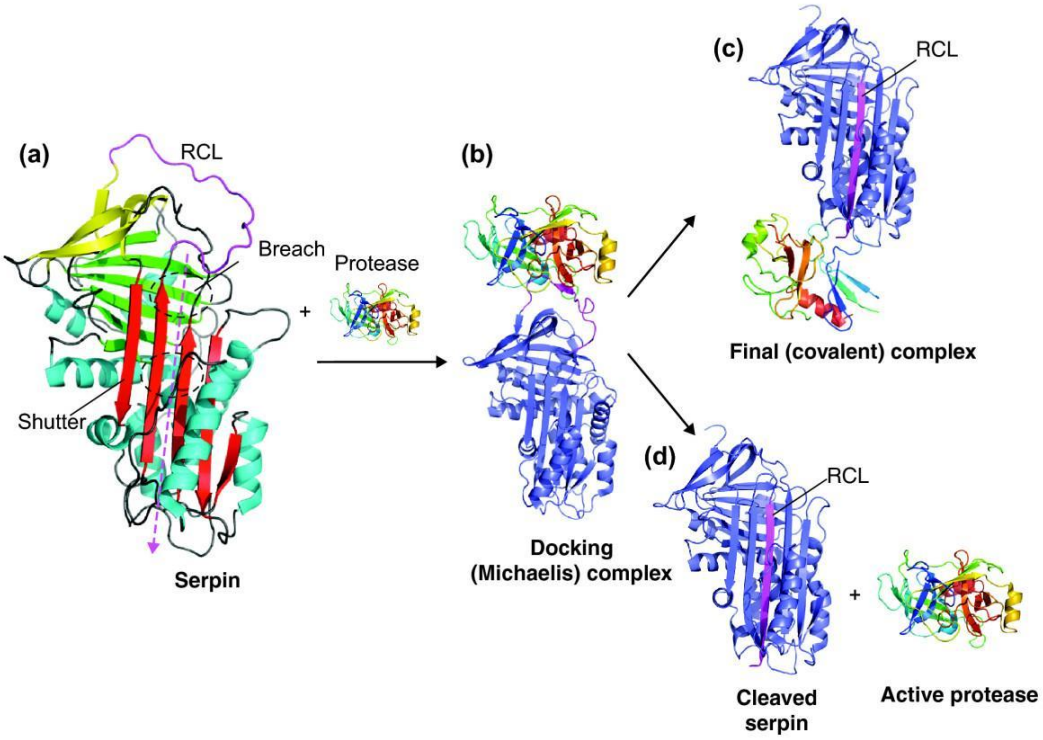
(Source: Kaiseman et al., 2006)

Figure 1.11 The structure of native SERPINA1 from *Homo sapian* species.

Many serpins in various organisms have been reported so far, such as *Homo sapien* and other mammalians. Mammalian serpins have functions in inflammation and blood coagulation (Marshall, 1993). In insects such as *Manduca sexta*, *Bombyx mori*, *Drosophila melanogater, and Ixodes Scappularis,* serpins in hemolymph play roles in regulating innate immune pathways, including proPO activation system and Toll pathway (Zou et al., 2010). Serpins acting as negative regulators of proPO activation system have been reported in various invertebrates.

In *D. melanogaster*, Serpin-27A is required to restrict the phenoloxidase activity at the site of injury or infection, preventing the insect from excessive melanization (Gregorio et al., 2002). In 2008, Scherfer reviewed a model in which Spn28D confines

PO availability by controlling its initial release, while Spn27A is rather limiting the melanization reaction at the wound site (Scherfer et al., 2008).



(Source: Law et al., 2006)

Figure 1.12 Inhibition mechanism of serpin.

In *M. sexta*, serpin-6 strongly inhibited PAP-3 but not PAP-1 or PAP-2, suggesting that the proPO activation by PAPs is differentially regulated by multiple serpins (Wang and Jiang, 2004). In 2010, An and Kanost reviewed that serpin-4 and serpin-5 formed SDS-stable complexes with HP6 *in vitro*, and they inhibited the activation of proHP8 and proPAP1 to modulate proPO activation and antimicrobial peptide production during immune responses of *M. sexta* (An and Kanost, 2010). Serpin-

1J functions to inhibit HP8 and thereby modulates the concentration of active Spätzle to regulate the Toll pathway response in *M. sexta* (An et al., 2011). Recently, serpin-3 complexes with proteinases identified by immunoblot analysis as prophenoloxidase-activating proteinase (PAP)-1, PAP-2, PAP-3, and hemolymph proteinase 8 (HP8). HP8 can cleave and activate the Toll ligand, Spätzle, leading to synthesis of antimicrobial peptides (Christen et al., 2012).

In *Anopheles gambiae*, serpins regulate the activation of prophenoloxidase and thus melanization, contribute to malaria parasite lysis, and likely Toll pathway activation (Gulley et al., 2013).

A few serpins in shrimp have been recently reported. First, the *PmSERPINB3* from *Penaeus monodon* hemocyte of *V. harveyi*-infected shrimp was identified by differential display PCR technique (Somboonwiwat et al., 2006). In 2009, a serpin has identified from hemocyte of *F. chinensis* (*Fc*-serpin) (Liu et al., 2009). Eight more *PmSERPIN* genes have been identified from *P. monodon* EST database (http://pmonodon.biotec.or.th) (Homvises et al., 2010). From previous report, *Pm*SERPIN6 expressed in response to infections at the late phase of bacterial and viral infections implicating its roles in regulation of shrimp immune response (Homvises et al., 2010). Recently, *Pm*SERPIN8 was found to be up-regulated upon *Vibrio harveyi* infection and can inhibit proPO activation (Somnuk et al., 2012). Also, Fc-serpin was expressed in response to bacterial and viral infections. Both *Pm*SERPIN6 and 8 were found in hemocytes.

1.8 Objectives of this thesis

So far, a little is known about function of serpin in shrimp. Therefore, the aim of this study is to characterize *Pm*SERPIN3 gene from the black tiger shrimp, *Penaeus monodon.* First, the full-length of *Pm*SERPIN3 cDNA and its genomic gene were identified. Its expression level in various tissues, developmental stages and in response to *V. harveyi*, WSSV, and YHV challenges were analyzed by RT-PCR. The recombinant mature *Pm*SERPIN3 protein (r*Pm*SERPIN3) was successfully produced in *Escherichia coli*. The native *Pm*SERPIN3 expressed in shrimp hemocyte was determined. The purified recombinant *Pm*SERPIN3 protein was used to determine the *in vitro* inhibitory activities on blood coagulation, proteinases and proPO activation as well as the effect on bacterial clearance in shrimp.

CHAPTER II

MATHERIALS AND METHODS

2.1 Chemicals and Equipments

2.1.1 Chemicals

- 2-Mercaptoethanol, C_2H_6OS (Fluka)
- Absolute ethanol, C_2H_5OH (Hayman)
- Absolute methanol, CH_3OH (Scharlau)
- Acetic acid glacial, CH_3COOH (Merck)
- Acrylamide,C_3H_5NO (Merck)
- Adenosine-5′-triphosphate potassium salt (ATP) (Sigma)
- Agar powder, Bacteriological (Hi-media)
- Agarose, (low EEO, Molecular Biology Grade (Research Organics)
- Amplicilin (bioBasic)
- Anti-His antiserum (Merck)
- Bovine Serum Albumin (BSA)
- Bromophenol blue (Merck)
- Calcium chloride ($CaCl_2$) (Merck)
- Casein Enzyme Hydrolysate, Type-I, Tryptone Type-I (Hi-media)
- Casein Peptone (Hi-media)
- Chloroform, $CHCl_3$ (Merck)
- Choramphinicol (Sigma)

- Coomasie brilliant blue G-250, (Fluka)
- DEPC (Diethyl pyrocarbonate), $C_6H_{10}O_5$ (Sigma)
- EDTA (Ethylene diamine tetraacetic acid disodium salt dehydrate), (Ajax)
- Ethidium bromide, (Sigma)
- GeneRuler™ 1 kb DNA ladder (Fermentas)
- GeneRuler™ 100 bp DNA ladder (Fermentas)
- Glycerol, $C_3H_8O_3$ (Ajax)
- Glycine, USO Grade, NH_2CH_2COOH (Research organics)
- Hydrochloric acid, (HCL) (Merck)
- Imidazole (Fluka)
- IPTG (Isopropyl-β-D-thiogalactoside), C9H18O5S (USBiological)
- Isoamylalcohol (Merck)
- Isopropanol, C_3H_7OH (Merck)
- Magnesium chloride, $MgCl_2$ (Merck)
- NBT (Nitroblue tetrazolium) (Fermentas)
- Ni Sepharose 6 Flas Flow (GE Healthcare)
- Paraformaldehyde (Sigma)
- Phenol: chloroform: isoamyl alcohol (Sigma)
- Prestain protein molecular weight markers (Fermentas)
- RNase A (Sigma)

- Skim milk powder (Hi-media)
- Sodium acetate, CH_3COONa (Carlo Erba)
- Sodium chloride, NaCl (Ajax)
- Sodium dihydrogen orthophosphate, $NaH_2PO_4H_2O$ (Carbo Erba)
- Sodium hydroxide, NaOH (Merck)
- TRIREAGENT®(Molecular Research Center)
- Tris (Vivantis)
- Triton® X-100 (Merck)
- Tryptic soy broth (Difco)
- Tween™-20 (Fluka)
- Unstained protein molecular weight markers (Fermentas)

2.1.2 Kits

- GenepHlow™ Gel/PCR Kit (Geneaid)
- QIAprep spin miniprep kit (Qiagen)
- RevertAID™ first strand cDNA synthesis kit (Fermentas)
- T&A cloning vector kit (RBC Bioscience)

2.1.3 Proteinases and its substrates

- 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)
- Subtilisin A (Sigma)
- Trypsin (Sigma)

2.1.4 Bacterial strain

- *Escherichia coli* strain BL21(DE3)
- *E. coli* strain XL-1-Blue
- *Vibrio harveyi* strain 639

2.1.5 Software

- BlastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi)
- ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/)
- ExPASy ProtParam (http://web.expasy.org/compute_pi/)
- GENETYX version 7.0 program (Software Development Inc.)
- MEGA4 version 4 (Tamura, Dudley, Nei, and Kumar 2007).
- NetNglyc software (http://www.cbs.dtu.dk/services/NetNGlyc/)
- *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/home.jsp)
- SECentral (Scientific & Educational Software)
- SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/)

2.1.6 Vector

- pET32b(+) (Novagen)
- pVR600
- T&A cloning vector (RBC Bioscience)

2.1.7 Equipments

- -20 °C Freezer (Whirlpool)
- -80 °C Freezer (Thermo Electron Corporation)
- 96-well cell culture cluster, flat bottom with lid (Costar)
- Autoclave model # MLS-3750 (SANYA E&E Europe (UK Branch) UK Co.)
- Automatic micropipette P10, P20, P100, P200 and P1000 (Gilson Medical Electrical)
- Balance PB303-s (Mettler Teledo)
- Biophotometer (Eppendorf)
- Centrifuge 5804R (Eppendorf)
- Centrifuge Avanti™ J-30I (Beckman Coulter)
- Force mini centrifuge (Select BioProducts)
- Gel Documention System (GeneCam FLEX1, Syngene)
- GelMate2000 (Toyobo)
- Gene pulser (Bio-RAD)
- Incubator 30 °C (Heraeus)
- Incubator 37 °C (Memmert)
- Innova 4080 incubator shaker (New Brunswick Scientific)
- Laminar Airflow Biological Safety Cabinets ClassII Model NU-440-400E (NuAire, Inc., USA)

- Microcentrifuge tube 0.6 ml and 1.5 ml (Axygen® Scientific, USA)
- Minicentrifuge (Costar, USA)
- Mini-PROTEAN® 3 cell (Bio-RAD)
- Minipulser electroporation system (Bio-RAD)
- 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)
- PD-10 column (GE Healthcare)
- pH-meter pH 900 (Precisa, USA)
- Pipette tips 10, 100 and 1000 µl (Axygen®Scientific, USA)
- Power supply, Power PAC3000 (Bio-RAD Laboratories, USA)
- Sonicator (Bandelin Sonoplus, Germany)
- SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices)
- Touch mixer Model#232 (Fisher Sciectific)
- Trans-Blot®SD (Bio-RAD Laboratories, USA)
- Water bath (Memmert)
- Whatman® 3 MM Chromatography paper (Whatman International Ltd., England)

2.2 Primer design

All primers used in this study were designed based on nucleotide sequences of template cDNA by SECentral programe (Scientific & Educational Software). The melting temperature, self-priming, GC content and primer-dimer formation were carefully considered (Table 2.1).

Table 2.1 Nucleotide sequences of the primers and annealing temperature for PCR reaction.

Name	Purpose	Sequence (5′-3′)	Annealing temperature (°C)
SPN3_RTF SPN3_RTR	RT-PCR and genome organization	CCTGATTCCTTCCGGCGTTCTA GCCAGCTTAGCTTCAACCTCAG	58
β-actin_RTF β-actin_RTR	RT-PCR	GGTGCTGGACAAGCTGAAGGC CGTTCCGGTGATCATGTTCTTGATG	55
EF1-α_RTF EF1-α_RTR	RT-PCR	GCTTGCTGATCCACATCTGCT ATCACCATCGGCAACGAGA	55
GSP_F NGSP_F	3′ RACE PCR	GACTTTGGTGAAAGTGAAGCCGTGCGC AGGACCTGATTCCTTCCGGCGTTCTAA	68
GSP_R NGSP_R	5′ RACE PCR	AGACTGAAGCTCCTGACTATGAGTTGCGG ATGTCAAGACTCTGGCTCTCAGTC	68
ORFSPN3_F ORFSPN3_R	Full-length cDNA identification and genome organization	ATGGCTGGTCCAGTCAGATTTGTGTT CTCGAGCTAAGGCTTGACAAATCGCCCA	67
rSPN3_F rSPN3_R	r*Pm*SERPIN3 production	CCATGGGCCAGGCCCCACTCTCCTTCCCA CTCGAGAGGCTTGACAAATCGCCCAGCAA AGTG	58

2.3 PCR product purification

NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) was used for DNA purification from PCR product and cut DNA band. The 200 µl NT buffer was added to dissolve 100 g gel and melted at 55 °C for 10-15 min. The mixed solution was transfer into a column. The column was centrifuged at 11,000 rpm for 1 min, washed by adding 600 µl of NT3 buffer and centrifuged at 11,000 rpm for 30 sec 2 times. The filtrate was poured off and the column was further centrifuged at 11,000 rpm for 2 min to dry membrane. The sterile deionized water (20-30 µl) was used as elution buffer. It was added into the membrane center, incubated at room temperature for 10 min and centrifuged at 11,000 rpm for 2 min to collect the purified DNA.

2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used for checking genomic DNA, RNA intrigrity and size of PCR and purified PCR products. The suitable percent of agarose gel was prepared in 1X TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). Approximately 60 °C gel solution was melt and poured onto a tray. The wells in the gel were formed by the comb teeth. The DNA samples mixed with 1X loading dye (5 mM Tris-HCl, 2.5 mg/ml bromophenol blue, 0.25 mg/ml xylene cyanol, 6% glycerol at pH 7.6) were run at 100 V for about 30-40 min. The size of DNA samples were determined by comparing to DNA ladder (100 bp or 1 kb markerss, Fermentas). The gel was stained in ethidium bromide solution for 30 sec and further destained in water for 30 min. The DNA samples were detected under UV transilluminator.

2.5 Competent cell preparation

E. coli strain BL21(DE3) and XL-1-Blue were restreaked onto the LB agar and LB agar containing tetracyclin (10 µg/µl) inoculated into LB and LB containing tetracycline (10 µg/µl) media, respectively, and shaked at 250 rpm at 37 °C for 16 h. The overnight culture was inoculated to fresh medium and cultured until OD_{600} reach 0.6 and the cells were then chilled on ice for 30 min. The cell pellet was collected by centrifugation at 4,000 x g for 15 min and washed once using 0.5 volumn of 10 mM $CaCl_2$ solution containing 10% (v/v) glycerol. The cells were chilled on ice for 30 min. The 100 µl of competent cells were aliquoted and stored at -80 °C.

2.6 Calcium chloride transformation

The 10 µl of ligation mixture was mixed with 100 µl of competent cell and chilled on ice for 30 min. The reaction was immediately incubated at 42°C for 1 min. One ml of LB medium was added to the mixture and incubated at 250 rpm at 37 °C for 1 h. After that, the cell was collected by centrifugation at 11,000 rpm for 2 min and subsequently spread onto the LB agar plate containing an appropriate antibiotic for recombinant done selection. The single colony of each transformant was cultured for plasmid extraction using the High-speed plasmid mini kit (Geneaid). The specific restriction enzymes were used for digestion to verify the size of DNA insert.

2.7 Identification of *PmSERPIN3* gene

2.7.1 Rapid Amplification of cDNA End (RACE)

Base on the partial sequence of *PmSERPIN3* gene from an EST clone no. SG5480 in the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/home.jsp), gene specific primers (GSP_F/R) and nested primers (NGSP_F/R) listed in Table 2.1 were designed for amplifying the 5′- and 3′-fragments by Rapid Amplification of cDNA Ends (RACE) techniques using SMARTer® RACE cDNA Amplification Kit (Clontech).

Total RNA of unchallenged shrimp hemocyte was used as a template for 3′-and 5′- RACE cDNA syntheses according to kit's instruction. For the 3′-RACE, the PCR reaction was mix in a total volume 50 µl as follows: 5 µl of 10× Advantage 2 PCR buffer, 5 µl of UPM (Universal Primer Mix) or NUP (Nested Primer Mix), 1 µl of the 10 mM GSP_F or NGSP_F primer (Table 2.1) for primary and secondary RACE PCR, respectively, 1 µl of each dNTP (10 mM), 2.5 µl of the 3′-RACE cDNA and 50-fold diluted 1st PCR product template for primary and secondary RACE PCR, respectively, 1 µl of 50× Advantage 2 Polymerase Mix and 34.5 µl of PCR grade water. The 5′- RACE PCR reaction was same as the 3′ RACE PCR reaction, but NUP was used instead of the UPM and GSP_R primer was used for primary 5′-RACE PCR only.

For the 3′- RACE PCR, all components are mixed, denatured for 5 cycles at 94°C for 30 sec, and subjected to 5 cycles of 95°C for 30 sec, 70°C for 30 sec, 72°C for 3 min. After another denaturation step, 20 PCR cycles were as follows: 94°C for 30 sec, 68°C for 30 sec, 72°C for 3 min. For the 5′- RACE PCR, all components are mixed, after

another denaturation steps as described in 3′- RACE PCR condition, 20 PCR cycles were as follows: 94°C for 30 sec, 68°C for 30 sec, 72°C for 3 min.

The RACE PCR products were analyzed by agarose gel electrophoresis, the amplified products were purified, isolated, cloned into a T&A vector (RBC Bioscience) and sequenced by a commercial service Macrogen Inc., Korea. The nucleotide sequences of EST clone and RACE PCR fragments were assembled and blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov).

The obtained full-length cDNA of *PmSERPIN3* was further analyzed by predicting the deduced amino acid sequences and the signal peptide by ExPASy tools (http://web.expasy.org/compute_pi/) and SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/), respectively. The calculated molecular mass and isoelectric point of the mature protein was predicted by ExPASy tools (http://web.expasy.org/compute_pi/).

2.7.2 Genome organization

The genomic DNA was extracted from the pleopods of black tiger shrimp using a standard phenol–chloroform extraction. The genomic *PmSERPIN3* gene was amplified from the genomic DNA using 2 pairs of primers designed from the cDNA sequence of *PmSERPIN3*such as, ORFSPN3_F and SPN3_RTR as well as SPN3_RTF and ORFSPN3_R (Table 2.1). Approximately 50 ng of genomic DNA was used as a template in 50 µl PCR reaction composing of 1× Advantage 2 buffer, 0.2 mM each dNTP, 2 µl of 5 µM forward and reverse primers, and 1 unit Advantage®2 Polymerase Mix (Clonetech). The reaction was started by heating for 2 min at 95 °C followed by 35

cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s, and ended with 10 min at 72 °C. The PCR products were analyzed by agarose gel electrophoresis, isolated, cloned into a T&A vector (RBC) and sequenced by a commercial service Macrogen Inc., Korea. The DNA sequence was aligned with that of *PmSERPIN3* cDNA to determine the introns, exons and splice sites.

