การระบุและลักษณะสมบัติของตัวยับยั้งซีรีนโปรตีเนสชนิดคาซอล SPIP*m*4 และ SPIP*m*5 จากเซลล์เม็ดเลือดของกุ้งกุลาดำ *Penaeus monodon*

นางสาวสุวัฒนา วิเศษนันท์

IDENTIFICATION AND CHARACTERIZATION OF KAZAL-TYPE SERINE

PROTEINASE INHIBITORS, SPI*Pm*4 and SPI*Pm*5, FROM HAEMOCYTES

OF BLACK TIGER SHRIMP *Penaeus monodon*

Miss Suwattana   Visetnan

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biochemistry

Department of Biochemistry

Faculty of Science

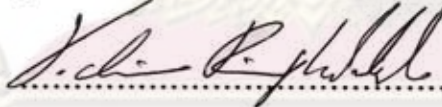**Chulalongkorn University**

Academic Year 2009

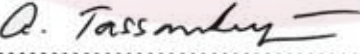|	
Thesis Title	IDENTIFICATION AND CHARACTERIZATION OF KAZAL-TYPE SERINE PROTEINASE INHIBITORS, SPI*Pm*4 AND SPI*Pm*5, FROM HAEMOCYTES OF BLACK TIGER SHRIMP *Penaeus monodon*
By	Miss Suwattana Visetnan
Field of Study	Biochemistry
Thesis Advisor	Associate Professor Vichien Rimphanitchayakit, Ph.D.
Thesis Co-Advisor	Professor Anchalee Tassanakajon, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree
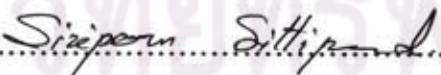
.... Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)
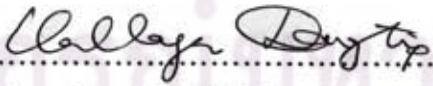
THESIS COMMITTEE

.... Chairman
(Associate Professor Piamsook Pongsawasdi, Ph.D.)

.... Thesis Advisor
(Associate Professor Vichien Rimphanitchayakit, Ph.D.)

.... Thesis Co-Advisor
(Professor Anchalee Tassanakajon, Ph.D.)

.... Examiner
(Associate Professor Siriporn Sittipraneed, Ph.D.)

.... External Examiner
(Kallaya Dangtip, Ph.D.)

สุวัฒนา วิเศษนันท์ : การระบุและลักษณะสมบัติของตัวยับยั้งซีรีนโปรตีเนสชนิดคาซอล SPIPm4 และ SPIPm5 จากเซลล์เม็ดเลือดของกุ้งกุลาดำ *Penaeus monodon* (IDENTIFICATION AND CHARACTERIZATION OF KAZAL-TYPE SERINE PROTEINASE INHIBITORS, SPIPm4 AND SPIPm5, FROM HAEMOCYTES OF BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. วิเชียร ริมพณิชยกิจ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม ศ.ดร.อัญชลี ทัศนาขจร, 135 หน้า.

ตัวยับยั้งซีรีนโปรตีเนสมีบทบาทสำคัญในระบบภูมิคุ้มกัน และมีบทบาทสำคัญอย่างยิ่งในกระบวนการพื้นฐานต่างๆ ทางสรีรวิทยา พบในสิ่งมีชีวิตหลายชนิด จากข้อมูล EST ของกุ้งกุลาดำ พบว่าสามารถจัดจำแนกตัวยับยั้งซีรีนโปรตีเนสชนิดคาซอลได้ทั้งหมด 9 ชนิด คือ SPIPm1-9 การศึกษาระดับการแสดงออกของตัวยับยั้งซีรีนโปรตีเนสชนิดคาซอล 7 ชนิด ที่ถูกกระตุ้นด้วย *V. harveyi* 639 ด้วยวิธี semi-quantitative RT-PCR พบว่า ระดับการแสดงออกของยีน *SPIPm*1, 2, 6, 7 และ 9 ในระดับ mRNA ไม่เปลี่ยนแปลงหลังจากการกระตุ้น สำหรับการศึกษา RT-PCR ของ SPIPm5 แสดงให้เห็นว่าระดับการแสดงออกของ SPIPm5 เพิ่มขึ้น และตอบสนองต่อความร้อน ส่วน SPIPm4 และ 5 ประกอบด้วยบริเวณ open reading frame ขนาด 387 และ 399 เบส แปลรหัสให้โปรตีนที่มีกรดอะมิโนจำนวน 128 และ 132 ตัว ซึ่งรวมส่วน signal peptide 21 และ 19 เรสซิดิวส์ของกรดอะมิโน และได้โปรตีนสมบูรณ์ที่ประกอบด้วยกรดอะมิโน 107 และ 113 หน่วย ตามลำดับ โปรตีนรีคอมบิแนนท์ rSPIPm4 และ 5 ที่ผลิตได้จากระบบ *E. coli* มีขนาด 12.862 และ 13.433 กิโลดาลตัน จากการศึกษาแอกทิวิตีการยับยั้งโปรตีเนส พบว่า SPIPm4 มีแอกทิวิตีในการยับยั้ง subtilisin ส่วน SPIPm5 มีแอกทิวิตีในการยับยั้ง subtilisin และ elastase ซึ่งชนิดของการยับยั้งเป็นแบบแข่งขัน โดย SPIPm4 มีค่าคงที่ในการยับยั้ง subtilisin เท่ากับ 14.95 nM และ SPIPm5 มีค่าคงที่ในการยับยั้ง subtilisin และ elastase เท่ากับ 4.19 และ 59.64 nM ตามลำดับ นอกจากนั้นพบว่าโปรตีนรีคอมบิแนนท์ของ SPIPm4 และ SPIPm5 ไม่มีฤทธิ์ทำลายแบคทีเรีย เมื่อทำการทดสอบด้วยเชื้อ *B. subtilis, B. megaterium, S. aureus, V. harveyi* 639 และ *E. coli* JM109

ภาควิชา......ชีวเคมี.................... ลายมือชื่อนิสิต..... สุวัฒนา วิเศษนันท์

สาขาวิชา......ชีวเคมี.................... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.................

ปีการศึกษา......2552.................... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.................

v

4972548423: MAJOR BIOCHEMISTRY
KEYWORDS KAZAL-TYPE/KAZAL-TYPE SERINE PROTEINASE INHIBITOR/*Vibrio harveyi*/BLACK TIGER SHRIMP/*Penaeus monodon*

SUWATTANA VISETNAN: IDENTIFICATION AND CHARACTERIZATION OF KAZAL-TYPE SERINE PROTEINASE INHIBITORS, SPI*Pm*4 and SPI*Pm*5, FROM HAEMOCYTES OF BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: ASSOC PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D., THESIS CO-ADVISOR: PROF ANCHALEE TASSANAKAJON, Ph.D., 135 p.

Serine proteinase inhibitors (SPIs) play important roles in physiological and immunological processes involving proteinases in all multicellular organisms. In black tiger shrimp *P. monodon*, nine different Kazal-type SPIs, namely SPI*Pm*1-9, were identified from the cDNA libraries. The semi-quantitative RT-PCR of seven Kazal-type SPI genes after *V. harveyi* 639 challenge showed that the expression of SPI*Pm*1 was significantly increased for 1.92 fold at 6 hpi. Other SPI genes: SPI*Pm*2, 6, 7 and 9 showed hardly any differences in mRNA expression level. RT-PCR study of the SPI*Pm*5 gene revealed that it was up-regulated in response to heat treatment. The SPI*Pm*4 and SPI*Pm*5 consist of open reading frames of 387 and 399 bp coding for polypeptides of 128 and 132 amino acids with putative signal peptides of 21 and 19 amino acid residues and mature SPIs of 107 and 113 amino acid residues, respectively. Recombinant expression in an *E. coli* expression system yielded recombinant proteins, rSPI*Pm*4 and rSPI*Pm*5, with molecular masses of 12.862 and 13.433 kDa, respectively. The SPI*Pm*4 inhibitor exhibited potent inhibitory activity against subtilisin and SPI*Pm*5 against subtilisin and elastase. The inhibition was a competitive type with inhibition constants (K_i) of 14.95 nM for SPI*Pm*4 against subtilisin, 4.19 and 59.64 nM, respectively, for SPI*Pm*5 against subtilisin and elastase. They had no bacteriostatic effect against *B. subtilis*, *B. megaterium*, *S. aureus*, *V. harveyi* 639 and *E. coli* JM109.

Department.......Biochemistry.......... Student's signature...... Suwattana Visetnan

Field of study.......Biochemistry......... Advisor's signature.......

Academic year.......2009.................. Co-advisor's signature...... A. Tassanalyt

ACKNOWLEDGEMENTS

CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EtBr	ethidium bromide
h	hour
kb	kilobase
M	molar
mg	milligram
ml	millilitre
mM	millimolar
MT	metric ton
ng	nanogram
nm	nanometre
O.D.	optical density
^{o}C	degree Celcius

ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
sec	second
μg	microgram
μl	microlitre
μM	micromolar
UTR	untranslated region

CHAPTER I

INTRODUCTION

1.1   General introduction

Shrimp have been one of the most economically important aquatic species in aquaculture up till now. Due to their high world-wide demand and a gradually-experienced effort in the development of production technologies, they become very important export products for many countries along the Indo-Pacific coast. In Thailand, the shrimp species produced is the Pacific white shrimp, *Penaeus vannamei*, though it was once the black tiger shrimp, *Penaeus monodon*.

Thai shrimp farming started in the early 1980s and really began to expand in the mid 1980s. Since then, Thailand has been the world leader for exporting shrimp produces in the forms of frozen and value-added products to several countries, e.g. Japan, USA and the European Union. The industry has been worth approximately 300,000-400,000 metric tons annually providing an income of nearly 85,000 million baht yearly for the country (Figure 1.1) (Source: Office of Agricultural Economics in cooperation with the Customs Department).

From 2001 till these days, the once successful shrimp farming industry has been seriously affected by many dramatically factors, for example, the outbreaks of bacterial and viral diseases, the water quality problem and the very rare high-quality broodstock. Consequently, the shrimp farming has been switched to the other species, namely the Pacific white shrimp, *Penaeus vannamei*. Because it is a genetically

improved strain, *P. vannamei* contains several great advantages over the *P. monodon* including the rapid growth rate, high stocking density tolerance, low salinities and temperatures tolerance, lower protein requirements (and, therefore, production costs), certain disease resistance (if specific pathogen resistance stocks are used), and high survival rate during larval rearing (50-60% comparing to 20–30% for *P. monodon*). However, the *P. vannamei* may possess certain disadvantages for it is an alien species for Thailand. It possibly acts as a carrier of various pathogens new to the culture areas in Thailand. Its broodstocks have to be imported mainly from the strain stocking institute at the Hawaii Marine Institute. From these reasons, the shrimp farming of native rather than the alien shrimp species should be considered as essential for Thailand.