2.7.3 Phylogenetic analysis

The deduced amino acid sequences of mature peptide of *Pm*SERPIN3 and other serpins found in the GenBank database were selected for amino acid alignment using ClustalX program (Larkin et al., 2007). The phylogenetic analysis was performed by the neighbor-joining (NJ) distance algorithm in the MEGA4 software (Tamura et al., 2007) with bootstrap trial 1000 replicates.

Table 2.2 Gene names and GenBank accession numbers of serpins used for the phylogenetic analysis.

Gene name	Accession no.	Gene name	Accession no.
Ms serpin-1	ACC47342	*Dr* glia-derived nexin	NP956478
Bm Antitrypsin isoform 1	ACT36276	*Hs* glia-derived nexin isoform 9	NP006207
Ms serpin-2	AAB58491	*Ss* glia-derived nexin	NP001133589
Dm Spn43A	AAQ64953	*Ec* nexin-1 isoform 1	XP001495988.1
Dm serpin6	NP_524953	*Is* serpin-7	XP002407493
Cq alaserpin	XP1865071	*Hs* antithrombin	CAA48690
Tt LICI-3	BAA12795	*Is* serpin-2	XP0024344444
Ms serpin-6	AAV91026	*B. alba* proteinase inhibitor I4	ZP02001593
Pl serpin	CAA57964	*Is* serpin-3	XP002416641
*Pm*SERPIN8	ADC42879	*Pm*SERPIN3	KC577446
*Pm*SERPIN6	GQ260129	*Bt* serpin B10	NP001092395
*Pm*SERPIN7	GU358487	*Ss* leukocyte elastase inhibitor I4	AC133239
Fc-serpin	ABC33916	*Hs* serpin B4	NP002965
Cq nexin	XP001866682	*Pm*SERPINB3	GQ260130
Dm Serpin27A	AAF24518	*Tt* LICI-2	BAA06909
Of RbPN-1	HQ385323		

2.8 Shrimp and pathogen infection experiments

2.8.1 Shrimp

The shrimp sample at different developmental stages such as, nauplius IV, zoea III and mysis IV larvae were collected from Suratthani farm and the whole animal was used for total RNA extraction.

The healthy black tiger shrimp, *P. monodon*, approximately 17-20 g body weight were bought from local shrimp farms in Chachoengsao Province, Thailand. The shrimp were maintained in the laboratory tank under room temperature for a few days before experiments.

2.8.2 *Vibrio harveyi* challenge

V. harveyi strain 639 was grown on a Tryptic soy agar (TSA) plate containing 2% NaCl, then, incubate at 30 °C for overnight. A single colony of *V. harveyi* was incubated into Tryptic soy broth (TSB) containing 2% NaCl and cultured at 30 °C with shaking at 250 rpm for overnight. The culture was diluted 1/100 in the sterile TSB containing 2% NaCl and cultured until A_{600} reached 0.6 where bacterial cell densities was 10^8 CFU/ml. The culture was diluted to 100 µl of 10^5 CFU of *V. harveyi* strain 639 in 0.85% (w/v) NaCl for shrimp injection. The control shrimp were injected with 100 µl 0.85% (w/v) NaCl.

2.8.3 WSSV and YHV challenges

For WSSV challenge, the stock of WSSV was diluted in lobster hemolymph medium (LHM) at 1:8000 dilutions. The 100 µl of diluted WSSV in LHM, dosages that killed 100% of shrimp within 4 days, was injected into shrimp.

For YHV challenge, the stock of YHV was dilute in lobster hemolymph medium (LHM) at 1:10^6 dilutions. The 100 µl of diluted YHV in LHM, dosages that killed 100% of shrimp within 4 days, was injected into shrimp.

2.9 Gene expression analysis of *PmSERPIN3* gene

2.9.1 Tissue distribution analysis

2.9.1.1 Tissue collection

For tissues distribution analysis of *PmSERPIN3* transcripts, various tissues of unchallenged shrimp such as gill, hepatopancreas, hemocyte, heart, nerve, epipodite, lymphoid organ, antennal gland, stomach, eyestalk, intestine and muscle were dissected and stored immediately in liquid nitrogen for further processing. Alternatively, hemocyte was prepared by collecting of hemolymph under 1/10 vol 10% sodium citrate and centrifugation at 800 ×g for 10 min at 4°C. The hemocyte pellet was stored in liquid nitrogen until further processing.

2.9.1.2 Total RNA preparation and cDNA synthesis

Total RNA was isolated according to the manufacturer's instructions. Briefly, the tissue samples were homogenized in 1 ml TRIREAGENT® (Molecular Research Center). Then, 200 µl of chloroform was added into the homogenate and 10 times of inversion were performed, incubated at room temperature for 5 min before 12,000 rpm of centrifugation at 4°C for 15 min. The upper solutions were transferred to new 1.5 ml tube and total RNA was precipitated with 1 vol of isopropanol. The pellet of RNA was collected by centrifugation at 12,000 rpm for 15 min at 4 °C and washed in 1 ml of 75% ethanol and stored at -80 °C until used. After centrifugation, 75% ethanol

was removed and the RNA pellet was air-dried and dissolved with diethyl pyrocarbonate (DEPC)-treated water and stored at -80 °C. The quantity and quality of total RNA was determined by meaning A_{260} and A_{280}. Equal amount of total RNA from 3 shrimp was pooled and treated with RQ1 RNase-free DNase (Promega) to remove any contaminating DNA. One microgram of total RNA was used as a template for first strand cDNA synthesis using the RevertAID™ First Strand cDNA Synthesis Kit (Fermentas).

2.9.1.3 Semi-quantitative Reverse Transcription-PCR (RT-PCR)

The gene-specific SPN3RTF/R primers (Table 1) were designed from the original EST clone, SG5480, which amplified a 303 bp fragment of the *PmSERPIN3* gene. PCR reactions in a total volume of 25 μl contained 3 μl of 10-fold diluted cDNA as a template, 5 μM of each forward (SPN3_RTF) and reverse (SPN3_RTR) primers (Table 2.1), 1.5 mM $MgCl_2$, 100 μM of each dNTP and 1 U *Taq* polymerase (RBC Bioscience). The PCR profile was 94 °C for 2 min followed by 29 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 30 sec.

For tissue distribution analysis, *β-actin* gene which is used as an internal control gene, was amplified as a 337-bp fragment using the β-actinRT_F/R primers and annealing temperature shown in Table 2.1. PCR product was analyzed by 1.5% agarose gel electrophoresis.

2.9.2 Expression analysis of *PmSERPIN3* gene in different shrimp developmental stage

2.9.2.1 Total RNA preparation and cDNA synthesis

Shrimp at nauplius IV, zoea III and mysis IV stages were homogenized in TRIREAGENT® and then extracted for total RNA as described in section 2.9.1.2. The 2.5 µg of total RNA was used as template for first strand cDNA synthesis as described in section 2.9.1.2.

2.9.2.2 Semi-quantitative Reverse Transcription-PCR (RT-PCR)

The gene-specific SPN3RTF/R primers and the PCR reactions, described in section 2.9.1.3 were used for amplification. The cDNA templates were prepared from the shrimp at nauplius IV, zoea III and mysis IV stages. The elongation factor-1α (*EF-1α*) was used as an internal control. The *EF-1α* was amplified as a 150-bp fragment using EF-1αRT_F/R primers at the annealing temperature shown in Table 2.1. PCR product was analyzed by 1.5% agarose gel electrophoresis.

2.9.3 Expression analysis of *PmSERPIN3* gene in response to pathogen infection

2.9.3.1 Total RNA preparation and cDNA synthesis

For the expression analysis of *PmSERPIN3* gene in response to pathogen infection, the shrimp were divided into 6 groups of 3 pathogen-challenged groups; *Vibrio harveyi*, white spot syndrome virus (WSSV) and yellow head virus (YHV)-challenged groups, and 3 control groups for the pathogen challenged groups. At 0, 6, 12, 24 and 48 h after pathogen injection, the hemolymph from 3 individual shrimp was collected using 10% (w/v) trisodium citrate as an anticoagulant. Hemocytes were

separated by centrifugation at 800 × g for 10 min at 4 °C and subjected to total RNA preparation. Subsequently, first strand cDNA was synthesized.

2.9.3.2 Semi-quantitative Reverse Transcription-PCR (RT-PCR)

The gene-specific SPN3RTF/R primers and the PCR reaction as described in section 2.9.1.3 were used for amplification. The cDNA templates were prepared from the hemocytes of pathogen challenge shrimps at various time points. The hemocytes of *V. harveyi* and WSSV challenged shrimp were collected at 0, 6, 24 and 48 hpi. Whereas, the hemocytes of YHV challenged shrimp were collected at 0, 6, 12, 24, 48 and 72 hpi. The *EF-1α* was amplified as a 150-bp fragment using EF-1αRT_F/R primers at the annealing temperature shown in Table 2.1. PCR product was analyzed by 1.5% agarose gel electrophoresis.

2.10 Expression and purification of recombinant *Pm*SERPIN3

2.10.1 Construction of expression vector for recombinant *Pm*SERPIN3 protein production

Two specific primers were designed to amplify a nucleotide sequences coding for the *Pm*SERPIN3 mature peptide. The *Nco*I and *Xho*I restriction sites were added to the 5′-end of forward primer rSPN3_F and reverse primer rSPN3_R (Table 2.1), respectively, for cloning into an expression vector (Figure. 2.1). The gene fragment was obtained by standard PCR. The amplified fragment was purified, then digested with *Nco*I and *Xho*I, and cloned into the pET-32b(+) (Novagen) and pVR600 (pET-28b(+) derivative) vectors. Then, the *Pm*SERPIN3 fragment was ligated with pET-32b(+) and pVR600 vectors cut with same restriction enzymes by incubating the ligation mixture at

room temperature (25 °C) for 3 h. Followed by transformation (Heat shock method), the ligation mixture was transformed into the *E.coli* strain XL-1-blue $CaCl_2$-treated competent by heat shock method. The recombinant clones were selected on LB agar plates containing 100 µg/µl ampicillin for pET-32-SERPIN3 and 50 µg/µl kanamycin for pVR600-SERPIN3. The single colony was cultured at 37 °C for overnight for plasmid extraction. The recombinant plasmids, pET-32-SERPIN3 and pVR600-SERPIN3) obtained were transformed into an expression host, *E. coli* strain BL21(DE3).

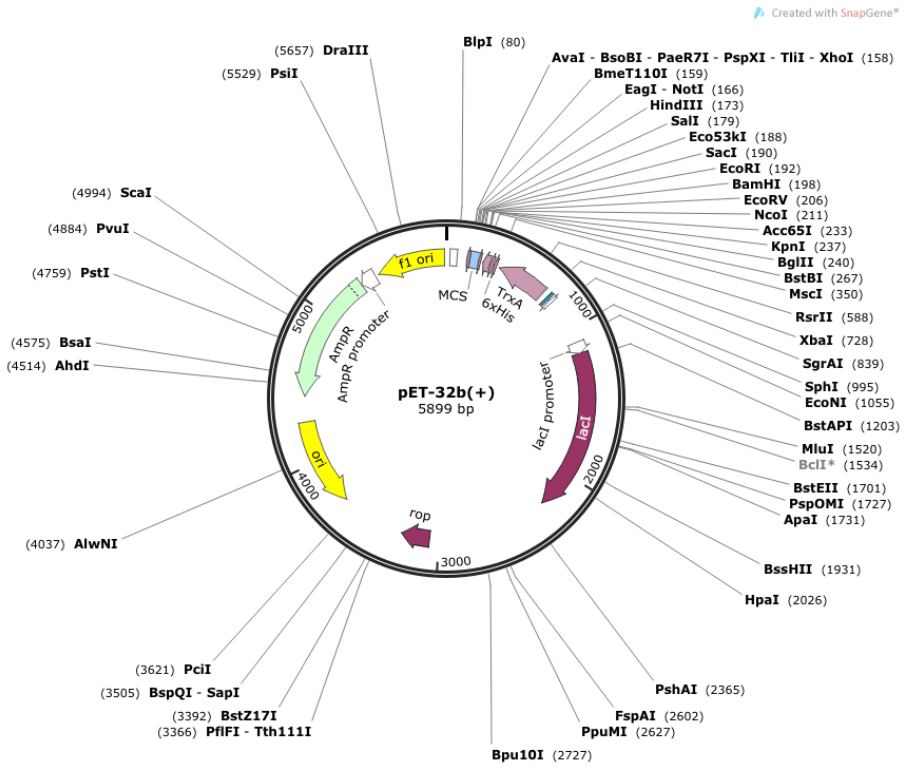


Figure 2.1 The pET-32b(+) vector map (Novagen®, Germany)

2.10.2 Expression of the recombinant *Pm*SERPIN3 protein

The recombinant *Pm*SERPIN3 protein (r*Pm*SERPIN3) was produced in an *Escherichia coli* expression system. To over-produce the *Pm*SERPIN3, a colony of *E. coli* BL21(DE3) containing the recombinant plasmid was cultured in LB broth containing an appropriate antibiotic. For r*Pm*SERPIN3 produced from pvR-600-SERPIN3 recombinant clones, r*Pm*SERPIN3 producing clone was cultured in LB broth containing kanamycin at 100 µg/µl, whereas the r*Pm*SERPIN3 from pET-32-SERPIN3 recombinant clone, ampicillin antibiotic at 100 µg/µl was used for recombinant clone selection. The culture was induced for the expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.

After induction, the cells were harvested at appropriate times and resuspended in 10 mM sodium phosphate buffer pH 7.4. The cell suspension was freeze-thawed three times before the cells were completely lysed by sonication. The cell lysate and inclusion bodies were separated by centrifugation. The r*Pm*SERPIN3 production was verified by separating either whole cells or fractions of cell lysate and inclusion bodies on 10% SDS-PAGE.

2.10.3 Purification of the recombinant *Pm*SERPIN3 protein

The r*Pm*SERPIN3 from pET-32-SERPIN3 was prepared from the inclusion bodies by solubilizing with 100 mM NaOH for 1 h and dialyzed against 20 mM Tris–HCl pH 8.0 for overnight. The r*Pm*SERPIN3 from pVR600-SERPIN3 was purified from the soluble cell lysate fraction. The crude soluble proteins were purified via Ni-NTA column (GE Healthcare).

For the crude r*Pm*SERPIN3 protein from pET-32-SERPIN3 was incubated with Ni-NTA bead pre-equilibrated with binding buffer (20 mM Tris-HCl pH 8.0, 20 mM Imidazole and 0.3 M NaCl). After that, the bead was washed with 10 column volumes of binding buffer. The step of elution was carried out using 20 mM Tris-HCl pH 8.0, 0.3 M NaCl and 100 mM Imidazole. Whereas, the crude protein of r*Pm*SERPIN3 from pVR600-SERPIN3 was incubated with affinity bead pre-equilibrated with binding buffer (1× Phosphate buffer pH 7.4, containing 50 mM imidazole). After that, the bead was washed with 10 column volumes of binding buffer. The steps of elution were carried out using 1× Phosphate buffer pH 7.4, containing 250 mM Imidazole imidazole.

The purified r*Pm*SERPIN3 protein was traced by 10% SDS-PAGE and Western blot using anti-His antibody (Merck). The fractions containing the purified r*Pm*SERPIN3 were dialyzed against 1× Phosphate buffer pH 7.4. The protein content was determined by the Bradford assay.

2.10.4 Protein detection by Western Blot analysis

After r*Pm*SERPIN3 was successfully produced, the western blotting was performed using anti-His antibody (Merck) and mouse polyclonal antiserum specific to r*Pm*SERPIN3 protein. After 10% SDS-PAGE running for the expected r*Pm*SERPIN3, the gel, nitrocellulose membrane and filter papers were soaked in transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 15-20 min. The gel, nitrocellulose membrane and filter paper were placed on Trans-Blot® SD (Bio-Rad) as the blotting sandwich. Protein transferred was carried out at a constant 90 mM for 60 min. After finished, 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 shaking for overnight. After washing out the blocking solution by PBS/Tween20 for 3 times, the membrane was incubated with primary antibody (anti-His antibody or anti-r*Pm*SERPIN3 polyclonal antiserum) in PBS/Tween20 containing 1% (w/v) skim milk for alkaline phosphatase conjugated 3 h at 1:10,000 dilution and 37°C. Before incubation with secondary antibody (goat anti-mouse IgG antibody (Millipore)) at room temperature for an hour, the membrane was washed three times with PBS/Tween20. The secondary antibodywas diluted in 1% (w/v) skim milk in PBS/Tween20 at 1:10,000 dilution. The membrane was washed three times with PBS/Tween20 before the protein detection by color development using NBT and BCIP (Fermentas) at the final concentration of 375 and 188 µg/ml, respectively, in 100 mM Tris–HCl, pH 9.5.

2.10.5 The anti-r*Pm*SERPIN3 polyclonal antibody production and purification

The purified r*Pm*SERPIN3 from pVR600-SERPIN3 was used to immunize a mouse in order to generate anti-*Pm*SERPIN3 polyclonal antiserum at the Biomedical Technology Research Unit, Chiangmai University, Thailand. The impurity mouse polyclonal antiserum specific to *Pm*SERPIN3 protein was purified on protein A column by incubated with protein A bead (GE Healthcare) pre-equilibrated with binding buffer (100 mM Tris-HCl pH 8.0). After that, the bead was washed with 10 column volumes of binding buffer and 10 mM Tris-HCl pH 8.0. The step of elution was carried out using 100 mM glycine pH 3.0 and 50 µl of 1M Tris pH 8.0 was added to each fraction.

2.11 Detection of *Pm*SERPIN3 protein in hemocyte of shrimp

To detection of *Pm*SERPIN3 protein in hemocyte of shrimp using SDS-PAGE and western blotting techniques. First, the hemolymph from 3 normal shrimps was collected under equal volume of MAS solution (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose and 9 mM EDTA, pH 7.0) and separated the hemocytes by centrifugation at 800 × g for 10 min at 4 °C. The hemocyte pellet was washed three times with MAS solution, then resuspended and homogenized in MAS solution. The supernatant was collected after centrifugation at 14,000 × g for 10 min. The protein content of hemocyte lysate was measured by Bradford assay. BSA was used as a standard protein. The 50 µg of hemocyte lysate was separated on a 12.5% (w/v) acrylamide SDS-PAGE. The western blot analysis was performed as described in section 2.10.4.

2.12 Testing for antibody specificity

The 2 µg of recombinant *Pm*SERPIN3 and *Pm*SERPIN8 were run on duplicate 10% (w/v) acrylamide SDS-PAGE. The western blot analysis was performed as described in section 2.10.4 but the anti-*Pm*SERPIN3 and anti-*Pm*SERPIN8 antibodies were used both in two reactions as primary antibody at 1:10,000 dilution. Before incubation with secondary antibody at room temperature for an hour, the membrane was washed three times with PBS/Tween20. The secondary conjugated with alkaline phosphatase (AP) was diluted in 1% (w/v) skim milk in PBS/Tween20 for 1 h at 1:10,000 dilution. The membrane was washed three times with PBS/Tween20 before detection by adding NBT and BCIP (Fermentas) at the final concentration of 375 and 188 µg/ml.

2.13 Immunolocalization of *Pm*SERPIN3 protein in shrimp hemocytes

The hemocyte of 0.85% NaCl and *V. harveyi* 639 (5×10^6 CFU) injected shrimp at 0, 30 min and 3 hour after challenges was collected and fixed in 4% (w/v) paraformaldehyde for 10 min on ice. The fixed hemocytes were separated by centrifuge at 1,000 × g at 4 °C for 10 min. The hemocytes were resuspended in 1X PBS and coated onto the poly-L-lysine slide (Thermo Scientific) by centrifugation at 1,000 × g at 4°C for 10 min. The cells were blocked with 10% fetal bovine serum (FBS) in PBS at room temperature for 1 h and probed with the purified mouse polyclonal antibody specific to *Pm*SERPIN3 in 1% FBS in PBS at the dilution of (1:10) at 4°C for 12 h. The negative control were cells incubated with 1% FBS in PBS. The cells were extensively washed for 3 times with PBS/Tween20 to remove non-specific binding and the slides were then probed with the secondary antibody, goat anti-mouse antibodies conjugated with Alexa Fluor 568 (Invitrogen) in 1% FBS in PBS at the dilution of 1:1000 at room temperature for 1 h. The cells were extensively washed as above before mounting with ProLong® Gold antifade reagent with DAPI (Molecular Probes®). The fluorescent staining was observed under a confocal laser scanning microscope (Nikon).