Although the overall biological systems of *P. monodon* are progressively studied at the molecular level, the immune system should be intensively studied considering its importance in fighting the shrimp diseases. Most of the studies are to characterize the factors involved in shrimp response to the infection by the pathogens. The knowledge is not only applied for the protection of shrimp from the diseases but also for the selective breeding for healthy shrimp for the industry in Thailand.
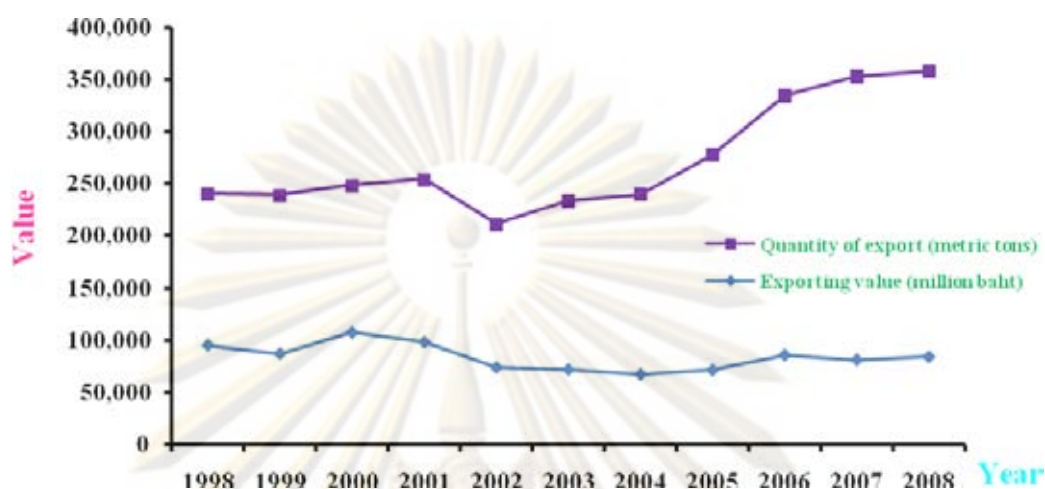
Figure 1.1   Cultured shrimp production in Thailand from 1998 to 2008**.**
(**Source**: Office of Agricultural Economics in cooperation with the Customs Department)

1.2   Shrimp diseases

Bacterial and viral diseases are known to be the major constraint for the progress of semi-intensive and intensive shrimp cultures throughout the world. The major disease outbreaks from both viruses and *Vibrio* spp. have been detrimental to the industry from time to time in recent years. Viral disease outbreaks are generally more severe. The viral outbreaks often result from various stress factors, such as overcrowding, abnormal temperatures and low dissolved oxygen. Most bacterial infections result from extreme stress. The most common bacterial infection in marine shrimp is *Vibrio*. *Vibrio* infections often occur following the environmental stresses or viral diseases, and are not the primary disease problem (Nash, 1990).

Five viral pathogens are considered the main hazards to the cultivated *P. monodon* in Thailand. These include white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvovirus (HPV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and monodon baculovirus (MBV). Only the WSSV and YHV are presented herein.

1.2.1　White spot syndrome virus (WSSV)

White spot syndrome first appeared in northeast Asia in 1992-93, and rapidly spread to several shrimp farming countries in Asia and the Indo-Pacific. This is a lethal disease with 90-100% mortality caused by white spot syndrome virus (WSSV). WSSV is a viral pathogen of crustaceans with a wide host range. It can infect both freshwater and marine species, for instance shrimp, crab and crayfish (Chou et al., 1995; Wang et al., 1998). This viral infection has been observed in several commercial penaeid species, including *P. monodon*, *Marsupenaeus japonicus*, *P. chinensis*, *P. indicus*, *P. merguiensis*, *P. vannemei*, *P. stylirostris*, *P. penicillatus* and *P. setiferus*. The virus has been given various names, including baculoviral hypodermal and haematopoietic necrosis virus (Chou et al., 1995), rod shaped virus of *M. japonicus* (Takahashi, 1996), systemic ectodermal and mesodermal baculovirus (Wongteerasupaya et al., 1995) and white spot baculovirus (WSBV) (Chou et al., 1995; Lo et al., 1996)

In shrimp, the disease is characterized by the presence of white spots of about 5 mm on the cuticle and sometimes is accompanied with a reddish coloration on the body. Having lethargy and a rapid reduction in food consumption, the infected shrimp swim slowly near the pond surface and eventually sink to the bottom and die.

The high mortality rate (100%) occurs within 3 to 10 days after the first signs of this disease (Karunasasagas, 1997).

The mechanism of infection and spreading of WSSV in crustacean hosts is not clear. It is believed that the envelop protein of virus play important roles in virus infection (Zhang, 2004; Wu, 2005). The experimental transmission of WSSV indicates that the white spot disease could be transferred by cohabitation with or ingestion of WSSV-infected animals (Kanchanaphum et al., 1998; Supamattaya et al., 1998). WSSV can be detected in early larvae stages of *P. monodon* but significant mortality was observed in post-larvae and juveniles shrimp (Yoganandhan et al., 2003).

Detection of virus at an early stage is necessary to reduce damage from the WSSV infection. Several diagnostic methods have been described such as PCR (Lu et al., 1996), *in situ* hybridization (Wang et al., 1998), negative staining of tissues (Inouye, 1994) and reverse passive latex agglutination (RPLA) method (Okumura et al., 2005). To protect the shrimp or other crustaceans against WSSV, the WSSV subunit vaccine, the WSSV envelop proteins VP19 and VP28, have been studied. The VP19 and VP28 fused to the maltose binding protein (MBP) provide significant better survival rate for the treated shrimp than the control shrimp (Witteveldt et al., 2005).

1.2.2 Yellow head virus (YHV)

In Thailand, the yellow head disease is called 'hua leung' (Chantanachookin et al., 1993; Lightner, 1996). It is caused by the yellow head virus. It has caused significant losses of cultured shrimp *P. monodon* throughout Asia (Chantanachookin et al., 1993; Flegel, 1997). Extensive characterization of yellow

head virus (YHV) genome (Cowley et al., 2000; Sittidilokratna et al., 2002; Jitrapakdee et al., 2003) has clearly shown that this virus can be classified into a new genus *Okavirus* and family *Roniviridae* in the order *Nidovirales* (Mayo, 2002). The yellow head virus principally infects pond-reared black tiger shrimp *P. monodon*. This syndrome occurs in the juvenile to sub-adult stages of shrimp 5 to 15 grams in size, especially at 50-70 days of grow out (Lightner, 1996). The affected shrimp exhibit light yellow coloration in the cephalothorax area and a generally pale or bleached appearance. The shrimp die within a few hours afterwards. By the following day, the number of similarly affected shrimp increases dramatically, and by the third day after the first appearance of moribund shrimp, the entire crop is typically lost (Chantanachookin et al., 1993; Lightner, 1996).

Several diagnostic methods are invented to detect YHV infection in shrimp, for example antibody (Lu et al., 1996; Nadala et al., 2000; Sithigorngul et al., 2000; Sithigorngul et al., 2002; Soowannayan et al., 2003), *in situ* hybridization (Tang et al., 1999; Tang et al., 2002; Spann et al., 2003) conventional RT-PCR (Wongteerasupaya et al., 1997; Cowley et al., 1999; Tang et al., 1999; Cowley et al., 2000) and real-time RT-PCR (Dhar et al., 2002).

1.2.3 Bacterial disease

Species of *Vibrio* are commonly found in aquatic environments as the bacterial flora and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). The virulence of this species has been recognized in a small but expanding list of penaeid cultures in Asia and Australia (Vandenberghe et al., 1998). The *Vibrio* spp., especially the luminous *V. harveyi*, has been implicated as the main

bacterial pathogens of shrimps (Baticados, 1990) *V. harveyi* is considered the most devastating cause of disease resulting in the extreme losses of cultured *P. monodon* in hatcheries and shrimp farms. This bacterial outbreak causes up to 100% mortality of the affected shrimps whether they are larvae, post-larvae, juveniles, sub-adults or adults (Lightner, 1983).

The *V. harveyi* is a rod shape, Gram-negative bacterium with 0.5-0.8 μm in width and 1.4-2.6 μm in length. This bacterium is able to emit a blue-green color light. Presumptive diagnosis can be made on the basis of clinical signs and culture of the suspensions of hepatopancreas or blood on trytic plate supplemented with 2% (w/v) NaCl. The colonies of *V. harveyi* show strong luminescence in dim light. Other gross features of the infected shrimps are the milky white body and appendages, weakness, disoriented swimming, lethargy and loss of appetite. Eventually, it leads to death. Antibiotics have been used in several attempts to control the bacteria but it might have led to the problems of drug resistance. The development and use of a probiotics, a marine bacterial strain *Pseudomonas* I-2, can alleviate the infection for it can produced a compound with inhibitory property against shrimp pathogen.

1.3   Immunity

All multicellular organisms need to protect themselves from the invasion of potentially harmful non-self substances. Traditionally, the immune system can be divided into two parts, innate and adaptive immunity (Kim, 2006).

1.3.1   Adaptive immunity

Adaptive immunity is a newcomer in the evolutionary scene. It appeared about 500 million years ago in vertebrates (Kim, 2006). The adaptive immune system

is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogenic challenges. Thought to have arisen in the first jawed vertebrates, the adaptive or "specific" immune system is activated by the "non-specific" and evolutionarily older innate immune system (which is the major system of host defense against pathogens in nearly all other living things). The adaptive immune response provides the vertebrate immune system with the ability to recognize and remember specific pathogens (to generate immunity), and to mount stronger attacks each time the pathogen is encountered. It is adaptive immunity because the body's immune system prepares itself for future challenges.

1.3.2 Innate immunity

The innate immune system is the first line of defense against bacterial, fungal, and viral pathogens (Hoebe et al., 2004) that helps to limit infection at an early stage. This defense system is essential for the survival and perpetuation of all multicellular organisms (Hoffmann et al., 1999; Salzet, 2001). The vertebrates possess both adaptive and innate immune system whereas the invertebrates have only the innate immunity. Nevertheless, the invertebrates survive in a pathogen-laden environment without an adaptive immune system. The recognition of pathogens depends on a limited number of germ-line encoded receptors, which recognize conserved pathogen-associated molecular patterns (PAMPs) found in microorganisms such as bacterial lipopolysaccharide (LPS), peptidoglycan and β-1, 3-glucan (Figure 1.2) (Janeway Jr, 1998). Innate immune responses include phagocytosis, complement, antimicrobial peptides and proteinase cascades, which lead to melanization and coagulation (Kim, 2006).
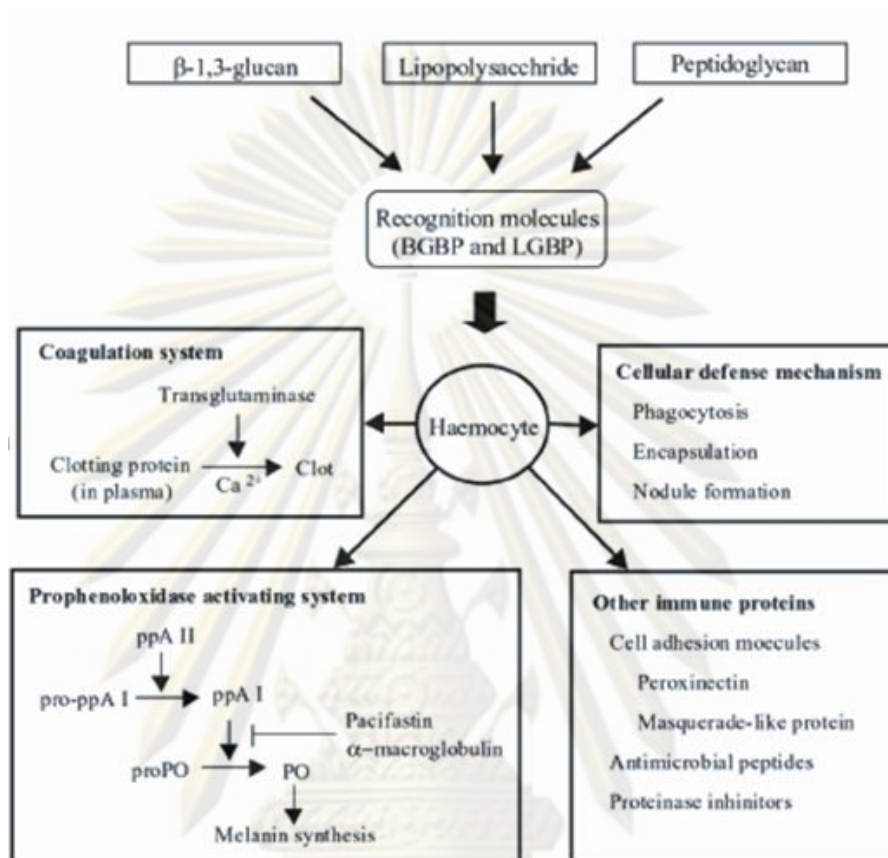
Figure 1.2 Schematic overview of crayfish defense reactions.