2.14 Proteinase inhibitory activity assay

The inhibitory activity of r*Pm*SERPIN3 from pET-32-SERPIN3 against 4 commercial proteinases such as subtilisin Carlsberg (*Bacillus licheniformis*, Sigma), α-chymotrypsin (type II bovine pancreas, Sigma), trypsin (bovine pancreas, Sigma) and elastase (porcine pancreas, Pacific Science) was carried out as described by Hergenhahn et al. (Hergenhahn et al., 1987). The reaction mixture contained 50 mM Tris-HCl pH 8.0,

0.04 μM proteinase and a chromogenic substrate: 80 μM of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as a substrate for subtilisin and α-chymotrypsin, 110 μM of *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide hydrochloride as a substrate for trypsin, and 166 μM of *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide as a substrate for elastase. The r*Pm*SERPIN3 was added to make various proteinase to inhibitor molar ratios from 1:0 to 1:25. The reaction mixture was mixed, incubated at 30 ℃ for 15 min and measured the product at the absorbance of 410 nm. The remaining activity was calculated as a percentage of the absorbance reduction comparing to the negative control, a reaction without r*Pm*SERPIN3. The control reaction was performed using recombinant thioredoxin instead of r*Pm*SERPIN3 (for subtilisin containing reaction only). All reactions were done in triplicate.

2.15 Prophenoloxidase (ProPO) activation inhibitory activity assay

2.15.1 Hemocyte lysate (HLS) preparation

Inhibition of prophenoloxidase system was assayed using a method modified from Somnuk et al. (Somnuk et al., 2012). The shrimp hemocyte lysate supernatant (HLS) was prepared from the hemocytes of healthy shrimp. The hemocytes were separated from the hemolymph by centrifugation at 800 × g for 10 min at 4 ℃. Hemocyte pellet was washed 3 times with CAC buffer 500 μl (10 mM sodium cacodylate pH 7.0, 10 mM $CaCl_2$) and homogenized in 500 μl CAC buffer (Hung et al., 1997). The HLS was separated by centrifugation at 12,000 rpm for 10 min at 4 ℃. The protein concentration of HLS was determined using Bradford assay. Note that, HLS was always kept on ice during experiment.

2.15.2 Assay for inhibitory activity on proPO activation

To assay for proPO inhibitory activity, 30 µl of HLS (approximately 300 µg protein) was mixed with r*Pm*SERPIN3 to the final concentrations of 2 and 5 µM in the final volume of 85 µl adjusted with CAC buffer. The proPO reaction was activated by adding 40 µl of 1 mg/ml lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Sigma) and incubated at room temperature for 5 min. Twenty-five microliters of 3 mg/ml L-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma) was added and the PO activity was spectrophotometrically measured at 490 nm using a microplate reader SpectraMaxM5 (Molecular Devices*)* every 10 min for the duration of 1 h. For the positive inhibition control, 10 µl of 17 mg/ml phenylthiourea (PTU) (Sigma) was added instead of r*Pm*SERPIN3. For the control of normal proPO reaction, neither r*Pm*SERPIN3 nor PTU was added to the reaction mixture.

2.16 Effect of r*Pm*SERPIN3 on bacterial clearance

To examine the involvement of *Pm*SERPIN3 on shrimp bacterial clearance mechanism, shrimp (~3 g, fresh weight) were intramuscularly injected with 100 µl of sterile 0.85% NaCl containing 5 µM of r*Pm*SERPIN3, mixed with the highly pathogenic *V. harveyi* 639 (5×10^6 CFU). Shrimp injected with 100 µl 0.85% NaCl mixed with the *V. harveyi* 639 (5×10^6 CFU), or only 0.85% NaCl were used as control groups. The hemolymph samples were collected individually at 5 and 30 min after challenge and serially diluted in sterile 0.85% NaCl. The 10 µl diluted hemolymph samples were, then, plated onto the LB-agar containing 2% NaCl and TCBS-agar containing 2% NaCl and further grown at 30 °C overnight. The bacterial colonies were counted and calculated as

CFU/ml. All experiment tests were done in triplicate and statistical analysis was performed using t-test.

2.17 Assay for inhibitory activity of blood clotting

The hemolymph from 3 individual shrimp were collected under 10% (w/v) trisodium citrate as an anticoagulant and then pooled into the one tube. The clotting reactions of hemolymph containing anticoagulant (positive control), hemolymph containing anticoagulant, 20 mM Tris-HCl pH 8.0 and 40 mM $CaCl_2$, hemolymph containing anticoagulant, 20 mM Tris-HCl pH 8.0, 40 mM $CaCl_2$ and 22 µg of BSA as internal control, hemolymph containing anticoagulant, and 40 mM $CaCl_2$ (D) as internal control, and hemolymph containing anticoagulant, 20 mM Tris-HCl pH 8.0, 40 mM $CaCl_2$ and 22 µg of r*Pm*SERPIN3 (E). The 10 µl of 40 mM $CaCl_2$ was added for activate theclot formation process of shrimp. The result of blood cloting was observed in 5 min.

CHAPTER III

RESULTS

3.1 Sequence analysis of *PmSERPIN3* gene

Previously, 9 *PmSERPIN* genes have been identified in *P. monodon*, *PmSERPINB3* gene with differential display-PCR and the other eight *PmSERPIN1-8* genes with *P. monodon* EST approach (Somboonwiwat et al., 2006; Homvises et al., 2010). Amino acid sequence comparison of their partial sequences revealed that *PmSERPIN2* and *PmSERPIN3* were close related to *PmSERPINB3* but different from other *PmSERPIN*s (data not shown). The *PmSERPIN3* gene was, then, chosen for this study.

The *PmSERPIN3* gene was identified as a partial nucleotide sequence of about 720 bp in the *P. monodon* EST library (clone SG5480). To obtain the full-length *PmSERPIN3* cDNA, the 5'- and 3'- RACE techniques were performed. From the 5'- and 3'-RACE PCR, the PCR products were 480 bp (Figure 3.1) and 800 bp (Figure 3.2), respectivelty. After cloned and sequencing, we compared and combined them with a partial nucleotide sequences from *P. monodon* EST database. A total cDNA sequence obtained was 1,456 bp which contained a 5′-UTR of 145 bp, an open reading frame of 1,233 bp (Figure 3.3) encoding 410-amino acid protein and a 3′-UTR of 78 bp. The N-terminal portion before Gln24 of deduced amino acid sequence showed a typical profile of secretory signal peptide as determined by SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The calculated molecular mass and isoelectric point of mature peptide was 46.2 kDa and

5.73, respectively. Two *N*-linked glycosylation sites were predicted by NetNGlyc 1.0 server at Asn31 and Asn47 (http://www.cbs.dtu.dk/services/NetNGlyc/) (Figure 3.4).

The homology searching using blastp and blastx against the NCBI GenBank database revealed that the *Pm*SERPIN3 showed the highest 44% amino acid sequence homology to the SERPINs from tick, *Ixodes scapularis*, putative serpin 2 (XP_002434444) and serpin 7 (XP_002407493) (Table 3.2). To make multiple amino acid sequence comparison and phylogenetic analysis, the SERPIN sequences from various organisms were retrieved from the GenBank. Only the mature SERPIN sequences were used.

Amino acid sequence comparison among the SERPINs from shrimp, other crustaceans and insects was performed using the ClastalX program. The comparison revealed that the *Pm*SERPIN3, like other SERPINs, contained conserved amino acid sequences such as the hinge region and serpin signature (Figure 3.5). The putative P1-P1′ amino acids were predicted to be Arg-Met. A phylogenetic tree was made and the result revealed that the shrimp SERPINs can be grouped into 3 different clusters; *Pm*SERPIN6-8 and Fc-serpin (Liu et al., 2009; Homvises et al., 2010; Somnuk et al., 2012) were grouped together while the *Pm*SERPIN3 and *Pm*SERPINB3 (Somboonwiwat et al., 2006) were in different clusters (Figure 3.5).

Table 3.1 The *Pm*SERPIN data summary from *P. monodon* EST database.

SERPIN	Contig or singleton	Number of clones	Representative clone (ORF) and GenBank accession no.	ORF length (amino acids)	Predicted signal sequence (amino
*Pm*SERPIN1	CT2488	2	Incomplete ORF	-	-
*Pm*SERPIN2	CT1501	3	Incomplete ORF	-	-
***Pm*SERPIN3**	**SG5480**	**1**	**Complete ORF**	**410**	**23**
*Pm*SERPIN4	SG7094	1	Incomplete ORF	-	-
*Pm*SERPIN5	CT1116	5	Incomplete ORF	-	-
*Pm*SERPIN6	CT1604	5	Complete ORF	415	19
*Pm*SERPIN7	CT1087	5	Complete ORF	411	19
*Pm*SERPIN8	SG5654	1	Complete ORF	417	19
*Pm*SERPINB3	-	-	Complete ORF	410	17

Table 3.2 Top 5 hit lists of homology search result of *PmSERPIN3* gene against NCBI database using blastX program.

Accession	Description	Max ident
XP_002434444.1	serpin 2 precursor, putative [*Ixodes scapularis*]	43%
XP_002407493.1	serpin 7 precursor, putative [*Ixodes scapularis*]	43%
ZP_02001593.1	Proteinase inhibitor I4, serpin [*Beggiatoa sp. PS*]	43%
NP_001117987.1	leukocyte elastase inhibitor [*Oncorhynchus mykiss*]	41%
XP_003225623.1	PREDICTED: LOW QUALITY PROTEIN: serpin B10-like [*Anolis carolinensis*]	40%

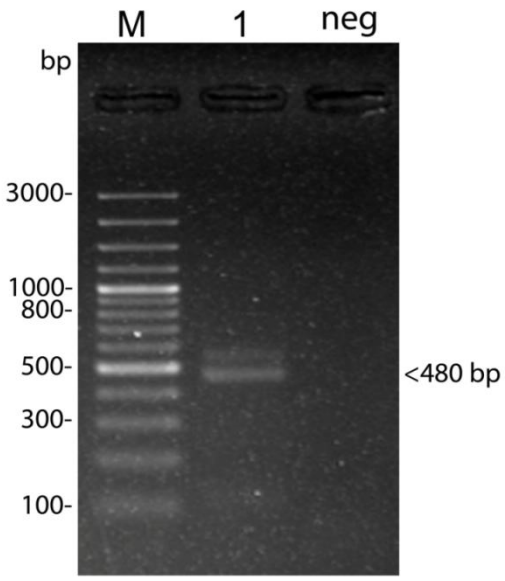


Figure 3.1 The primary 5′-RACE PCR product of *PmSERPIN3* cDNA. The 5'-RACE cDNA of shrimp hemocyte was used as template for 1st PCR using GSP_R (Table 2.1) and UPM (Universal Primer Mix) for RACE PCR amplification.

Lane 1: 5′-RACE PCR product of *PmSERPIN3* cDNA

Lane neg: Negative control.

Lane M: 100 bp DNA ladder markerss (GeneRuler™ 100 bp DNA ladder, Fermentas).

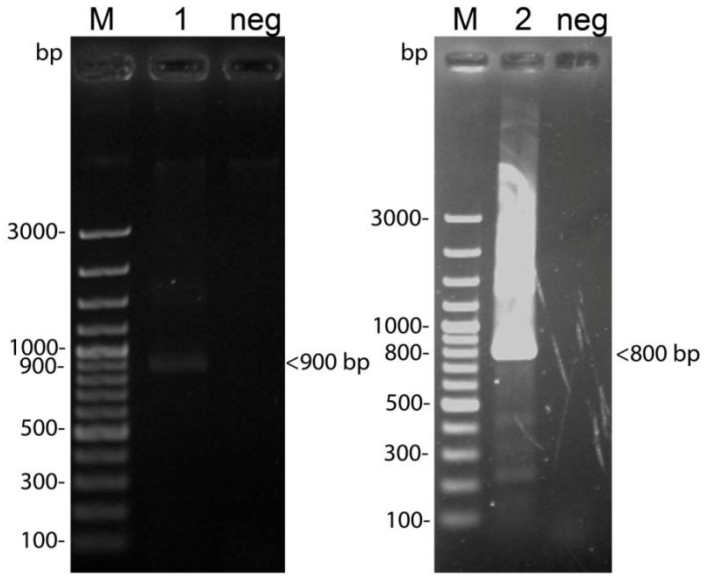


Figure 3.2 The 3′-RACE PCR product of *PmSERPIN3* cDNA. The 3′-RACE cDNA was used as template for 1st PCR using GSP_F primer (Table 2.1) and UPM. Next, 50-fold diluted 1st PCR product was used as template for nested PCR using NGSP_F primer (Table 2.1) and NUP.

Lane 1: The primary 3′-RACE PCR product of *PmSERPIN3* gene

Lane 2: The nested 3′-RACE PCR products of *PmSERPIN3* gene

Lane neg: Negative control

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

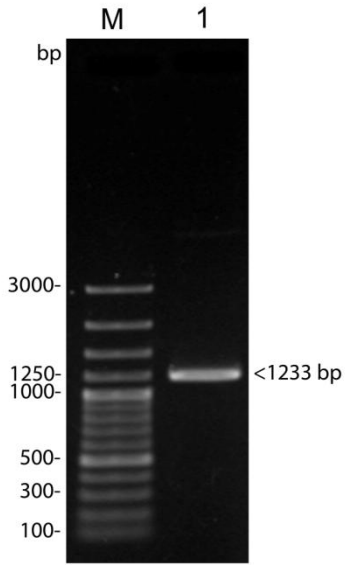


Figure 3.3 Amplification of the full-length *PmSERPIN3* cDNA. The cDNA of unchallenged *P. monodon* was used as a template for amplification of *Pm*SERPIN3 ORF using ORFSPN3_F and ORFSPN3_R primer (Table 2.1).

Lane 1: The purified PCR product of *PmSERPIN3* ORF gene fragment.

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

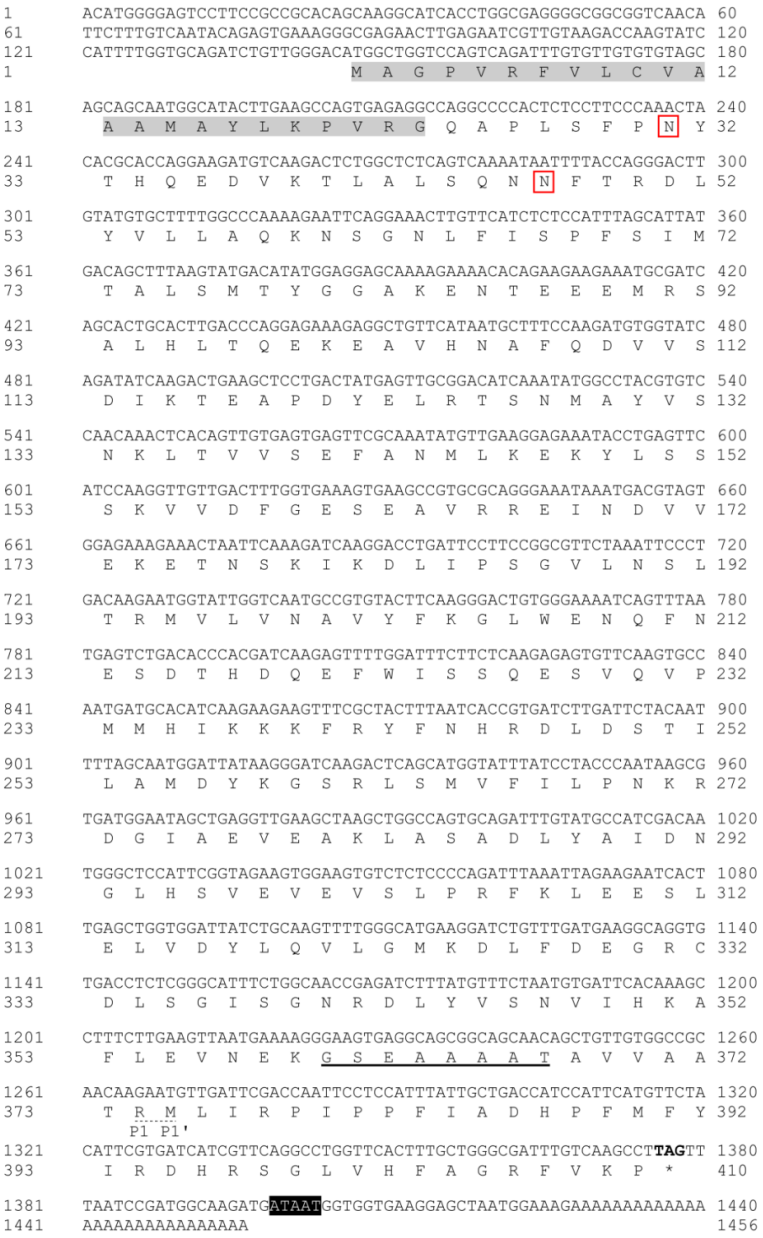


Figure 3.4 The nucleotide and the deduced amino acid sequences of *PmSERPIN3* gene. The signal peptide predicted by SignalP 4.0 server is highlighted in gray. The predicted *N*-linked glycosylation sites are shown in the boxes. The hinge region is underlined. The predicted P_1-P_1' residues are marked under the amino acid residues. Asterisk indicates stop codon. The putative polyadenylation signal is highlighted in black.