1.4 The crustacean immune system

The major defense systems of crustaceans are the innate immune response based on humoral and cellular components of the circulatory system. After pathogen infection, the recognition molecules may interact with and activate the haemocytes. Haemocytes are the effectors of the cellular immune response and they are also involved in the synthesis of the majority of humoral effectors (Figure 1.2). The direct participation of blood cells are demonstrated in phagocytosis, encapsulation, cell-

mediated cytotoxicity and clotting. The humoral factors comprise molecules that act in the defense without direct involvement of cells, although many of the factors are originally synthesized and stored in the blood cells such as clotting proteins, agglutinins (e.g. lectins), hydrolytic enzymes and antimicrobial peptides.

1.4.1 Cell-mediated defense reactions

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

1.4.2 The prophenoloxidase (proPO) system

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

In vitro studies have shown that phenoloxidase (PO) exists as an inactive precursor, prophenoloxidase (proPO), which is activated by a stepwise process involving serine proteases activated by microbial cell wall components, such as low quantities of lipopolysaccharides or peptidoglycans from bacteria and β-1,3-glucans from fungi, through pattern-recognition proteins (PRPs) (Ariki et al., 2004). An enzyme that is able to activate the proPO *in vivo* is termed prophenoloxidase activating enzyme (factor) (ppA, PPAE, PPAF) (Figure 1.3). In crayfish, ppA is a

trypsin-like proteinase present as an inactive form in the haemocyte granules. After degranulation, the enzyme is released together with proPO and becomes an active form in the presence of microbial elicitors. The active ppA will convert proPO to an active form, phenoloxidase (PO) (Aspan, 1991; 1995). PO is a copper-containing protein and a key enzyme in melanin synthesis (Soderhall et al., 1998; Shiao et al., 2001). It both catalyses O-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can polymerise non-enzymatically to melanin. PO is a sticky protein and can adhere to the surface of parasites, which will lead to melanisation of the pathogen.

Melanisation is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites, such as the crayfish plague fungus, *Aphanomyces astaci* (Soderhall et al., 1982). The production of forming insoluble melanin deposits involving in the process of sclerotisation, wound healing and encapsulation of foreign materials (Theopold et al., 2004). To prevent excessive activation of the proPO cascade, proteinase inhibitors are needed for its regulation.

The prophenoloxidase activating enzyme (PPA) is a zymogenic protein (proppA). The C-terminal half of the proppA is composed of a typical serine proteinase domain, with a sequence similar to other invertebrate and vertebrate serine proteinases. The N-terminal half contains a cationic glycine-rich domain, a cationic proline-rich domain and a clip-domain, in which the disulfide-bonding pattern is likely to be identical to those of the horseshoe crab big defensin and mammalian β-

defensins. The clip-domains in proppAs may function as antibacterial peptides (Wang et al., 2001).

In the penaeid shrimp, enzymes of the proPO system are localized in the semigranular and granular cell (Perazzolo et al.). This is in agreement with a recent study showing that *P.monodon* proPO mRNA is expressed only in the haemocytes (Sritunyalucksana et al., 2000).
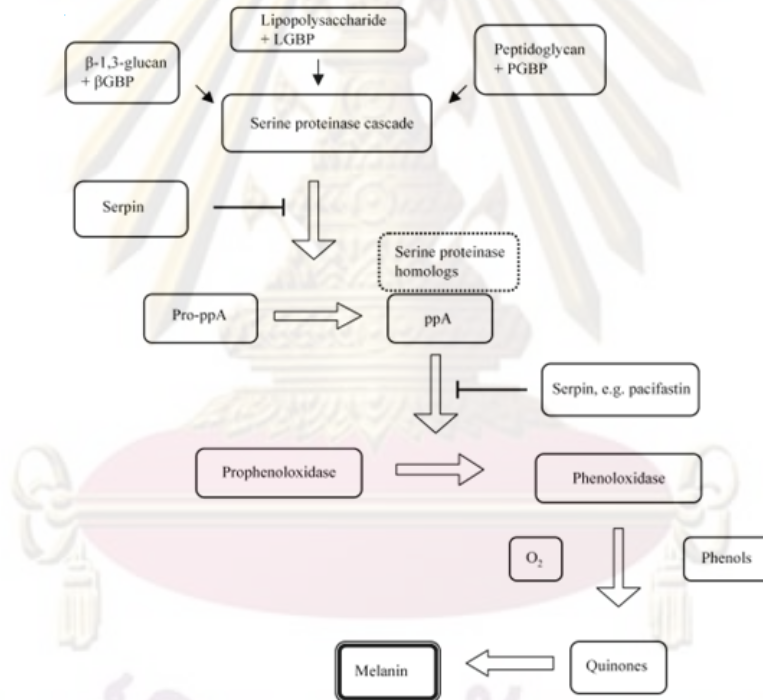
Figure 1.3   Overview of the arthropod prophenoloxidase (proPO)-activating system.

1.4.3   The coagulation system/the clotting system

Hemolymph coagulation is defense response of crustaceans that prevents both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al., 1991). It is a proteolytic cascade and is activated by microbial cell wall components. The coagulation system involves a plasma-clotting protein (CP) and a haemocyte-derived transglutaminase (TG) (Kopacek et al., 1993; Yeh et al., 1998). The crayfish CP is a dimeric protein, whose subunits have both free lysine and glutamine residue for covalently linking to each other by TGases. The CP is synthesized in the hepatopancreas and released into the hemolymph.

Transglutaminase (TG) are Ca^{2+}-dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on clotting protein molecules in the presence of calcium ion to form a soft gel at the wound sites (Wang et al., 2001). In addition, they have assumed a wide variety of functions during development, differentiation and immune responses and post-translational protein remodeling (Greenberg et al., 1991; Aeschlimann, 1994).