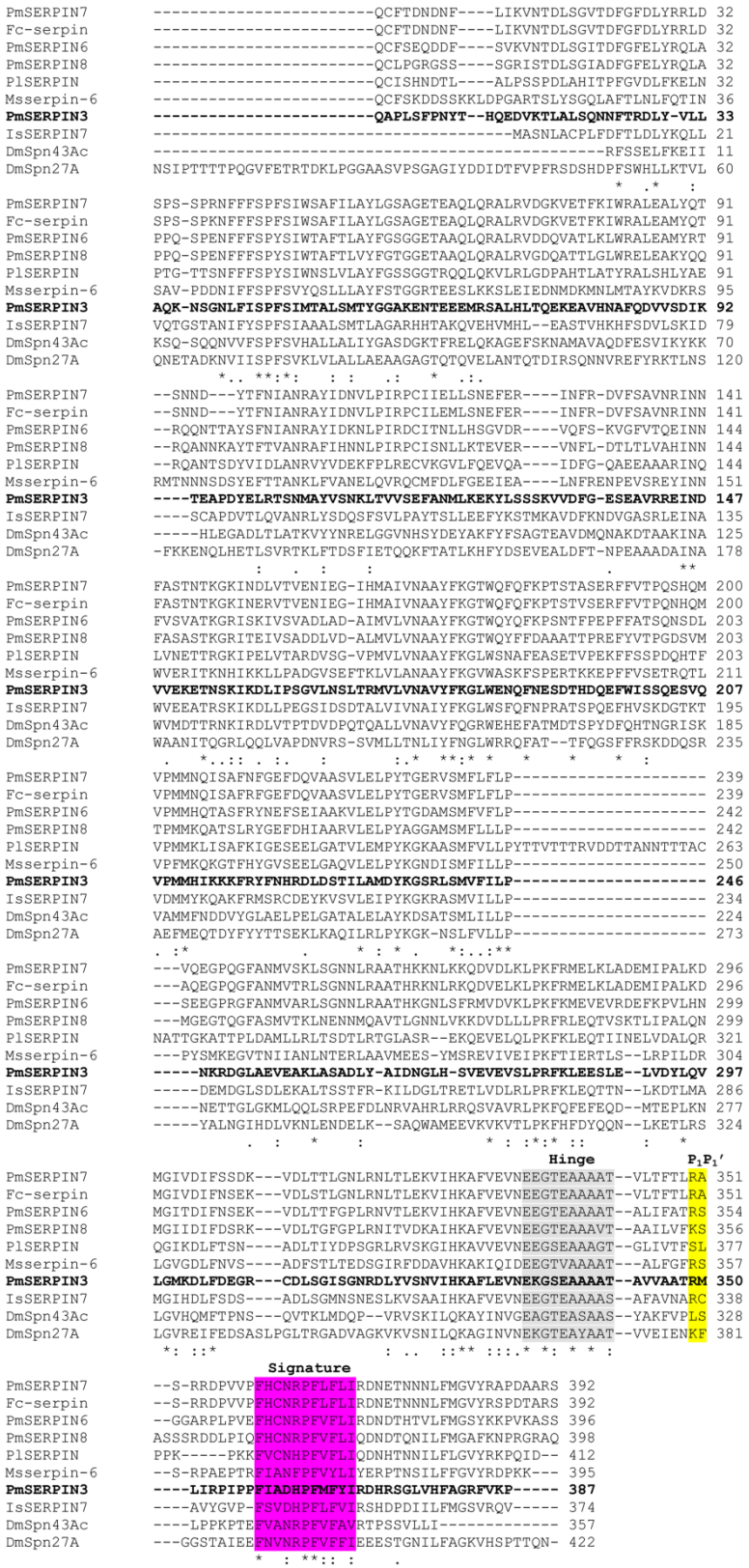

PmSERPIN7 -----------------------QCFTDNDNF----LIKVNTDLSGVTDFGFDLYRRLD 32
Fc-serpin -----------------------QCFTDNDNF----LIKVNTDLSGVTDFGFDLYRRLD 32
PmSERPIN6 -----------------------QCFSEQDDF----SVKVNTDLSGITDFGFELYRQLA 32
PmSERPIN8 -----------------------QCLPGRGSS----SGRISTDLSGIADFGFELYRQLA 32
PlSERPIN -----------------------QCISHNDTL----ALPSSPDLAHITPFGVDLFKELN 32
Msserpin-6 -----------------------QCFSKDDSSKKLDPGARTSLYSGQLAFTLNLFQTIN 36
PmSERPIN3 -----------------------QAPLSFPNYT--HQEDVKTLALSQNNFTRDLY-VLL 33
IsSERPIN7 ----------MASNLACPLFDFTLDLYKQLL 21
DmSpn43Ac -----------RFSSELFKEII 11
DmSpn27A NSIPTTTTPQGVFETRTDKLPGGAASVPSGAGIYDDIDTFVPFRSDSHDPFSWHLLKTVL 60
PmSERPIN7 SPS-SPRNFFFSPFSIWSAFILAYLGSAGETEAQLQRALRVDGKVETFKIWRALEALYQT 91
Fc-serpin SPS-SPKNFFFSPFSIWSAFILAYLGSAGETEAQLQRALRVDGKVETFKIWRALEAMYQT 91
PmSERPIN6 PPQ-SPENFFFSPYSIWTAFTLAYFGSGGETAAQLQRALRVDDQVATLKLWRALEAMYRT 91
PmSERPIN8 PPQ-SPENFFFSPYSIWTAFTLVYFGTGGETAAQLQRALRVGDQATTLGLWRELEAKYQQ 91
PlSERPIN PTG-TTSNFFFSPYSIWNSLVLAYFGSSGGTRQQLQKVLRLGDPAHTLATYRALSHLYAE 91
Msserpin-6 SAV-PDDNIFFSPFSVYQSLLLAYFSTGGRTEESLKKSLEIEDNMDKMNLMTAYKVDKRS 95
PmSERPIN3 AQK-NSGNLFISPFSIMTALSMTYGGAKENTEEEMRSALHLTQEKEAVHNAFQDVVSDIK 92
IsSERPIN7 VQTGSTANIFYSPFSIAAALSMTLAGARHHTAKQVEHVMHL--EASTVHKHFSDVLSKID 79
DmSpn43Ac KSQ-SQQNVVFSPFSVHALLALIYGASDGKTFRELQKAGEFSKNAMAVAQDFESVIKYKK 70
DmSpn27A QNETADKNVIISPFSVKLVLALLAEAAGAGTQTQVELANTQTDIRSQNNVREFYRKTLNS 120
PmSERPIN7 --SNND---YTFNIANRAYIDNVLPIRPCIIELLSNEFER----INFR-DVFSAVNRINN 141
Fc-serpin --SNND---YTFNIANRAYIDNVLPIRPCILEMLSNEFER----INFR-DVFSAVNRINN 141
PmSERPIN6 --RQQNTTAYSFNIANRAYIDKNLPIRDCITNLLHSGVDR----VQFS-KVGFVTQEINN 144
PmSERPIN8 --RQANNKAYTFTVANRAFIHNNLPIRPCISNLLKTEVER----VNFL-DTLTLVAHINN 144
PlSERPIN --RQANTSDYVIDLANRVYVDEKFPLRECVKGVLFQEVQA----IDFG-QAEEAAARINQ 144
Msserpin-6 RMTNNNSDSYEFTTANKLFVANELQVRQCMFDLFGEEIEA----LNFRENPEVSREYINN 151
PmSERPIN3 ----TEAPDYELRTSNMAYVSNKLTVVSEFANMLKEKYLSSSKVVDFG-ESEAVRREIND 147
IsSERPIN7 ----SCAPDVTLQVANRLYSDQSFSVLPAYTSLLEEFYKSTMKAVDFKNDVGASRLEINA 135
DmSpn43Ac -----HLEGADLTLATKVYYNRELGGVNHSYDEYAKFYFSAGTEAVDMQNAKDTAAKINA 125
DmSpn27A -FKKENQLHETLSVRTKLFTDSFIETQQKFTATLKHFYDSEVEALDFT-NPEAAADAINA 178
PmSERPIN7 FASTNTKGKINDLVTVENIEG-IHMAIVNAAYFKGTWQFQFKPTSTASERFFVTPQSHQM 200
Fc-serpin FASTNTKGKINERVTVENIEG-IHMAIVNAAYFKGTWQFQFKPTSTVSERFFVTPQNHQM 200
PmSERPIN6 FVSVATKGRISKIVSVADLAD-AIMVLVNAAYFKGTWQYQFKPSNTFPEPFFATSQNSDL 203
PmSERPIN8 FASASTKGRITEIVSADDLVD-ALMVLVNAAYFKGTWQYFFDAAATTPREFYVTPGDSVM 203
PlSERPIN LVNETTRGKIPELVTARDVSG-VPMVLVNAAYFKGLWSNAFEASETVPEKFFSSPDQHTF 203
Msserpin-6 WVERITKNHIKKLLPADGVSEFTKLVLANAAYFKGVWASKFSPERTKKEPFFVSETRQTL 211
PmSERPIN3 VVEKETNSKIKDLIPSGVLNSLTRMVLVNAVYFKGLWENQFNESDTHDQEFWISSQESVQ 207
IsSERPIN7 WVEEATRSKIKDLLPEGSIDSDTALVIVNAIYFKGLWSFQFNPRATSPQEFHVSKDGTKT 195
DmSpn43Ac WVMDTTRNKIRDLVTPTDVDPQTQALLVNAVYFQGRWEHEFATMDTSPYDFQHTNGRISK 185
DmSpn27A WAANITQGRLQQLVAPDNVRS-SVMLLTNLIYFNGLWRRQFAT--TFQGSFFRSKDDQSR 235
PmSERPIN7 VPMMNQISAFNFGEFDQVAASVLELPYTGERVSMFLFLP---------- 239
Fc-serpin VPMMNQISAFRFGEFDQVAASVLELPYTGERVSMFLFLP---------- 239
PmSERPIN6 VPMMHQTASFRYNEFSEIAAKVLELPYTGDAMSMFVFLP---------- 242
PmSERPIN8 TPMMKQATSLRYGEFDHIAARVLELPYAGGAMSMFLLLP---------- 242
PlSERPIN VPMMKLISAFKIGESEELGATVLEMPYKGKAASMFVLLPYTTVTTTRVDDTTANNTTTAC 263
Msserpin-6 VPFMKQKGTFHYGVSEELGAQVLELPYKGNDISMFILLP---------- 250
PmSERPIN3 VPMMHIKKKFRYFNHRDLDSTILAMDYKGSRLSMVFILP---------- 246
IsSERPIN7 VDMMYKQAKFRMSRCDEYKVSVLEIPYKGKRASMVILLP---------- 234
DmSpn43Ac VAMMFNDDVYGLAELPELGATALELAYKDSATSMLILLP---------- 224
DmSpn27A AEFMEQTDYFYYTTSEKLKAQILRLPYKGK-NSLFVLLP---------- 273
PmSERPIN7 ---VQEGPQGFANMVSKLSGNNLRAATHKKNLKKQDVDLKLPKFRMELKLADEMIPALKD 296
Fc-serpin ---AQEGPQGFANMVTRLSGNNLRAATHRKNLRKQDVELKLPKFRMELKLADEMIPALKD 296
PmSERPIN6 ---SEEGPRGFANMVARLSGNNLRAATHKGNLSFRMVDVKLPKFKMEVEVRDEFKPVLHN 299
PmSERPIN8 ---MGEGTQGFASMVTKLNENNMQAVTLGNNLVKKDVDLLLPRFRLEQTVSKTLIPALQN 299
PlSERPIN NATTGKATTPLDAMLLRLTSDTLRTGLASR--EKQEVELQLPKFKLEQTIINELVDALQR 321
Msserpin-6 ---PYSMKEGVTNIIANLNTERLAAVMEES-YMSREVIVEIPKFTIERTLS--LRPILDR 304
PmSERPIN3 -----NKRDGLAEVEAKLASADLY-AIDNGLH-SVEVEVSLPRFKLEESLE--LVDYLQV 297
IsSERPIN7 -----DEMDGLSDLEKALTSSTFR-KILDGLTRETLVDLRLPRFKLEQTTN--LKDTLMA 286
DmSpn43Ac -----NETTGLGKMLQQLSRPEFDLNRVAHRLRRQSVAVRLPKFQFEFEQD--MTEPLKN 277
DmSpn27A -----YALNGIHDLVKNLENDELK--SAQWAMEEVKVKVTLPKFHFDYQQN--LKETLRS 324
Hinge
P1P1′
PmSERPIN7 MGIVDIFSSDK---VDLTTLGNLRNLTLEKVIHKAFVEVNEEGTEAAAAT--VLTFTLRA 351
Fc-serpin MGIVDIFNSEK---VDLSTLGNLRNLTLEKVIHKAFVEVNEEGTEAAAAT--VLTFTLRA 351
PmSERPIN6 MGITDIFNSEK---VDLTTFGPLRNVTLEKVIHKAFVEVNEEGTEAAAAT--ALIFATRS 354
PmSERPIN8 MGIIDIFDSRK---VDLTGFGPLRNITVDKAIHKAFVEVNEEGTEAAAVT--AAILVFKS 356
PlSERPIN QGIKDLFTSN----ADLTIYDPSGRLRVSKGIHKAVVEVNEEGSEAAAGT--GLIVTFSL 377
Msserpin-6 LGVGDLFNVS----ADFSTLTEDSGIRFDDAVHKAKIQIDEEGTVAAAAT---ALFGFRS 357
PmSERPIN3 LGMKDLFDEGR---CDLSGISGNRDLYVSNVIHKAFLEVNEKGSEAAAAT--AVVAATRM 350
IsSERPIN7 MGIHDLFSDS----ADLSGMNSNESLKVSAAIHKAFVEVNEEGTEAAAAS--AFAVNARC 338
DmSpn43Ac LGVHQMFTPNS---QVTKLMDQP--VRVSKILQKAYINVGEAGTEASAAS--YAKFVPLS 328
DmSpn27A LGVREIFEDSASLPGLTRGADVAGKVKVSNILQKAGINVNEKGTEAYAAT--VVEIENKF 381
Signature
PmSERPIN7 --S-RRDPVVPFHCNRPFLFLIRDNETNNNLFMGVYRAPDAARS 392
Fc-serpin --S-RRDPVVPFHCNRPFLFLIRDNETNNNLFMGVYRSPDTARS 392
PmSERPIN6 --GGARPLPVEFHCNRPFVFLIRDNDTHTVLFMGSYKKPVKASS 396
PmSERPIN8 ASSSRDDLPIQFHCNRPFVFLIQDNDTQNILFMGAFKNPRGRAQ 398
PlSERPIN PPK-----PKKFVCNHPFVFLIQDNHTNNILFLGVYRKPQID-- 412
Msserpin-6 --S-RPAEPTRFIANFPFVYLIYERPTNSILFFGVYRDPKK--- 395
PmSERPIN3 ----LIRPIPPFIADHPFMFYIRDHRSGLVHFAGRFVKP----- 387
IsSERPIN7 ----AVYGVP-FSVDHPFLFVIRSHDPDIILFMGSVRQV----- 374
DmSpn43Ac ----LPPKPTEFVANRPFVFAVRTPSSVLLI------------ 357
DmSpn27A ---GGSTAIEEFNVNRPFVFFIEEESTGNILFAGKVHSPTTQN- 422

Figure 3.5 Amino acid sequence comparison among the mature proteins of SERPINs. The SERPIN amino acid sequences downloaded from the GenBank were compared using ClustalX. The hinge region is underlined. The predicted P_1-P_1' residues are marked under the amino acid residues. The accession number of each SERPIN was as follow: ABC33916 for Fc-serpin from *Fenneropenaeus chinensis*; KC577446, GQ260129, GU358487 and ADC42879 for *Pm*SERPIN3, 6, 7 and 8 from *Penaeus monodon*; AAF24518 and AAQ64953 for *Dm*Spn27A and *Dm*Spn43Ac from *Drosophila melanogaster*; AAV91026 for *Ms*serpin-6 from *Manduca sexta*; CAA57964 for *Pl*SERPIN from *Pacifastacus leniuscukus*; XP002407493 for *Is*SERPIN7 from *Ixodes scapularis.*

3.2 Genome organization of *PmSERPIN3* gene

Having obtained the cDNA full-length sequence of *PmSERPIN3* (GenBank accession KC577446), two pairs of specific primers were designed in order to amplify the homologous region from genomic DNA. The *PmSERPIN3* gene was amplified from the genomic DNA using 2 pairs of primers designed from the cDNA sequence of *PmSERPIN3*, the ORFSPN3_F with SPN3_RTR and SPN3_RTF with ORFSPN3_R (Table 2.1). The PCR products obtained were 600 and 750 bp, respectively (Figure 3.7). It was purified, cloned into T&A cloning vector and DNA sequenced. The genomic sequence of *PmSERPIN3* gene was, then, compared to the cDNA sequence. The result showed that the genomic *PmSERPIN3* gene had no intron (Figure 3.7).

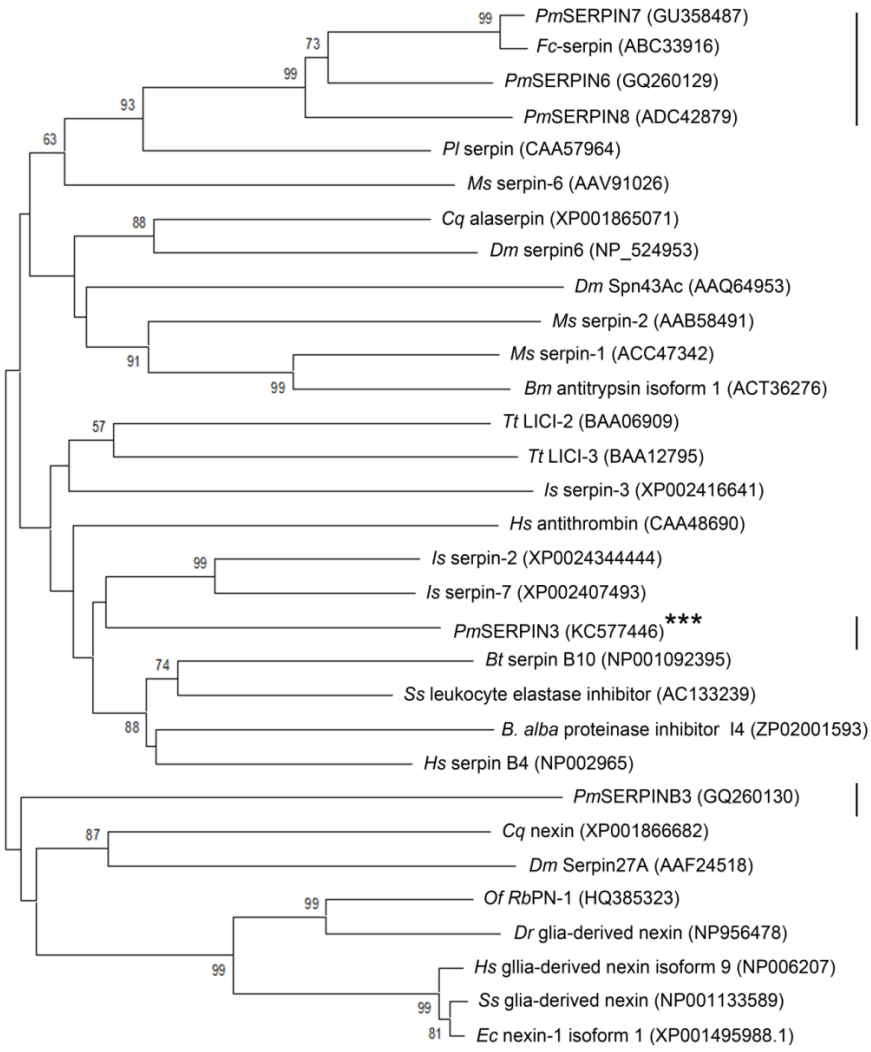


Figure 3.6 Phylogenetic analysis of *Pm*SERPINs and the SERPINs the from various organisms e.g. *Manduca sexta* (*Ms*), *Bombyx mori* (*Bm*), *Drosophila melanogaster* (*Dm*), *Culex quinquefasciatus* (*Cq*), *Pacifastacus leniusculus* (*Pl*), *Penaeus monodon* (*Pm*), *Fenneropenaeus chinensis* (*Fc*), *Oncopeltus fasciatus* (*Of*), *Danio rerio* (*Dr*), *Homo sapian* (*Hs*), *Salmo salar* (*Ss*), *Equus caballus* (*Ec*), *Ixodes scapularis* (*Is*), *Bos taurus* (*Bt*), *Beggiatoa alba* (*Ba*), and *Tachypleus tridentatus* (*Tt*). The numerals are neighbor-joining distances. The GenBank accessions are in the parentheses. Asterisks indicate the *Pm*SERPIN3.

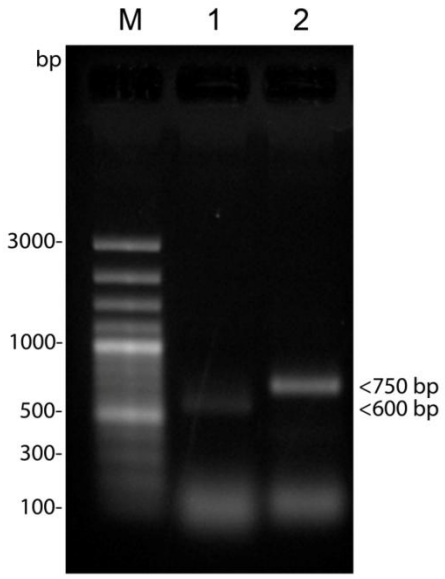


Figure 3.7 Amplification of homologous region from *PmSERPIN3* genomic DNA using two primer pairs such as ORFSPN3_F with SPN3_RTR and SPN3_RTF with ORFSPN3_R (Table 2.1).

Lane 1: The PCR products of genome fragment of *PmSERPIN3* from the first pair of primers (ORFSPN3_F with SPN3_RTR)

Lane 2: The PCR products of genome fragment of *PmSERPIN3* from the second pair of primers (SPN3_RTF with ORFSPN3_R)

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

3.3 Tissue distribution, expression at various developmental stages and in response to pathogenic infection

The semi-quantitative RT-PCR was employed in this experiment to determine the *PmSERPIN3* gene expression in different tissues of healthy shrimp. The *β-actin* gene was used as an internal control. The RT-PCR analysis revealed that the *PmSERPIN3* transcripts were expressed in all shrimp tissues tested such as gill, hepatopancreas, hemocyte, heart, nerve, epipodite, antennal gland, lymphoid organ, stomach, eyestalk, intestine, and muscle (Figure 3.8 A).

The expression of *PmSERPIN3* at various developmental stages such as nauplius IV, zoea III and mysis IV larvae of black tiger shrimp was determined as compared to the *EF-1α* gene expression, the results showed that *Pm*SERPIN3 transcripts were expressed at all developmental stages tested (Figure. 3.8 B) including subadult as shown in Figure 3.8 A.

To determine whether the expression of *PmSERPIN3* gene was influenced by pathogenic infection, the hemocytes were chosen for the analysis because they were directly involved in the defense mechanism. The hemocytes collected at different time points from *V. harveyi*-, WSSV-, and YHV-challenged shrimp were used for RT-PCR analysis. In these experiments, the *EF-1α* gene was used as an internal control. Surprisingly, the results showed that there was no significant change of the transcription level upon *V. harveyi*-, WSSV- and YHV-challenges (Figure 3.8 C-E).

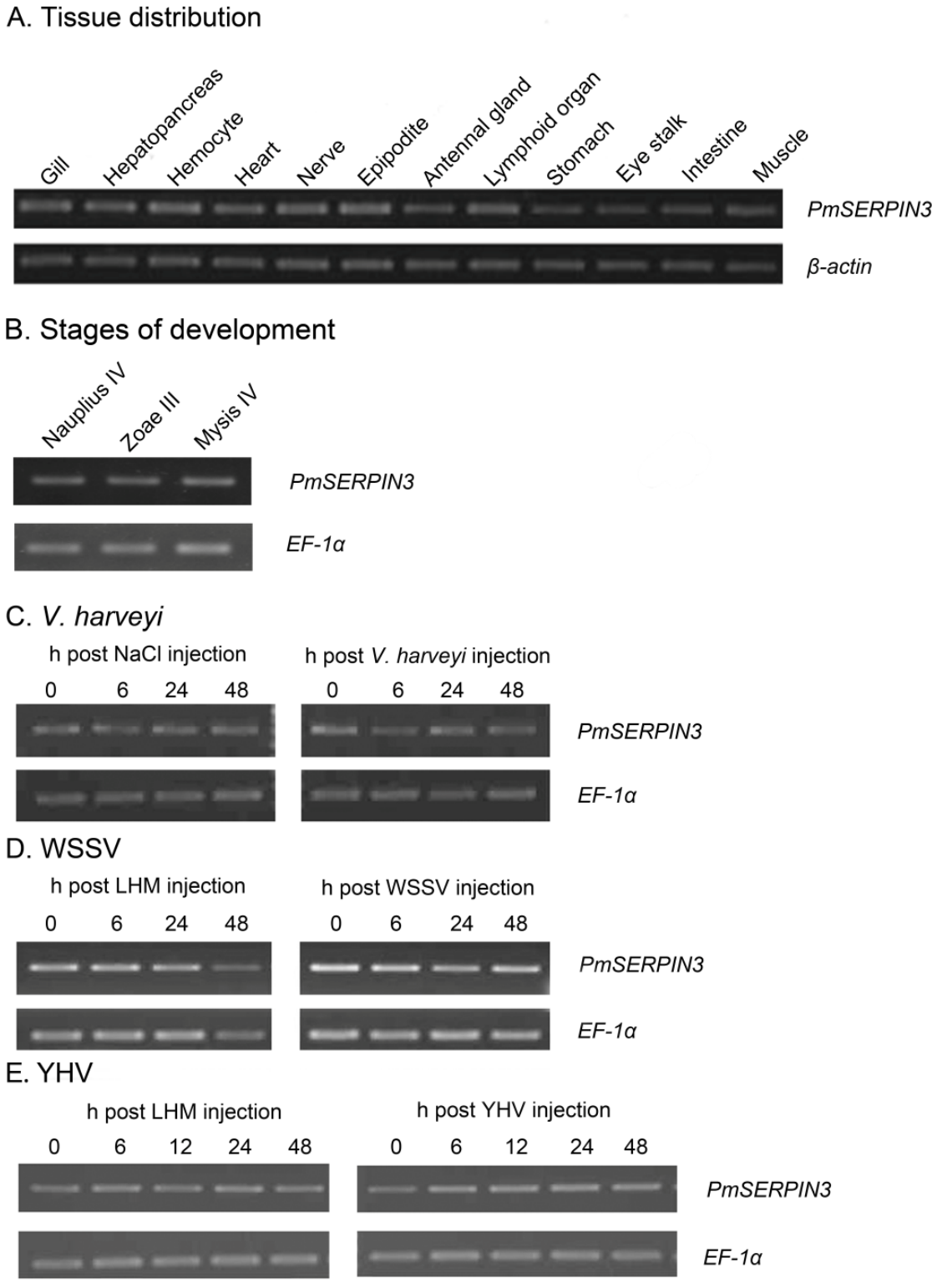

A. Tissue distribution
Gill
Hepatopancreas
Hemocyte
Heart
Nerve
Epipodite
Antennal gland
Lymphoid organ
Stomach
Eye stalk
Intestine
Muscle
PmSERPIN3
β-actin
B. Stages of development
Nauplius IV
Zoae III
Mysis IV
PmSERPIN3
EF-1α
C. V. harveyi
h post NaCl injection
0 6 24 48
h post V. harveyi injection
0 6 24 48
PmSERPIN3
EF-1α
D. WSSV
h post LHM injection
0 6 24 48
h post WSSV injection
0 6 24 48
PmSERPIN3
EF-1α
E. YHV
h post LHM injection
0 6 12 24 48
h post YHV injection
0 6 12 24 48
PmSERPIN3
EF-1α

Figure 3.8 RT-PCR analysis of *PmSERPIN3* expression in shrimp tissues, developmental stages and in response to pathogenic infection. (A) Tissue distribution of *PmSERPIN3* transcripts in twelve shrimp tissues. The *PmSERPIN3* gene was amplified from shrimp tissues such as gill, hepatopancreas, hemocyte, heart, nerve, epipodite, antennal gland, lymphoid organ, stomach, eyestalk, intestine, and muscle. The *β-actin* was used as an internal control. (B-E) RT-PCR analysis of *PmSERPIN3* gene expression in *P. monodon* hemocytes at different stages of development (B) and in response to *V. harveyi* (C), WSSV (D) and YHV (E) infection at different time points. The *EF-1α* was used as an internal control.

3.4 Expression and purification of recombinant *Pm*SERPIN3 protein

The purified recombinant proteins were used for functional characterization. To study the proteinase inhibitory activity and the involvement in proPO system of *Pm*SERPIN3, the recombinant *Pm*SERPIN3 protein (r*Pm*SERPIN3) was over-produced in an *E. coli* expression system using two different constructs of recombinant expression plasmids. The first construct was based on the pET-32b(+) and thus named pET-32_SERPIN3. The 62 kDa over-produced r*Pm*SERPIN3 protein, herein after called r*Pm*SERPIN3-1, had thioredoxin domain at its N-terminus and 6X His-tag at both termini. The second expression plasmid was constructed on an expression plasmid pVR600, a pET-28b(+) derivative whose N-terminal sequence from *Nco*I to *Bam*HI was deleted. The resulting expression plasmid was named pVR600_SERPIN3 which produced r*Pm*SERPIN3-2, a 44 kDa mature protein with a 6X His-tag at its C-terminus.

The *Pm*SERPIN3 nucleotide sequences corresponding to the mature peptide was amplified (Figure 3.9) by rSPN3_F and rSPN3_R primers (Table 2.1). The purified PCR product was futher ligated and cloned into T&A vector. The recombinant plasmid was checked by *Hind*III and *Bgl*II double digestion (Figure 3.10) and the plasmid lane 1 in Figure 3.10 was confirmed for the correctness of sequence by sequencing. The recombinant plasmids were digested with *Nco*I to *Xho*I for cloning into pET-32b(+) and pVR600 (pET-28b(+) derivative) cut with the same restriction enzymes. These two recombinant plasmids obtained were checked by digestion with *Nco*I to *Xho*I (Figure 3.11 and 3.12)

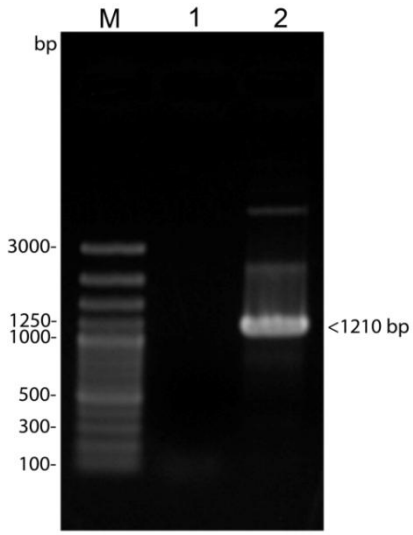


Figure 3.9 Amplification of the mature *Pm*SERPIN3 gene to be expressed in *E. coli* system. The rSPN3_F and rSPN3_R primers (Table 2.1) were used to amplify the mature *PmSERPIN3* gene.

Lane 1: Negative control

Lane 2: PCR product of mature *PmSERPIN3* gene (1,210 bp).

Lane M represents 100 bp DNA ladder markers (GeneRuler™ 100 bp DNA ladder, Fermentas)

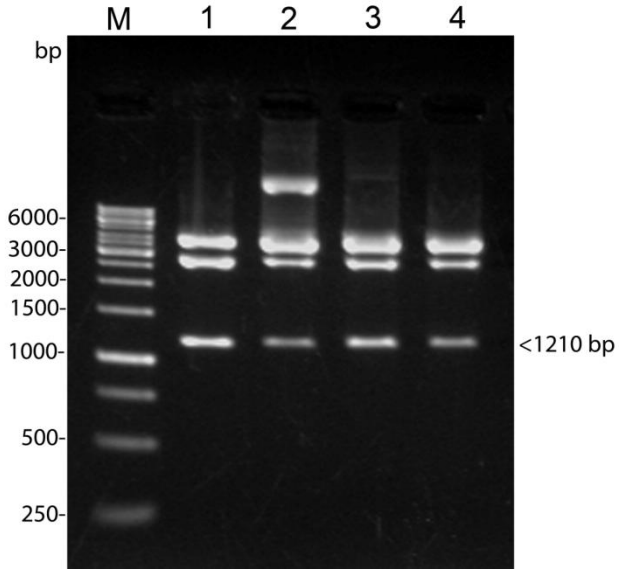


Figure 3.10 Screening of recombinant T&A-*Pm*SERPIN3 plasmids. The recombinant plasmids were extracted and digested with *Hind*III and *Bgl*II. The expected size of *Pm*SERPIN3 fragment was 1,210 bp.

Lane 1-4: All of digested product from 4 recombinant plasmids T&A-*Pm*SERPIN3.

Lane M: 1 kb DNA ladder markers (GeneRuler™ 1 kb DNA ladder, Fermentas)

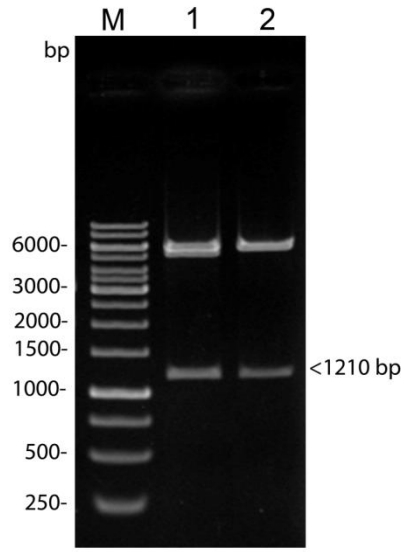


Figure 3.11 Screening of recombinant pET32b-*Pm*SERPIN3 plasmids. The recombinant plasmids were extracted and digested with *Nco*I and *BamH*I. The expected size of *Pm*SERPIN3 fragment was 1,210 bp.

Lane 1-2: Digestion products from 2 recombinant plasmids pET32b-*Pm*SERPIN3

Lane M: 1 kb DNA ladder markers (GeneRuler™ 1 kb DNA ladder, Fermentas)

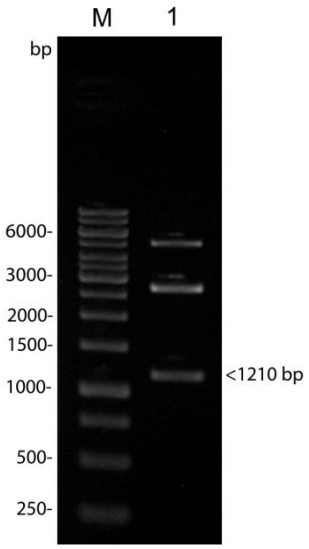


Figure 3.12 Screening of recombinant pVR600-*Pm*SERPIN3 plasmids. The recombinant plasmid was extracted and digested with *Nco*I and *BamH*I. The expected size of *Pm*SERPIN3 fragment was 1,210 bp.

Lane 1: The digested product from a recombinant plasmids pVR600-*Pm*SERPIN3

Lane M: 1 kb DNA ladder markers (GeneRuler™ 1 kb DNA ladder, Fermentas)

The r*Pm*SERPIN3-1 was over-produced upon induction with 1 mM IPTG (Figure 3.13). The cells were harvested and determined whether it was produced in the inclusion bodies or in the soluble forms. The results showed that r*Pm*SERPIN3-1 was expressed in the inclusion bodies from (Figure 3.13) The inclusion bodies was solubilized by immediately adding 100 mM NaOH and the r*Pm*SERPIN3 in the soluble fraction was immediately dialyzed against 20 mM Tris-HCl pH 8.0 for neutralization. The expected product was purified via Ni-NTA column. The purified r*Pm*SERPIN3-1 fraction eluted with 20 mM Tris-HCl pH 8.0, 0.3 M NaCl containing 100 mM Imidazole (Figure 3.14), after that analyzed for the expressed protein by 10% SDS-PAGE (coomassie staining) and western bloting (Figure 3.15). The purified r*Pm*SERPIN3-1 was used for the proteinase inhibitory activity assay.

Like r*Pm*SERPIN3-1, the r*Pm*SERPIN3-2 was successfully produced in *E. coli* BL21(DE3). The whole cells were collected at 0, 2, and 4 h after induction by adding 1 mM IPTG (Figure 3.16) and were then lyzed by sonication. Next, the soluble protein and inclusion bodies were separated. SDS-PAGE and western blot analysis revealed that the r*Pm*SERPIN3-2 was produced as soluble form (Figure 3.16). The crude soluble r*Pm*SERPIN3-2 was purified via Ni-NTA column according to material and method section 2.10.3. The purified r*Pm*SERPIN3-2 fraction eluted with 1× Phosphate buffer pH 7.4, containing 100, 150, and 250 mM Imidazole, respectively (Figure 3.17). Then the purified r*Pm*SERPIN3-2 protein was analyzed by 10% SDS-PAGE (coomassie staining) and western bloting (Figure 3.18). It was used for the prophenoloxidase inhibitory activity assay and the test on bacterial clearance.

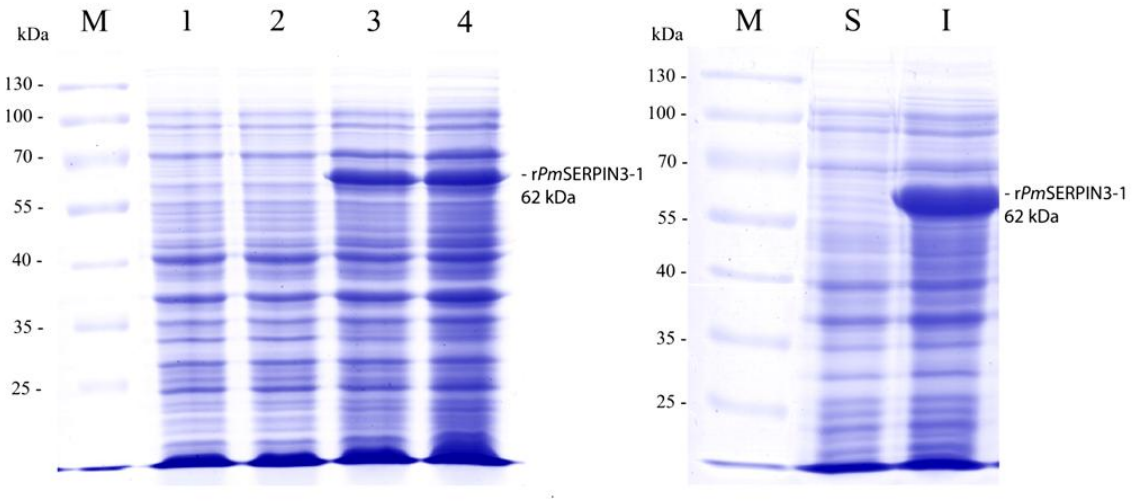


Figure 3.13 Expression of r*Pm*SERPIN3-1 in *E. coli* strain BL21(DE3). The recombinant clone was cultured and induced for r*Pm*SERPIN3-1 expression by 1 mM IPTG at 0, 2, and 4 h, respectively. The cells were collected and checked for protein expression by coomassie stained 10% SDS-PAGE. The expected size of r*Pm*SERPIN3-1 was 62 kDa containing fusion protein (Thioredoxin), which was 18 kDa in size. The soluble and inclusion bodies fractions obtained after sonication were analyzed by Coomassie stained 10% SDS-PAGE.

Lane 1: The whole cell at 0 h without IPTG induction

Lane 2: The whole cell at 0 h with IPTG induction

Lane 3: The whole cell at 2 h with IPTG induction

Lane 4: The whole cell at 4 h with IPTG induction

Lane S: The soluble fraction

Lane I: The inclusion fraction

Lane M: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

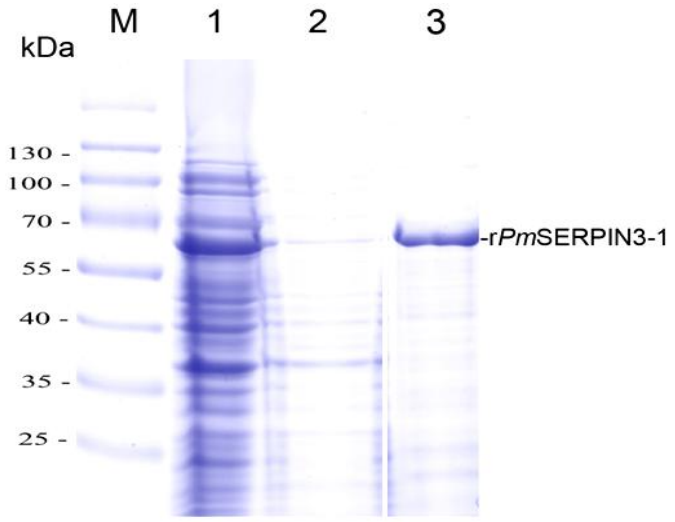


Figure 3.14 r*Pm*SERPIN3-1 protein purification using Ni-NTA column.

Lane 1: Flowthrough fraction

Lane 2: Wash fraction

Lane 3: Purified r*Pm*SERPIN3-1 fraction eluted with 20 mM Tris-HCl pH 8.0, 0.3 M NaCl containing 100 mM Imidazole.