In shrimp, TG is important for blood coagulation and post-translation remodeling of proteins. Synthesized and stored in young haemocytes (hyaline and semigranular cell), TG facilitates the instant release of TG protein and blood clotting following injury (Aono et al., 1996; Huang et al., 2004). TG activity was greatest in the hepatopancreas, then the heart, haemocytes and other organs (Huang et al., 2004). It sequence is homologous with that of factor XIIIa in the coagulation system. Recently, a shrimp second TG (STG II) was found from the tiger shrimp haemocyte

cDNA. The STG II was characterized as a haemocyte TG that is involved in coagulation (Chen et al., 2005).

1.4.4 Antimicrobial peptides (AMPs)

The peptide antibiotics are defined as anti-microbial agents made by animals, including humans, with a function that is important for the innate immunity of that animal. Most of the AMPs are small in size, generally less than 150-200 amino acid residues, amphipathic structure and cationic property. However, the anionic peptides also exist. Their small size makes them easy to synthesize without dedicated cells or tissues and they rapidly diffuse to the point of infection. For many of these peptides, there is evidence that one of the targets for the peptide is the lipid bilayer of the membrane. This is because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the organism by rupturing the membrane or perturbing the membrane lipid bilayer that allows the leakage of certain cellular components as well as dissipating the electrical potential of the membrane.

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

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

1.5   Serine proteinases (SPs)

Serine proteinase (SP) is a group of endopeptidase that cleaved peptide bond in protein (Neurath, 1985) in which one of the amino acid at the active site is serine (Phillips et al., 1992). They are found in both single-cell and complex organisms, in both cells with nuclei (eukaryotes) and without nuclei (prokaryotes). In previous study, serine proteinases were originally digestive enzymes. Nowadays, serine proteinases have been studied intensively and their role in a wide array of vital physiological processes, such as food digestion, blood clotting, embryogenesis and immune response (complement activation), has been well documented. Many of these

processes are in essence proteolytic cascades, which, once 'turned on', lead very rapidly and irreversibly to a specific cellular response. As a consequence, the normal physiology of an organism is likely to be impaired if these proteolytic cascades are not well regulated. Therefore, most organisms synthesize a set of proteinase inhibitors, whose function is to prevent (regulate) unwanted proteolysis (Simonet et al., 2002a).

Serine proteases are grouped into clans that share structural similarities (homology) and are then further subgrouped into families with similar sequences. The major clans found in humans include the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase, and signal peptidase clans. The SPs belong to one of the four protease families. Generally SPs can be classified to 6 clans (Othman et al.). The six clans are clan A to clan F. Especially, clan A contains a family that share a common origin with chymotrypsin such as trypsin, elastase and the enzymes of blood clotting system (Barrett et al., 1995). These enzymes typically are synthesized in inactive forms which require activation by cleavage of a peptide bond near the N-terminus (Neurath, 1989). In chymotrypsin, this is between Arg-15 and Ile16; the free, protonated amino group of Ile16 is important for the mechanism. The amino-terminal peptide with residues 1 through 13 stays attached to the rest of the protein through a disulfide bond. In trypsin, the activation cuts off an amino-terminal hexapeptide, which does not remain attached (Neurath et al., 1976) whereas thrombin does not have its amino terminal domain attached by a disulfide bond and goes free in the plasma to attack fibrinogen and generate clots (Dunn et al., 1982).

1.5.1 Chymotrypsin-like

The three serine proteases of the chymotrypsin-like clan that have been studied in greatest detail are chymotrypsin, trypsin, and elastase. All three enzymes are synthesized by the pancreatic acinar cells, secreted into the small intestine, and are responsible for catalyzing the hydrolysis of peptide bonds. All three enzymes are similar in structure, as shown through their X-ray structures. They share closely similar structures (tertiary as well as primary). In fact, their active serine residue is at the same position (Ser-195) in all three. The differing aspect lies in the peptide bond that is being cleaved; this is called the scissile bond. The different enzymes, like most enzymes, are highly specific in the reactions they catalyze. Each of these digestive serine proteases targets different regions of a polypeptide chain, based upon the side chains of the amino acid residues surrounding the site of cleavage (Kurth et al., 1997; Hung et al., 1998).

Chymotrypsin is responsible for cleaving peptide bonds following a bulky hydrophobic amino acid residue. Preferred residues include phenylalanine, tryptophan, and tyrosine, which fit into a snug hydrophobic pocket. Trypsin is responsible for cleaving peptide bonds following a positively-charged amino acid residue. Instead of having the hydrophobic pocket of the chymotrypsin, there exists an aspartic acid residue at the base of the pocket. This can then interact with positively-charged residues such as arginine and lysine on the substrate peptide to be cleaved. Elastase is responsible for cleaving peptide bonds following a small neutral amino acid residue, such as alanine, glycine, and valine. (These amino acid residues form much of the

connective tissues in meat). The pockets in "trypsin" and "chymotrypsin" can partially accommodate these smaller amino acid residues rendering it a mere depression.

1.5.2 Subtilisin-like

Subtilisin is a serine protease secreted by the bacterium *Bacillus subtilis*. Subtilisins found in higher eukaryotes fall into two families: the pyrolisins and kexins. Subtilisin-like serine proteases or subtilases constitute a protease superfamily that is prevalent in various organisms such as archaea, protozoa, bacteria, yeast, vertebrates and plants (Hamilton et al., 2003) having diverse roles. Subtilases occur in distinct parts of plant ranging from seeds to fruits in various plant species such as melon (Yamagata et al., 1994), lily (Kobayashi et al., 1994). *Alnus glutinosa* (Ribeiro et al., 1995), *Arabidopsis thaliana* (Neuteboom et al., 1999), tomato (Tornero et al., 1996) and soybean (Nelsen et al., 2004). Subtilisin-like serine proteases have been associated with many physiological processes such as microsporogenesis (Taylor et al., 1997), hypersensitive response (Taylor et al., 1997), signal transduction (Yano et al., 1999), cell differentiation (Batchelor et al., 2000) and lateral root development (Neuteboom et al., 1999). In performing their respective roles, LIM9 in lily (Taylor et al., 1997), *Arabidopsis* AIR3 (Neuteboom et al., 1999) and tomato P69A (Tornero et al., 1996) use structural proteins in the cell wall as their substrates. Although it has the same mechanism of action as the serine proteases of mammals, its primary structure and tertiary structure are entirely different. Subtilisin is evolutionary unrelated to the chymotrypsin-clan, but shares the same catalytic mechanism utilizing a catalytic triad, to create a nucleophilic serine. This is the classic example used to illustrate convergent evolution, since the same mechanism evolved twice independently during

evolution. The structure of subtilisin has been determined by X-ray crystallography. It is a 275 residue globular protein with several alpha-helices and a large beta-sheet.

1.6 Mechanism of action of serine proteinases

The peptide bond is cleaved by nucleophilic attack of the serine hydroxyl group on the scissile carbonyl bond, forming an acyl enzyme intermediate (Figure 1.4). The carbonyl carbon of this bond is position near the nucleophilic serine. The serine-OH attacks the carbonyl carbon, and the nitrogen of the histidine accepts the hydrogen from the -OH of the serine and a pair of electrons from the double bond of the carbonyl oxygen moves to the oxygen. As a result, a tetrahedral intermediate is generated. The bond joining the nitrogen and the carbon in the peptide bond is now broken. The covalent electrons creating this bond move to attack the hydrogen of the histidine, breaking the connection. The electrons that previously moved from the carbonyl oxygen double bond move back from the negative oxygen to recreate the bond, generating an acyl-enzyme intermediate. Now, water comes into the reaction. Water replaces the N-terminus of the cleaved peptide, and attacks the carbonyl carbon. Once again, the electrons from the double bond move to the oxygen making it negative, as the bond between the oxygen of the water and the carbon is formed. This is coordinated by the nitrogen of the histidine. This accepts a proton from the water. Overall, this generates another tetrahedral intermediate.

In a final reaction, the bond formed in the first step between the serine and the carbonyl carbon moves to attack the hydrogen that the histidine just acquired. The now electron-deficient carbonyl carbon re-forms the double bond with the oxygen. As

a result, the C-terminus of the peptide is now ejected. In trypsin, the catalytic triad is composed of Ser195, His57 and Asp102 (Phillips et al., 1992).
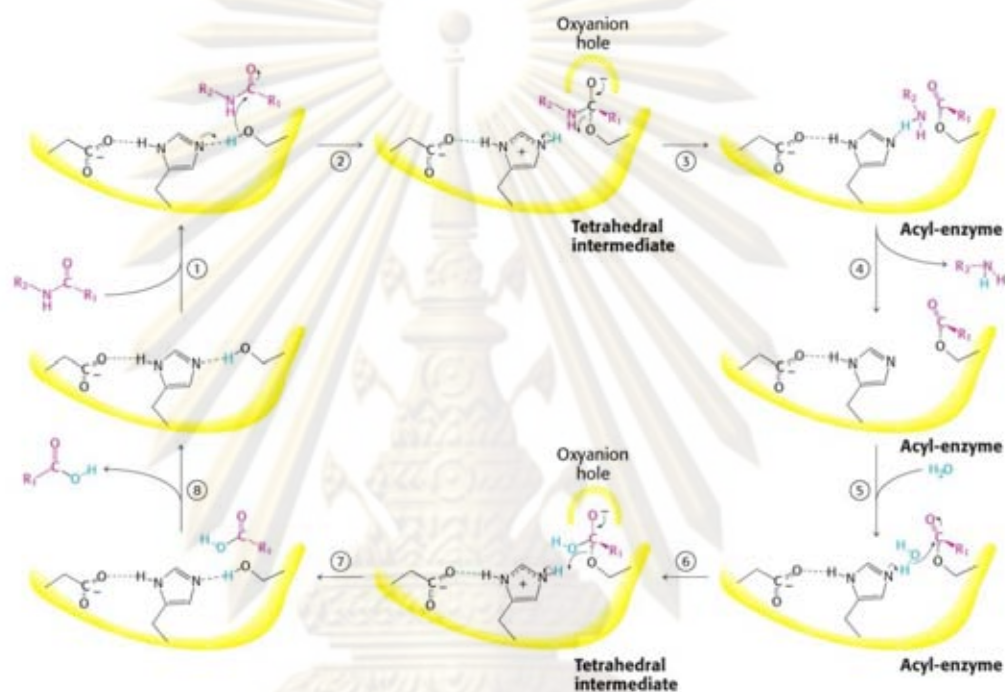
Figure 1.4 A detailed mechanism for the chymotrypsin-like SP reaction.
(Source: http://www.bmolchem.wisc.edu/courses/spring503/503-sec1/503-3a.htm)

1.7   Proteinase inhibitor

In multicellular organisms, serine proteinase inhibitors (SPIs) are essential factors involving in controlling the various proteinase mediated biological processes, such as the complement system, blood coagulation, melanization, apoptosis, etc. (Iwanaga et al., 2005; Jiravanichpaisal et al., 2006). Not only do they control the extent of deleterious protease digestion in such processes, they potentially fight as

part of the humoral defence of the innate immune system against the invading pathogens (Christeller, 2005). Injury and microbial infection in vertebrates lead to activation of the blood coagulation and proPO systems. Both of these systems employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides) resulting in rapid and efficient responses to the threats to health (O'Brien, 1993; Whaley, 1993). Blood clotting and phenoloxidase activation can also be harmful to the host if they are not limited as local and transient reactions. For this reason the proteinases in these systems are tightly regulated by proteinase inhibitors.

The SPIs are also involved in direct defense against proteinases from invading pathogens. For example, a subtilisin inhibitor, BmSPI, from *Bombyx mori* might function as an inhibitor to the microbial proteases and protected the silkworm pupae from infection by pathogens (Zheng et al., 2007). Some microbial pathogens and parasites use the SPIs to counterdefense the host protective proteinases. For example, the oomycete *Phytophthora infestans*, a cause of disease in potato and tomato, produces an extracellular protease inhibitor to counter-defense the plant defensive proteinases (Tian et al., 2004; Tian et al., 2005). The obligate intracellular parasite of human *Toxoplasma gondii* produces a serine protease inhibitor to protect itself from the digestive enzymes during its residency in small intestine (Morris et al., 2002).