Lane M: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

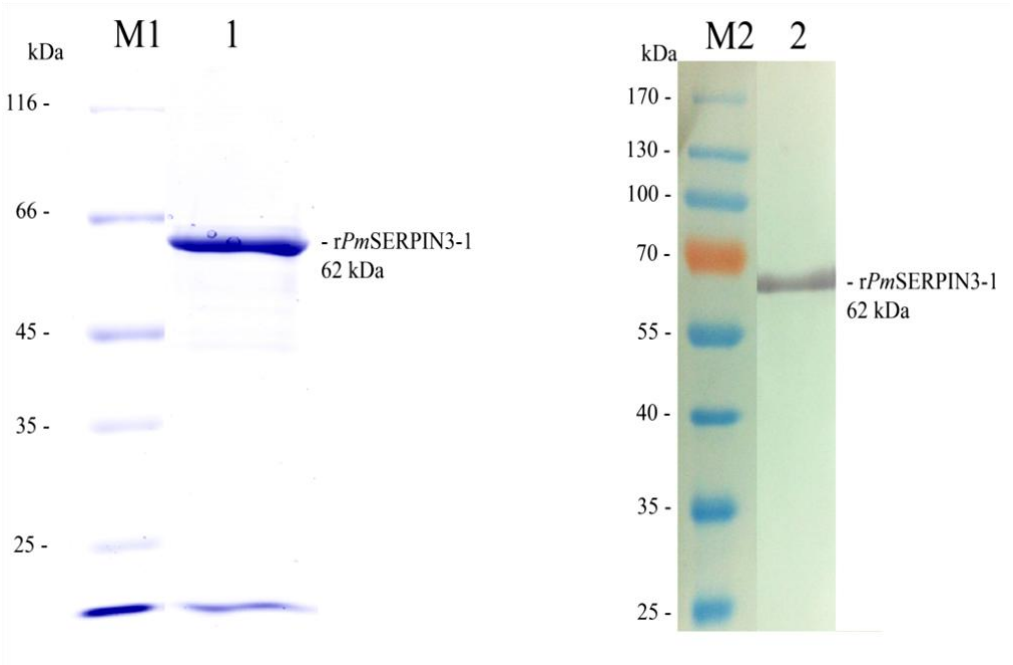


Figure 3.15 Analysis of the purified r*Pm*SERPIN3-1 protein. The crude recombinant protein r*Pm*SERPIN3-1 was purified through Ni-NTA column. The protein was run on 10% SDS-PAGE and detected by coomassie staining. Western blot analysis using anti-His_6 antibody as primary antibody

Lane 1: Coomassie staining of the purified r*Pm*SERPIN3-1 protein

Lane 2: Western blot analysis of the purified r*Pm*SERPIN3-1 protein

Lane M1: Unstained protein markers (PageRuler™ Unstained protein ladder, Fermentas)

Lane M2: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

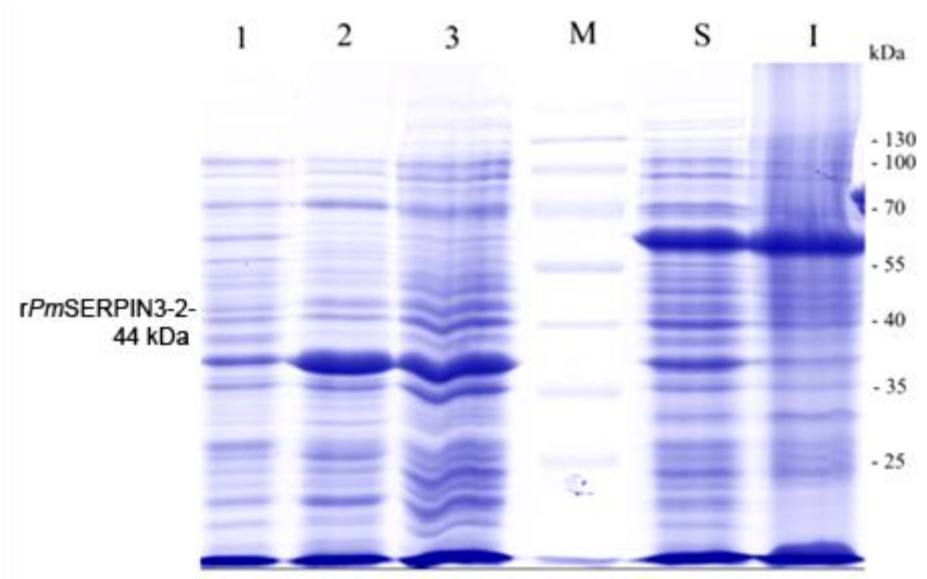


Figure 3.16 Expression of *Pm*SERPIN3-2 in *E. coli* strain BL21(DE3). The recombinant clone was cultured and induced for r*Pm*SERPIN3-2 expression by 1 mM IPTG at 0, 2, and 4 h, respectively. The cells were collected and checked for protein expression by Coomassie stained 10% SDS-PAGE. The expected size of r*Pm*SERPIN3-2 was 44 kDa. The soluble and inclusion bodies fractions obtained after sonication were analyzed by Coomassie stained 10% SDS-PAGE

Lane 1: The whole cell at 0 h with IPTG induction

Lane 2: The whole cell at 2 h with IPTG induction

Lane 3: The whole cell at 4 h with IPTG induction

Lane S: The soluble fraction

Lane I: The inclusion bodies fraction

Lane M: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

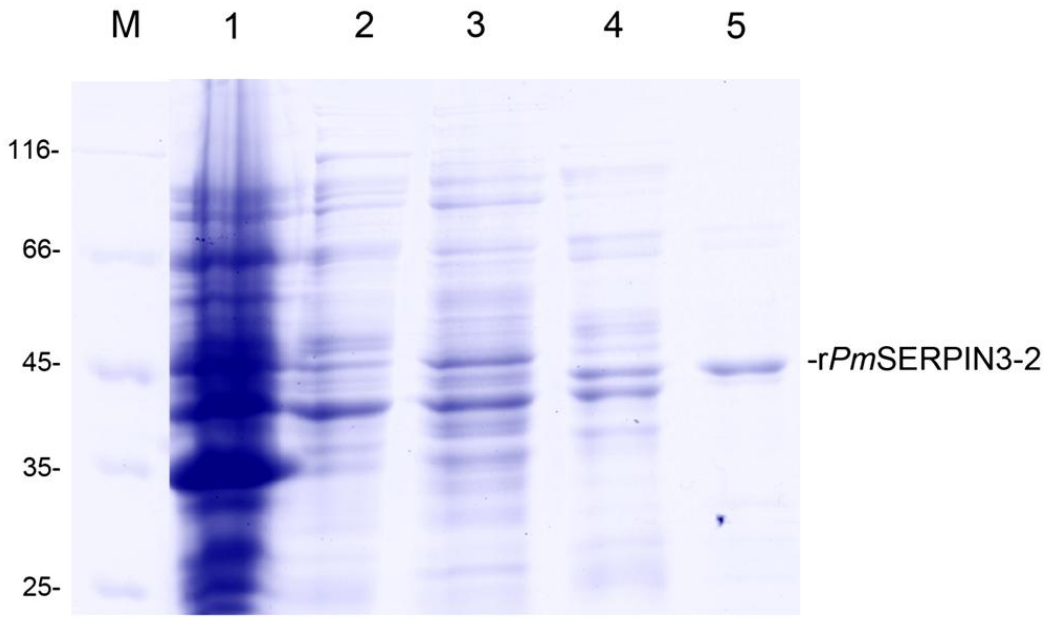


Figure 3.17 r*Pm*SERPIN3-2 protein purification using Ni-NTA column.

Lane 1: Flow through fraction

Lane 2: Wash fraction

Lane 3: Purified r*Pm*SERPIN3-2 fraction eluted with 1× Phosphate buffer pH 7.4, containing 100 mM Imidazole

Lane 4: Purified r*Pm*SERPIN3-2 fraction eluted with 1× Phosphate buffer pH 7.4, containing 150 mM Imidazole

Lane 5: Purified r*Pm*SERPIN3-2 fraction eluted with 1× Phosphate buffer pH 7.4, containing 250 mM Imidazole

Lane M: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

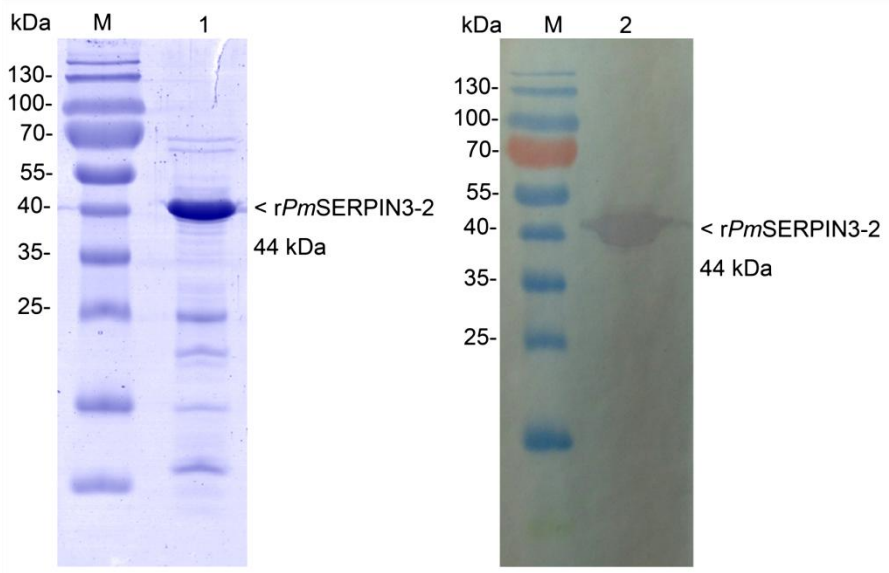


Figure 3.18 Analysis of the purified r*Pm*SERPIN3-2 protein. The crude recombinant protein r*Pm*SERPIN3-2 was purified by Ni-NTA affinity column and detected by western blot analysis using anti-His_6 antibody as primary antibody

Lane 1: Coomasie staining of the purified r*Pm*SERPIN3-2 protein

Lane 2: Western blot analysis of the purified r*Pm*SERPIN3-2 protein

Lane M: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

3.5 Specificity of anti-*Pm*SERPIN3 polyclonal antiserum

To verify specificity of anti-*Pm*SERPIN3 polyclonal antiserum to r*Pm*SERPIN3, anti-*Pm*SERPIN3 antiserum (Figure 3.19 A) and anti-*Pm*SERPIN8 antiserum (Figure 3.19 B) were used to probe the purified r*Pm*SERPIN3 and 8. Only the band corresponding to r*Pm*SERPIN3 was observed when using anti-*Pm*SERPIN3 antiserum as a primary antibody. Also, anti-*Pm*SERPIN8 antiserum specifically detected only r*Pm*SERPIN8. These indicated that, the anti-*Pm*SERPIN3 is highly specific to PmSERPIN3 and it has no cross-reactivity against other *Pm*SERPINs.

3.6 Detection of *Pm*SERPIN3 protein in hemocyte of shrimp

To confirm the presence of native *Pm*SERPIN3 protein in shrimp. The hemocyte lysate of unchallenged shrimp was prepared and 50 µg of hemocyte lysate was separated on a 12.5% (w/v) acrylamide SDS-PAGE. The western blot analysis was performed using the anti-*Pm*SERPIN3 polyclonal antiserum. The expected band of 44 kDa was observed in hemocyte lysate (Figure 3.20). This result indicated that there is native *Pm*SERPIN3 protein in the hemocyte of unchallenged shrimp.

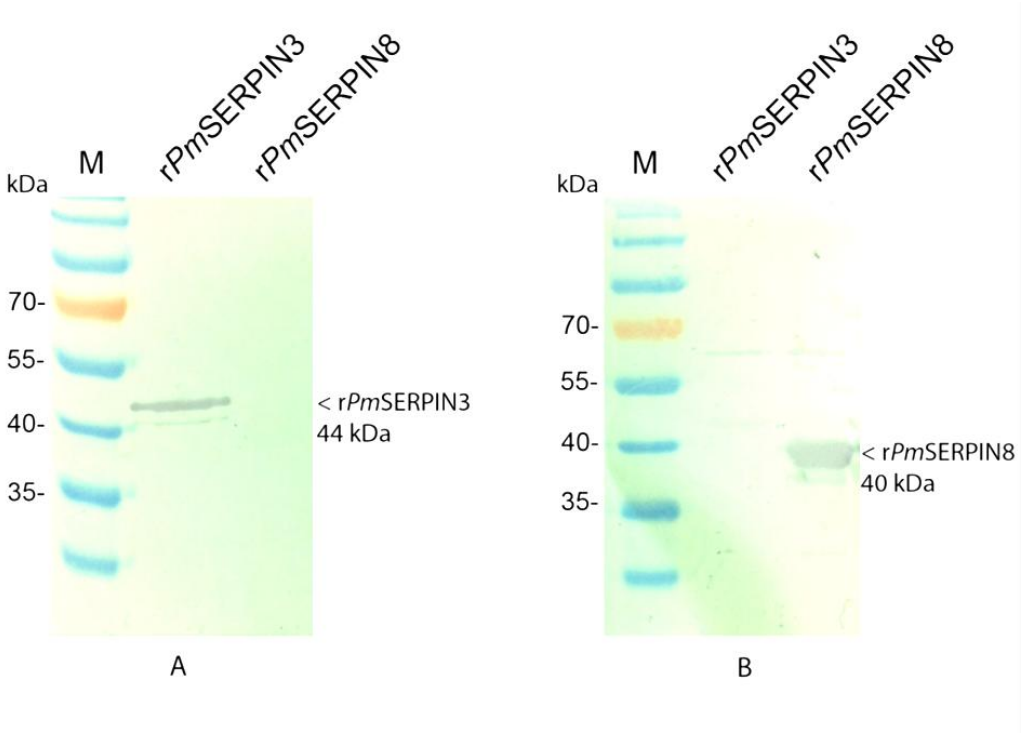


Figure 3.19 Specificity of antibody specific to *Pm*SERPIN. The anti-*Pm*SERPIN3 (A) and anti-*Pm*SERPIN8 antiserum (B) were use as primary antibody for detection r*Pm*SERPIN3 and 8 proteins by western blot analysis.

Lane r*Pm*SERPIN3: The purified r*Pm*SERPIN3 (44 kDa) was run on 10% SDS-PAGE and transfered onto nitrocellulose membrane.

Lane r*Pm*SERPIN8: The purified r*Pm*SERPIN8 (40 kDa) was run on 10% SDS-PAGE and transfered onto nitrocellulose membrane.

Lane M: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

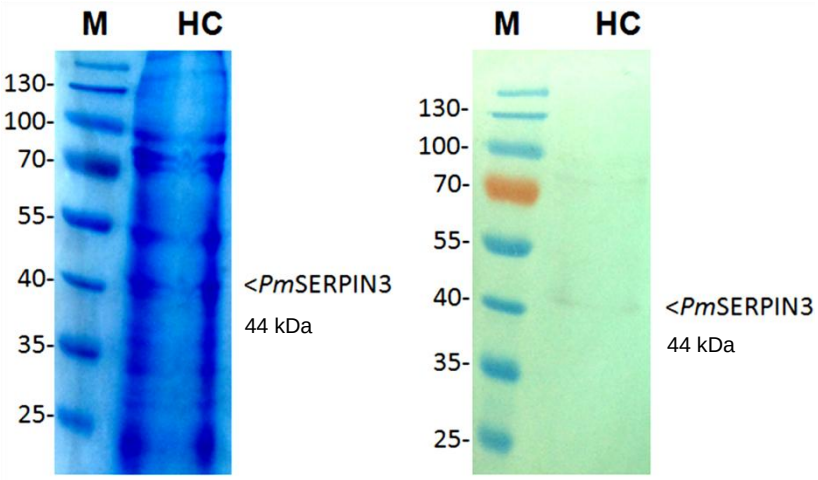


Figure 3.20 Detection of *Pm*SERPIN3 protein in hemocyte of shrimp. The hemocyte lysate of shrimp was separated by 12.5% (w/v) acrylamide SDS-PAGE and *Pm*SERPIN3 was detected by western blot analysis using anti-*Pm*SERPIN3 antiserum as a primary antibody.

Lane HC: Coomassie staining (left panel) and western blot analysis (right panel) of the hemocyte lysate.

Lane M: Prestained protein markers (PageRuler™ Prestained protein ladder, Fermentas)

3.7 Immunolocalization of *Pm*SERPIN3 protein in shrimp hemocytes

Previously, it was revealed that the *PmSERPIN3* gene and protein were expressed in *P. monodon* hemocytes. To further characterize *Pm*SERPIN3, the expression of *Pm*SERPIN3 protein in response to bacterial challenge was determined in the 0.85% NaCl and *V. harveyi* 639 (5×10^6 CFU) injected shrimp hemocyte at 0, 30 min and 3 hours after challenge. *Pm*SERPIN3 protein was probed with a purified anti-*Pm*SERPIN3 antibody and Alexa Fluor 568-conjugated secondary antibody (red fluorescence) and the cellular DNA was stained with DAPI (blue fluorescence). Using immunofluorescent staining observed under confocal laser scanning microscope, the result revealed that *Pm*SERPIN3 was expressed in both of hemocytes of challenged and unchallenged shrimps, but it expression was up-regulated in *V. harveyi* challenged group (Figure 3.21). Considering the type of hemocyte that can expressed *Pm*SERPIN3, we found that all 3 types of hemocyte such as hyaline, semigranular, and granular hemocytes expressed *Pm*SERPIN3 (Figure 3.22). It is true that with the signal peptide, the protein is destined to be secreted. What we believe is that the protein is stored in the secretory granules and secreted upon hemocyte activation possibly by the pathogens.

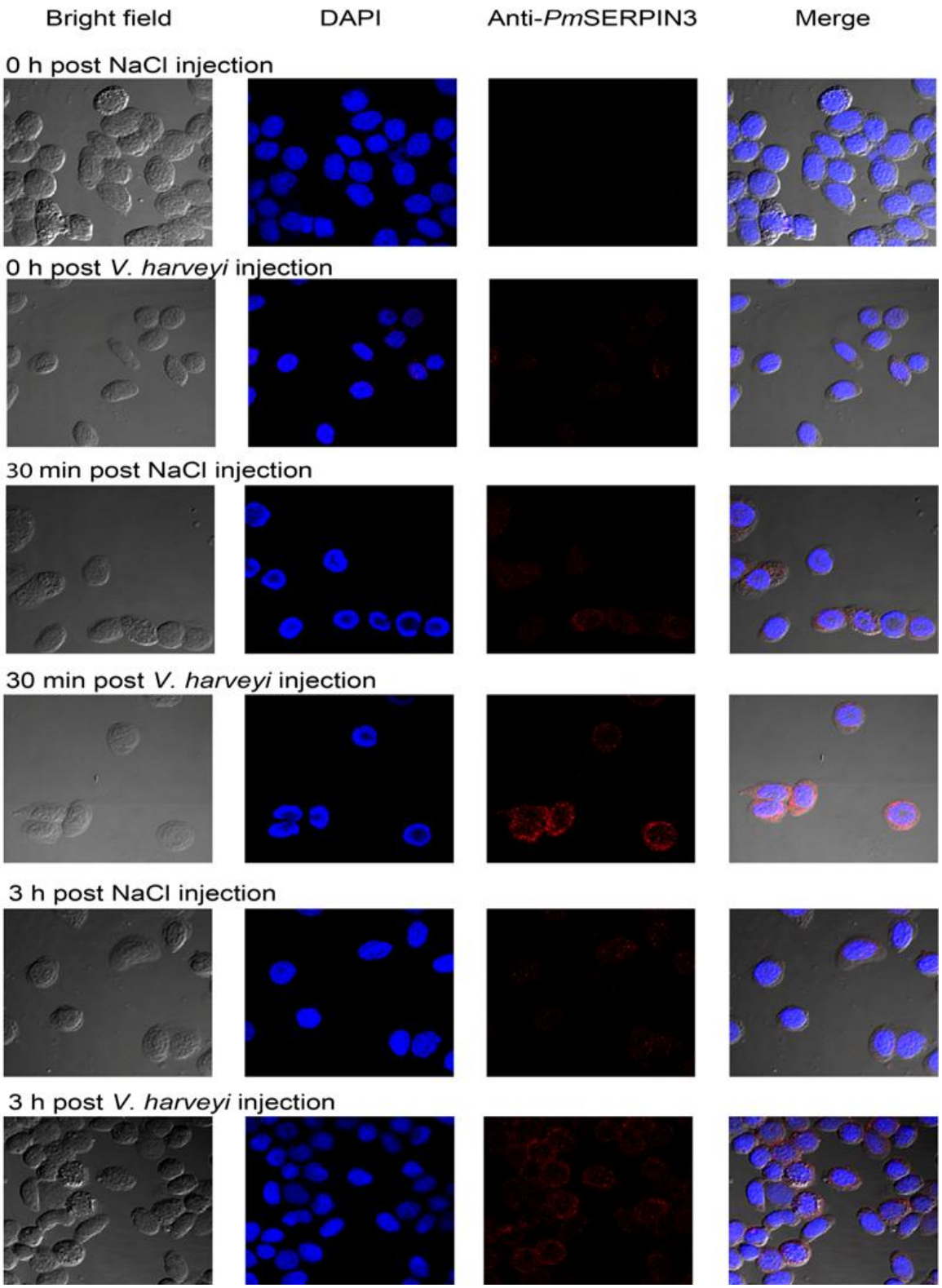

Bright field
DAPI
Anti-*Pm*SERPIN3
Merge
0 h post NaCl injection
0 h post *V. harveyi* injection
30 min post NaCl injection
30 min post *V. harveyi* injection
3 h post NaCl injection
3 h post *V. harveyi* injection

Figure 3.21 Immunofluorescent staining analysis of the *Pm*SERPIN3 protein in the hemocytes of 0.85% NaCl and *V. harveyi* 639 (5×10^6 CFU) injected shrimps at 0, 30 min and 3 hour after challenge. Fixed hemocytes were incubated with antibody specific to *Pm*SERPIN3 protein (red signal). The hemocytic nuclei were labeled with DAPI (blue signal). Images are representative of 3 fields of views.

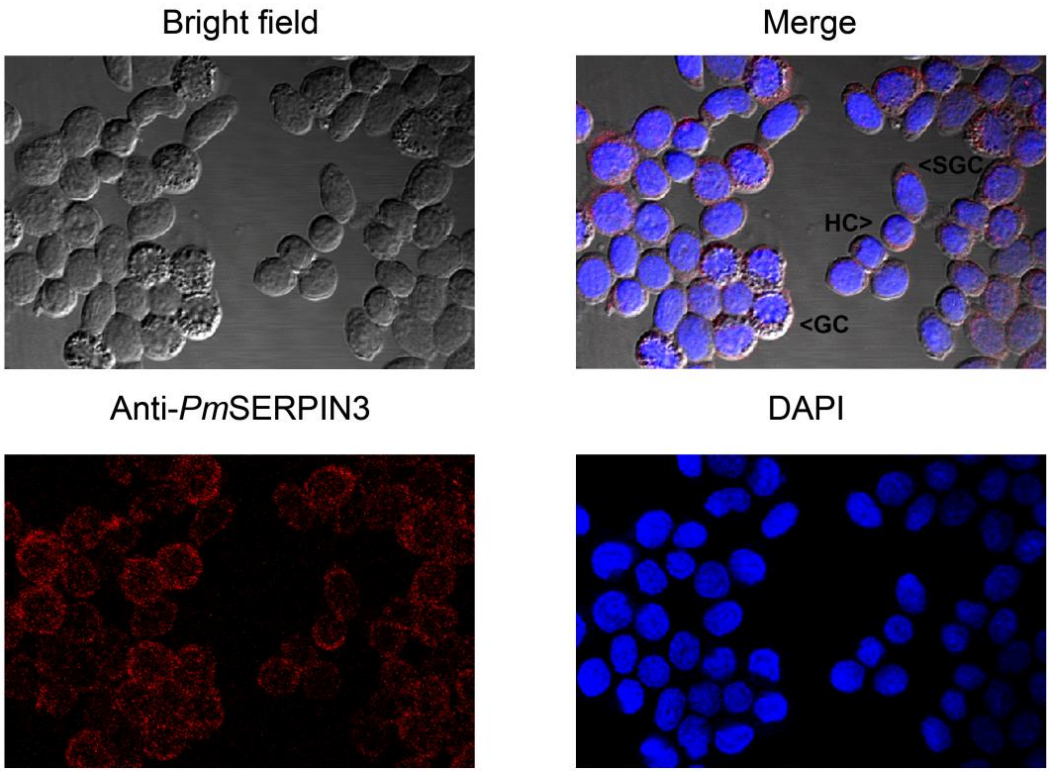


Figure 3.22 Immunofluorescent staining analysis of the *Pm*SERPIN3 protein in shrimp hemocytes represent in three different types of shrimp hemocytes (hyaline, semigranular, and granular hemocytes) monitored under confocal laser scanning microscope. Hemocytes of 3 h *V. harveyi* infected shrimp was collected and fixed with 4% Paraformaldehyde and processed for immune detection. Hemocytes were incubated with antibody specific to *Pm*SERPIN3 protein (red signal). The hemocytic nuclei were labeled with DAPI (blue signal). Images are representative of 3 fields of view. The HC, SGC, GC are hyaline, semigranular, and granular hemocytes.

3.8 Proteinase inhibitory activity assay

It is well known that SERPINs are proteinase inhibitors. The r*Pm*SERPIN3 was, therefore, assayed for its proteinase inhibitory activity. The purified r*Pm*SERPIN3-1 was tested against 4 commercial proteinases: trypsin, α-chymotrypsin, elastase and subtilisin, by measuring the remaining proteinase activity in the presence of r*Pm*SERPIN3-1 at different inhibitor:proteinase mole ratios. Considering at 1:25 mole ratio, the assay revealed that the r*Pm*SERPIN3-1 could inhibit subtilisin for 90% but did not inhibit trypsin, α-chymotrypsin and elastase though at much higher mole ratios did the r*Pm*SERPIN3-1 probably interfere with the proteinase activity (Figure 3.23). As a control, the thioredoxin was tested with subtilisin and showed no inhibition at all (Figure 3.24).

3.9 Prophenoloxidase inhibitory assay

In literatures, the SERPINs have been shown to regulate the activation of prophenoloxidase (proPO) activating system (An and Kanost, 2010). The involvement of *Pm*SERPIN3 in regulating the proPO activating system was verified by measuring the activity of phenoloxidase (PO) in the LPS-induced hemocyte lysate supernatant (HLS) in the presence of *Pm*SERPIN3. At final concentrations of 2 and 5 μM, the r*Pm*SERPIN3 was found to inhibit the activation of shrimp prophenoloxidase system for about 75% at 60 min reaction time point as compared to the buffer control (Figure 3.25). Therefore, the *Pm*SERPIN3 might play an important regulatory function in the shrimp prophenoloxidase system.

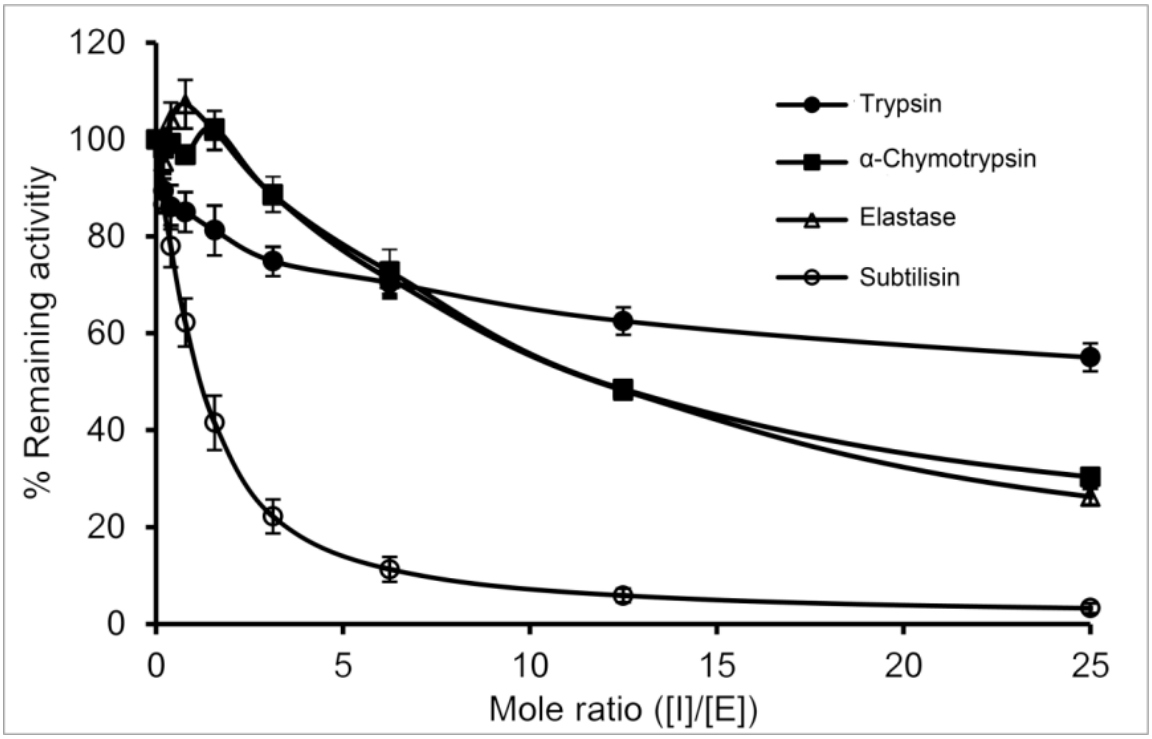


Figure 3.23 Proteinase inhibitory activity of r*Pm*SERPIN3-1 against commercial proteinases. The r*Pm*SERPIN3-1 was incubated with each proteinase: trypsin (●), chymotrypsin (■), elastase (Δ) or subtilisin (○), at various inhibitor:proteinase mole ratios in the reaction containing appropriate chromogenic substrate. After 15 min of incubation, the remaining activity of proteinase was determined. The results are means of three replicates ± SD.

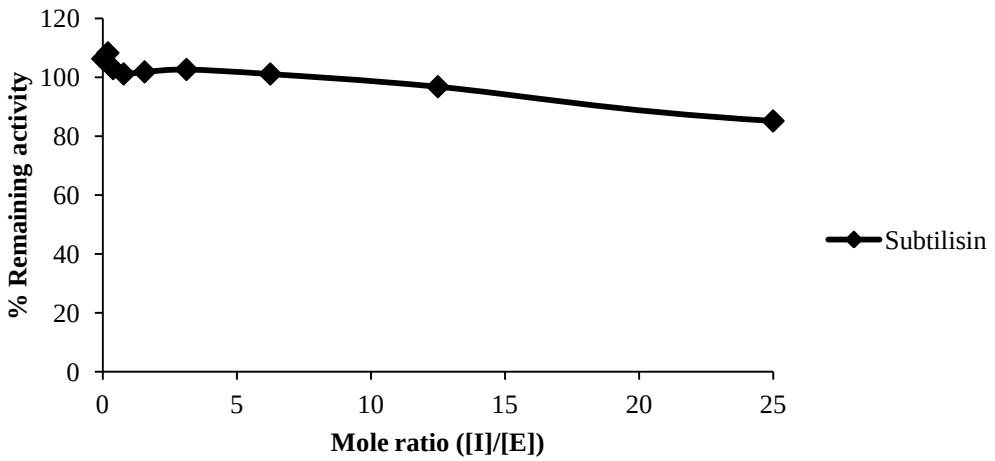


Figure 3.24 Proteinase inhibitory activity of thioredoxin against subtilisin. The r*Pm*SERPIN3-1 was incubated with one proteinase, subtilisin (◆) at various inhibitor:proteinase mole ratios in the reaction containing appropriate chromogenic substrate. After 15 min of incubation, the remaining activity of proteinase was determined.

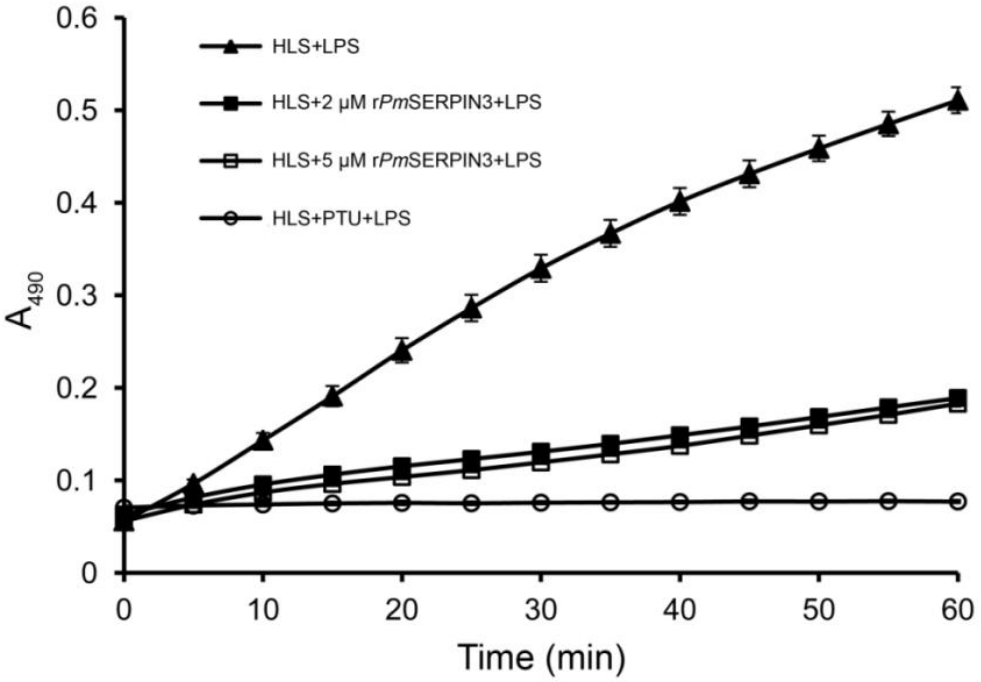


Figure 3.25 Inhibition of prophenoloxidase system by r*Pm*SERPIN3. The hemocyte lysate supernatant (HLS) was mixed with the r*Pm*SERPIN3 at the final concentrations of 2 (■) and 5 (□) μM. Buffer was added instead for the negative control (△). The positive control was the reaction containing PTU at the final concentration of 6.8 μM (○). The experiment was done in triplicate. The results are means with standard deviation.

3.10 Effect of r*Pm*SERPIN3 on bacterial clearance

To make certain that the *Pm*SERPIN3 plays an important role in shrimp immune system, the *Pm*SERPIN3 was tested for its role in bacterial clearance in the live shrimp. The pathogenic bacteria, *V. harveyi* 639 (5×10^6 CFU) pre-incubated with 5 µM r*Pm*SERPIN3 were injected into the shrimp and the shrimp were reared normally for sometimes. At 5 and 30 min after injection, a number of total bacteria and *Vibrio* species in the hemolymph were determined by plating the hemolymph on LB-agar containing salt and TCBS-agar containing salt, respectively, and calculated as CFU per ml of hemolymph. The result showed that a number of total bacterial and *Vibrio* species counts were not significantly different at 5 min after injection but at 30 min they were significantly increased by 3.5- and 2.9-fold higher than the control shrimp injected with *V. harveyi* only (Figure 3.26). Therefore, the inhibitory activity of r*Pm*SERPIN3 could take part in the bacterial clearance process, probably via regulating the proPO system, in the shrimp hemolymph.

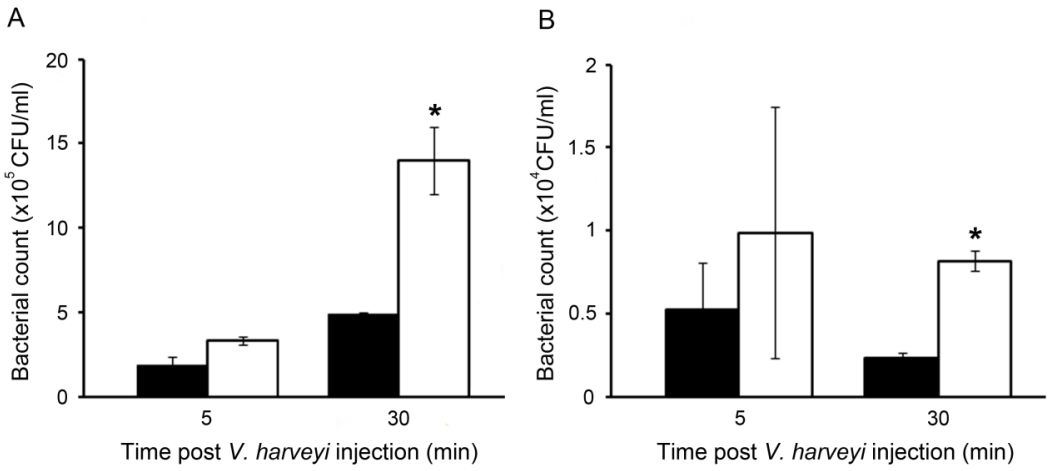


Figure 3.26 Effect of r*Pm*SERPIN3 on bacterial clearance in shrimp. The number of total bacterial (A) and *Vibrio* species (B) (CFU/ml) after injection with *V. harveyi* (shaded bar) or mixture of *V. harveyi* and 5 μM r*Pm*SERPIN3 (Opened bar) are shown. At 5 and 30 min after injection, hemolymph was drawn, diluted and plated onto the LB-NaCl (A) and TCBS-NaCl (B) agar plates. The results are means of triplicate results with SD. Asterisks indicate significant difference at $P < 0.05$ as compared to the control injection with *V. harveyi* only.

3.11 Assay for inhibitory activity of r*Pm*SERPIN3 on blood clotting

Other than proPO system, blood clotting system is one of the immune reaction of shrimp that restricts microbial invasion. According to the homology search result, *PmSERPIN3* was similar to serpins from tick that have been reported to be involved in regulating blood clotting. Therefore, we tested for the inhibitory activity of r*Pm*SERPIN3 on clot formation of shrimp hemolymph. The clotting reaction of shrimp hemolymph was mixed and activated by adding 10 µl of 40 mM $CaCl_2$ followed by flipping the tubes and observing the viscosity of hemolymph in 5 min. The result showed that in the presence of r*Pm*SERPIN3, the clot was formed (Figure 3.27 lane B) as compared to the clot forming control reaction (Figure 3.27 lanes C-D). Whereas in the negative control without adding $CaCl_2$, the clot was not observed (Figured 3.27 lane A).

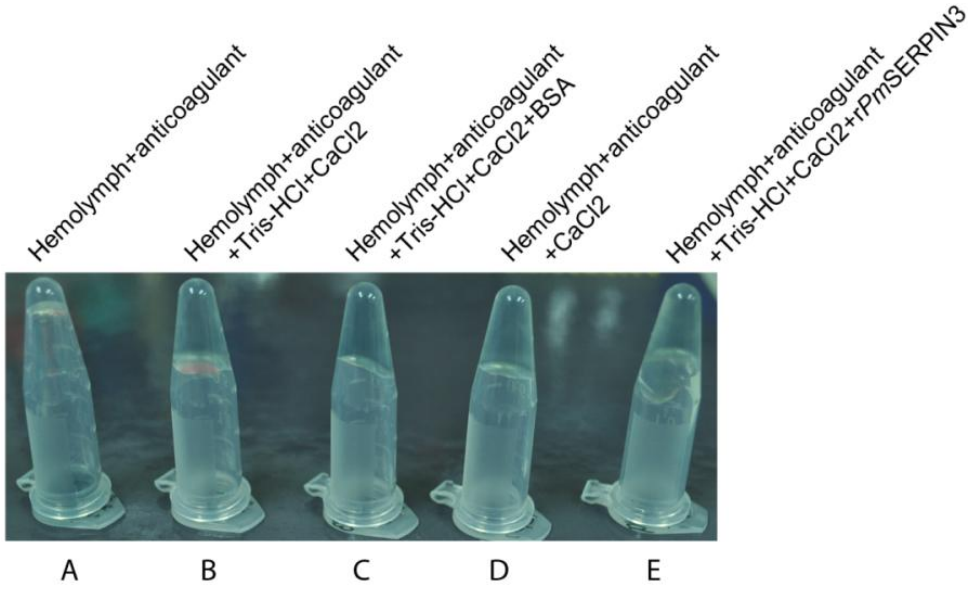


Figure 3.27 The effect of r*Pm*SERPIN3 on the shrimp blood coagulation system.

A: Hemolymph containing anticoagulant

B: Hemolymph containing anticoagulant, 20 mM Tris-HCl pH 8.0 and 40 mM $CaCl_2$

C: Hemolymph containing anticoagulant, 20 mM Tris-HCl pH 8.0, 40 mM $CaCl_2$ and 22 µg of BSA

D: Hemolymph containing anticoagulant, 20 mM Tris-HCl pH 8.0 and 40 mM $CaCl_2$

E: Hemolymph containing anticoagulant, 20 mM Tris-HCl pH 8.0, 40 mM $CaCl_2$ and 22 µg of r*Pm*SERPIN3

CHAPTER IV

DISCUSSION

The immunity of shrimp contains many processes to defende against of microbial infection, first the physical defence barriers, then the cellular and humoral defense reactions. Among these, there are many proteinase cascades that catalyzed changes of inactive serine proteinase to active serine protinase. There are many families of proteinase inhibitors such as Kazal, Kunitz, Serpin, α-macroglobulin and pacifastin that function as the regulator of proteinase in such cascade (Liang and Söderhäll, 1995; Liang et al., 1997; Kanost, 1999).

In arthropods, a group of regulator called serpins are synthesized and reported for their functions in an important immune defense system, prophenoloxidase activating system (proPO-system). proPO-system is mediated by serine proteinase cascades and regulated by serpins in the haemolymph (Zou and Jiang, 2005). Being interested in the immune modulators in the black tiger shrimp (*P. monodon*), the *Pm*SERPINB3 gene was first identified by differential display technique and found to be responded to bacterial infection (Somboonwiwat et al., 2006). We also studied for other potential serpins by searching the *P. monodon* EST database. In 2010, Homvises revealed that, eight more serpin genes were found from *P. monodon* EST database such as *PmSERPIN1-8*. Totally there are 9 *PmSERPIN* genes existed in *P. monodon*. The *Pm*SERPIN7 was found to be highly similar to *Fc*-serpin, with a 94% amino acid sequence identity, suggesting that *Pm*SERPIN7 is an orthologue of *Fc*-serpin *(F. chinensis* DQ318857) (Liu et al., 2009). The completed ORF of *Pm*SERPIN6, 7 and 8 were revealed and the *Pm*SERPIN6 was

characterized (Homvises et al., 2010). 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) (Zou and Jiang, 2005). Until now the full-length cDNA of *Pm*SERPINB3 and *Pm*SERPIN6 and 8 were identified (Homvises et al., 2010) (Somnuk et al., 2012).

In this study, the full-length cDNA of *Pm*SERPIN3 was obtained. *Pm*SERPIN3 contained an ORF of about 1,233 bp encoded for 410 amino acid residues protein with 23 residues signal peptide (Figure 3.4). It had a conserved reactive centre loop (RCL) near the carboxyl-terminal. The RCL, exposed at the surface of the protein, is the site of interaction between serpin and its target serine protease. The RCL contains a scissile bond between two residues, called P1–P1′, which is cleaved by the target protease (Wilczynska et al.,1995). According to blastX result, the *Pm*SERPIN3 shared high similarity with other insect serpins, 43% homology with *Is*SERPIN2, 7 (*I. Scapularis* XP_002434444.1, XP002407493) (Mulenga et al., 2009) and 41% with alaserpin (*C. quinquefasciatus* nexin XP001865071). Moreover, we compared the mature peptide of *Pm*SERPIN3 with other *Pm*SERPIN and other serpins in others arthopods such as *Pm*SERPIN6, *Pm*SERPIN8, *Pm*SERPINB3, *Pl*SERPIN, *Fc*-serpin, *Ms*Serpin6, *Is*Serpin7, *Dm*Spn27A and *Dm*Spn43Ac by alignment process. The putative P1-P1′ residues of *Pm*SERPIN3 were predicted as Arg-Met, respectively (Figure 3.5). The *Pm*SERPIN3 shared the similarity reactive site peptide bond (P1–P1′) with *Is*Serpin7, *Ms*Serpin6 and *Pm*SERPIN6. An Arg, at the P1 position of the reactive site indicated that *Pm*SERPIN3 may have inhibitory activity against bovine plasmin, bovine trypsin (Lui et

al., 2009) PAP (Jiang et al., 1996) and (Jiang et al., 1997), Limulus factor C, factor G (Miura et al., 1994), clotting enzyme and human tissue plasminogen activator (Agarwala et al., 1996). However, our result showed that the r*Pm*SERPIN3-1 used for the proteinases inhibitory activity assay could completely inhibit subtilisin but not other serine proteinases tested.

The phylogenic tree constructed based on the mature peptides of all *Pm*SERPINs and serpins from other crustaceans (Figure 3.6) showed that *Pm*SERPIN3 was in the different cluster to other *Pm*SERPINs. Meanwhile, *Pm*SERPIN3 was in the same cluster with antithrombin inhibitor. This suggested that *Pm*SERPINs does not have a single origin in gene evolution. The *PmSERPIN3* was the first *Pm*SERPIN identified as an intronless gene. This genome organization revealed the different genome structure to *PmSERPIN6* (Unpublished data) and *PmSERPIN8* (Somnuk et al., 2012).

Unlike other *Pm*SERPINs, the expression of *Pm*SERPIN3 did not respond to microbial challenges. *Pm*SERPIN6 expression was altered upon bacterial and viral infection (Homvises et al., 2010) whereas *Pm*SERPIN8 was up-regulated upon *V. harveyi* challenge (Somnuk et al., 2012). Tissue distribution analysis showed that *PmSERPIN3* was expressed in all 12 tissues tested and it also expressed at all developmental stages from nauplius IV, zoea III, mysis IV to sub-adult. The expression of *PmSERPIN3* in hemocytes suggests that *Pm*SERPIN3 might be involved in immune response of *P. monodon.* The expression result is also reported in *F. chinensis* by Liu (Liu et al., 2009). The *Pm*SERPIN3 in shrimp hemocyte was confirmed by western blot analysis. We also used immunofluorescent lebeling technique in conjunction with a confocal laser

scanning microscope to localize the *Pm*SERPIN3 in the hemocyte cells. It was found that *Pm*SERPIN3 waslocated in cytoplasm very likely to be in all 3 types of hemocytes cell such as, hyaline, semigranular and granular hemocytes for the fluorescence was more intense there (Figure 3.22). Moreover, the up-regulation of *Pm*SERPIN3 protein upon *V. harveyi* challenge was clearly observed.

Serpins acting as negative regulator of proPO activation system has been reported in various invertebrates. In insects, serpins in hemolymph have a role in regulating innate immune pathways, including proPO activation system and Toll pathway (Zou et al., 2010). In *Drosophila melanogaster*, Serpin-27A (Spn27A) restricts the phenoloxidase activity at the site of injury or infection, preventing the insect from excessive melanization while the Spn28D confines PO availability by controlling its initial release (Gregorio et al., 2002; Scherfer et al., 2008). In *M. sexta*, serpin-6 strongly inhibits prophenoloxidase-activating proteinase-3 (PAP-3) but not PAP-1 or PAP-2, suggesting that the proPO activation by PAPs is differentially regulated by multiple serpins (Wang and Jiang, 2004). Serpin-4 and serpin-5 can form complexes with hemolymph proteinase 6 (HP6) *in vitro* and inhibits the activation of proHP8 and proPAP1 to modulate proPO activation and antimicrobial peptide production (An and Kanost, 2010). To regulate the Toll pathway response, serpin-1J inhibits HP8 which can cleave and activate the Toll ligand, Spätzle and thereby modulates the concentration of active Spätzle and the synthesis of antimicrobial peptides (An et al., 2011; Christen et al., 2012). Recently, proteinases complexed with serpin-3 were identified as PAP-1, PAP-2, PAP-3, and HP8 (Christen et al., 2012). In mosquitoes, two serpins were shown to inhibit PO activity and

melanization (Gulley et al., 2012). In shrimp, only *Pm*SERPIN8 has been shown to be able to inhibit proPO activation (Somnuk et al., 2012). This study showed that *Pm*SERPIN3 can also inhibit the activation of proPO system by 75% in which this activity is stronger than that of *Pm*SERPIN8. Although, *Pm*SERPIN3 sequences was close related for antithrombin inhibitor but we have shown here that r*Pm*SERPIN3 was not able to inhibit the clot formation in shrimp hemolymph.

It should be noted that both r*Pm*SERPIN3 and 8 cannot completely inhibited activation of proPO system. However, how *Pm*SERPINs regulate the proPO system in shrimp is still elusive. Anyway, in this study, we showed that introducing r*Pm*SERPIN3 into shrimp resulted in the decrease in bacterial clearance capability of shrimp.

Taken together, it can be concluded that r*Pm*SERPIN3 transcript was potentially a housekeeping gene suggesting that *PmSERPIN3* is essential for controlling shrimp proPO system to prevent improper activation that is harmful to host itself. Inhibition of proPO activation by *Pm*SERPIN3 affected the bacterial clearance ability of shrimp.

CHAPTER V

CONCLUSIONS

The *Pm*SERPIN3 gene contained an open reading frame of 1,233 bp encoding for 410 amino acid residues protein. Sequence analysis revealed conserved structure of serpin including hinge region, reactive center loop (RCL) and signature sequences. The RCL of *Pm*SERPIN3 was predicted and its putative P1 and P1' residues were Arg and Met, respectively. At the transcriptional level, *Pm*SERPIN3 gene expressed in all tissues and developmental stages tested and the expression level of *Pm*SERPIN3 gene did not respond to *V. harveyi*, WSSV and YHV challenges. The *Pm*SERPIN3 was up-regulated upon *V. harveyi* infection at the translational level and was found in 3 main types of hemocytes such as hyaline, semigranular, and granular hemocytes of unchallenged and *V. harveyi*-challenged *P. monodon*.

The recombinant *Pm*SERPIN3 protein was successfully produced in *E. coli*. Testing for inhibitory activities indicated that it can completely inhibit subtilisin but not other commercial proteases tested. Moreover, the r*Pm*SERPIN3 can inhibit the activation of shrimp prophenoloxidase system; the phenoloxidase activity was down for about 75% at 60 min. The number of total bacterial and *V. harveyi* (CFU/ml) in shrimp hemocyte after injected with *V. harveyi* with r*Pm*SERPIN3 were 3.5- and 2.9- fold higher than the control shrimp injected with *V. harveyi* only. It was shown here that r*Pm*SERPIN3 was not an inhibitor of coagulation system of shrimp. Taken together, the r*Pm*SERPIN3 might function as an inhibitor of proPO system. Its inhibitory activity takes part in the bacterial clearance efficacy of shrimp.

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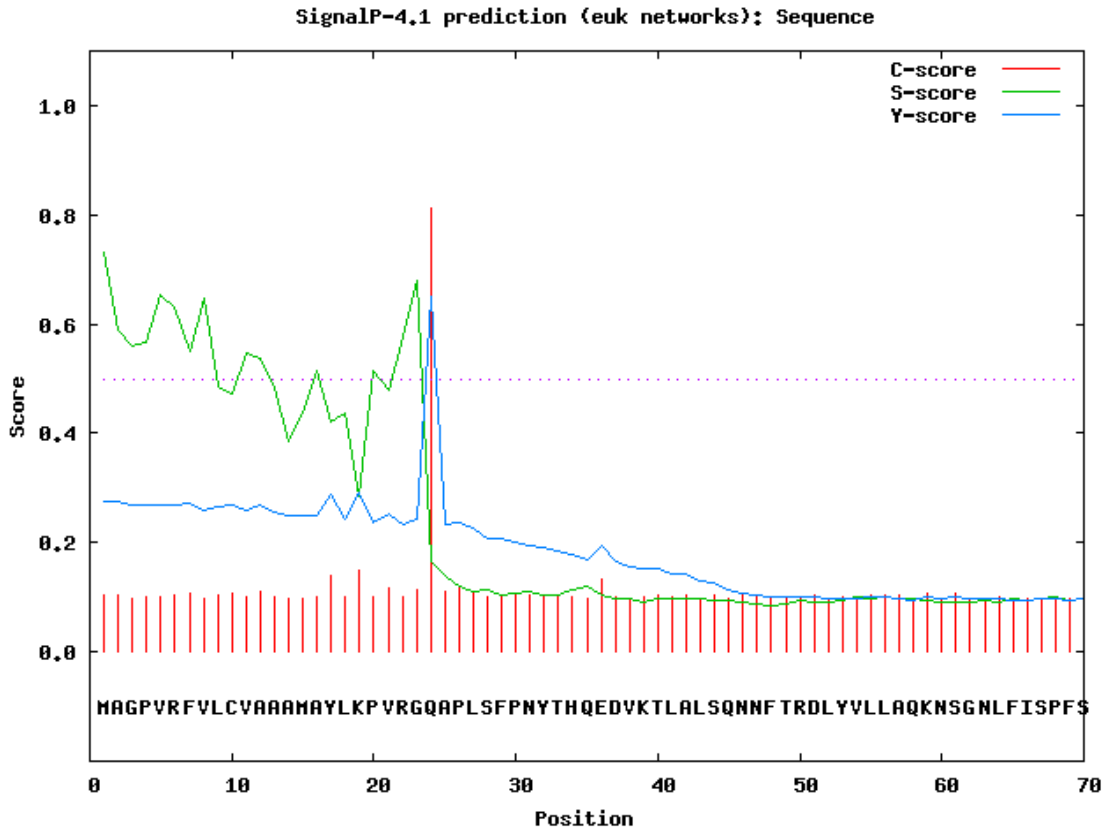
APPENDIX

CENTERFOR BIOLOGICAL SEQUENCE ANALYSIS CBS

Signal peptide prediction by SignalP-4.1 Server

SignalP-4.1 euk predictions
>**Sequence**



Measure Position Value Cutoff signal peptide?

max. C 24 0.813

max. Y 24 0.650

max. S 1 0.731

mean S 1-23 0.530

D 1-23 0.585 0.450 YES

Name=Sequence SP='YES' Cleavage site between pos. 23 and 24: VRG-QA D=0.585 D-cutoff=0.450 Networks=SignalP-noTM

The N-Glycosylation sites prediction with NetNGlyc 1.0

Server

Asn-Xaa-Ser/Thr sequons in the sequence output below are highlighted in **blue**.

Asparagines predicted to be N-glycosylated are highlighted in **red.**

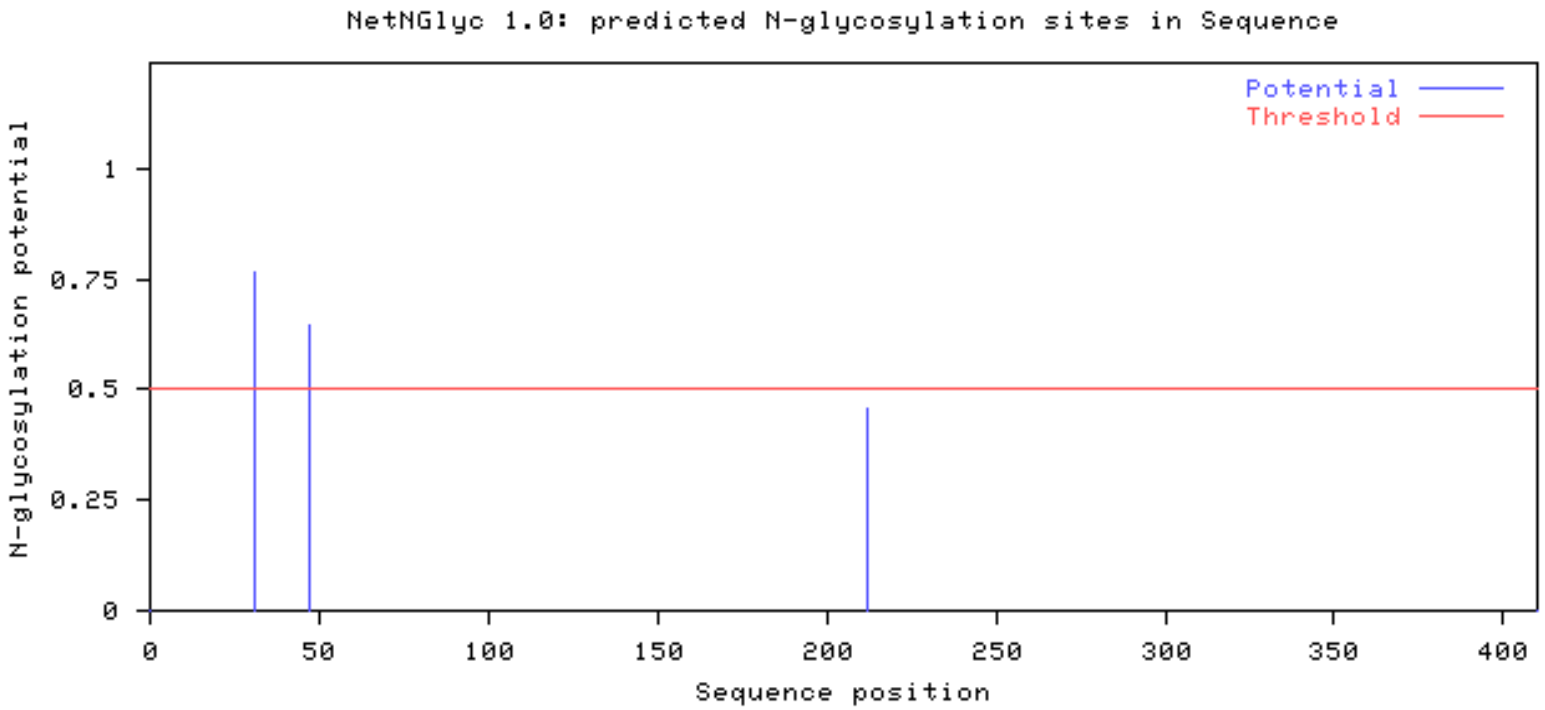
Output for 'Sequence'

Name: Sequence Length: 410

```
MAGPVRFVLCVAAAMAYLKPVRGQAPLSFPNYTHQEDVKTLALSQNNFTRDLYVLLAQKNSGNLFISPFSIMTALSMTYG      80
GAKENTEEEMRSALHLTQEKEAVHNAFQDVVSDIKTEAPDYELRTSNMAYVSNKLTVVSEFANMLKEKYLSSSKVVDFGE     160
SEAVRREINDVVEKETNSKIKDLIPSGVLNSLTRMVLVNAVYFKGLWENQFNESDTHDQEFWISSQESVQVPMMHIKKKF     240
RYFNHRDLDSTILAMDYKGSRLSMVFILPNKRDGIAEVEAKLASADLYAIDNGLHSVEVEVSLPRFKLEESLELVDYLQV     320
LGMKDLFDEGRCDLSGISGNRDLYVSNVIHKAFLEVNEKGSEAAAATAVVAATRMLIRPIPPFIADHPFMFYIRDHRSGL     400
VHFAGRFVKP
.............................N..............N..............................      80
................................................................................     160
................................................................................     240
................................................................................     320
................................................................................     400
..........                                                                           480
```

(Threshold=0.5)

SeqName	Position	Potential	Jury agreement	N-Glyc result
Sequence	31 NYTH	0.7680	(9/9)	+++
Sequence	47 NFTR	0.6430	(8/9)	+
Sequence	212 NESD	0.4599	(7/9)	-


NetNGlyc 1.0: predicted N-glycosylation sites in Sequence
Potential
Threshold
N-glycosylation potential
1
0.75
0.5
0.25
0
0
50
100
150
200
250
300
350
400
Sequence position

BIOGRAPHY

Miss Natthiya Wetsaphan was born on October 25, 1987 in Angthong. She graduated with the degree of Bachelor of Science from the department of Biochemistry, Faculty of Science, Chulalongkorn University in 2009. She has studied for the degree of Master of Science at the Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2010. In her third years of research work, she oral presented on the topic of "Characterization of *Pm*SERPIN3 gene from black tiger shrimp, *Penaeus monodon*" at the 13th FAOBMB International Congress of Biochemistry and Molecular Biology, the 17th Biological Sciences Graduate Congress 2012 and the 2012 Malasia – Thailand Graduate Forum in Life Science, Food Science and Agriculture.

Awards and Conference experiences

2010 : The consolation prize for poster presentation in "The Science Forum 2010" Faculty of Science, Chulalongkorn University on the topic of "Exon – intron organization of the *Pm*SERPIN6 gene in the black tiger shrimp, *Penaeus monodon*"

2012 : The winner award for poster presentation in "The Science Forum 2010" Faculty of Science, Chulalongkorn University on the topic of "Characterization of *Pm*SERPIN3 gene from black tiger shrimp, *Penaeus monodon*"

2012 : Oral presentation on the topic of "Characterization of *Pm*SERPIN3 gene from black tiger shrimp, *Penaeus monodon*" at

- The 13th FAOBMB International Congress of Biochemistry and Molecular Biology

- The 17th Biological Sciences Graduate Congress 2012

- 2012 Malasia – Thailand Graduate Forum in Life Science, Food Science and Agriculture

2013 : The 1st runner up award for oral presentation in "The Science Forum 2013" Faculty of Science, Chulalongkorn University on the topic of "Characterization of *Pm*SERPIN3 gene from black tiger shrimp, *Penaeus monodon*"