Some other SPIs are involved in reproductive processes. A male reproduction-related SPI is isolated from *Macrobrachium rosenbergii* with inhibitory activity on sperm gelatinolytic activity (Li et al., 2009). Another reproductive SPI was

from the turkey male reproductive tract (Slowinska et al., 2008). For haematophagous insects such as *Dipetalogaster maximus* and *Triatoma infestans*, they secrete potent thrombin inhibitors dipetalogastin and infestin, respectively, to prevent blood clotting during blood meal (Campos et al., 2002; Mende et al., 2004).

Based on the primary and three-dimensional structures, topological functional similarities (Laskowski et al., 1988; Bode, 1992) and inhibition mechanisms, the proteinase inhibitors are classified into at least 18 families according to Laskowski and Qasim (Laskowski Jr et al., 2000). Among them, the following six families: Kazal, BPTI-Kunitz, α-macroglobulin, serpin, pacifastin and bombyx (Pham et al., 1996; Kanost, 1999; Simonet et al., 2002b) have been described in invertebrate haemolymph or also in saliva. Although the primary structure, with the number of amino acids ranging from 29 to approximately 400, and the structural properties of these inhibitors differ significantly, only two fundamentally different inhibiting mechanisms exist. Most inhibitors bind to their cognate enzyme(s) according to a common, substrate-like standard mechanism. They are all relatively small (from 29 to 190 amino acids) and share an exposed, rigid binding loop with a very characteristic 'canonical' conformation (Laskowski et al., 1988; Bode, 1992).

One of the well known SPIs is the Kazal-type SPIs which are grouped into family I1 (http://merops.sanger.ac.uk/) (Rawlings et al., 2004; Rawlings et al., 2008). The Kazal inhibitors are usually multi-domain proteins containing more than one Kazal domain. Each domain of 50–60 amino acid residues contains six well-conserved cysteine residues capable of forming three intra-domain disulphide bridges resulting in a characteristic three-dimensional structure (Van de Locht et al., 1995).

Each domain binds tightly and competitively via its reactive site loop to the active site of cognate proteinase rendering the proteinase inactive. Structural studies reveal that there are several contact positions responsible for the interactions between Kazal domains and the proteinases (Lu et al., 1997; Bode et al., 2000). However, the inhibitory specificity is determined mainly by the P1 amino acid residue resided at the second amino acid residue after the second cysteine residue of the domain.

1.8   Objectives of the thesis

- To identify the serine proteinase inhibitors from the *Penaeus monodon* EST database.

- To determine the expression of Kazal-type serine proteinase inhibitor genes in response to Gram-negative bacteria, *V. harveyi* 639, challenge.

- To determine the expression of SPI*Pm*5 gene in response to heat stress.

- To characterize two two-domain Kazal-type serine proteinase inhibitors, SPI*Pm*4 and 5.

CHAPTER II

MATERIALS AND METHODS

2.1   Materials

2.1.1   Equipments

96-well plate (Bio-RAD)

Amicon Ultra-4 concentrators (Millipore)

Autoclave model # LS-2D (Rexall industries Co. Ltd., Taiwan)

Automatic micropipettes P10, P100, P200 and P1000 (Gilson Medical

Electrical S.A., France)

Balance Satorius 1702 (Scientific Promotion Co.)

Gel documentation (SYNGENE)

Gene pulser (Bio-RAD)

Hyperfilm MP (Amersham International, England)

Incubator 37 ºC (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets Class II Model NU-440-400E

(NuAire, Inc., USA)

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

Microtiter plate reader (BMG Labtech)

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

PCR thermal cycler: Gene Amp PCR System 2400 (Perkin Elmer)

PCR thermal cycler: DNA Engine (MI Research, USA)

PCR thin wall microcentrifuge tubes 0.2 ml (Perkin Elmer)

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

PD-10 column (GE Healthcare)

pH meter model # SA720 (Orion)

Pipette tips 10, 20, 200, and 1000 µl (Bio-RAD Laboratories, USA)

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

Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Sterring hot plate (Fisher Scientific)

Transilluminator 2011 Macrovue (LKB)

Trans-Blot® SD (Bio-Rad)

Vacuum blotter Model # 785 (Bio-RAD Laboratories, USA)

Vacuum pump (Bio-RAD Laboratories, USA)

Vertical electrophoresis system (Hoefer™ miniVE)

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

England)

White/UV transilluminator: UVP ImageStore 7500 (Mitsubishi Electric

Corporation, Japan)

2.1.2 Chemicals and Reagents

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

2-Mercaptoethanol, C_2H_6OS (Fluka)

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

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

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

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

Agarose (Sekem)

Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson

ImmunoResearch Laboratories, Inc.)

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

Anti-His antiserum (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast ext ract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bovine serum albumin (Fluka)

Bromophenol blue (Merck, Germany)

Calcium chloride, $(CaCl_2)$ (Merck)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

Dextran sulfate (BioBasic Inc.)

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

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

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

Dithiothreitol (Pharmacia)

Ethidium bromide (Sigma)

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

Formaldehyde (BDH)

GeneRuler™ 100bp DNA ladder (Fermentas)

Glycerol, $C_3H_8O_3$ (BDH)

Isoamylalcohol (Merck)

Isopropanol (Merck)

Isopropyl-β-D-thiogalactoside (IPTG)

N, N'-methylene-bisacrylamide, $C_7H_{10}N_2O_2$ (USB)

Nytrans® super charge nylon membrane (Schleicher & Schuell)

Phenol crystals, C_6H_5OH (Carlo Erba)

RNase A (Sigma)

RNA markers (Promega)

Sodium acetate, CH_3COONa (Merck)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

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

Tryptic soy broth (Difco)

Trizol reagent (Gibco BRL)

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

X-Gal (5-Bromo-4-Chloro-3-Idolyl-β-D-Galactopyranose)

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

2.1.3 Kits

ImProm-II™ Reverse Transcription system kit (Promega)

NucleoSpin® Extract II kit (Macherey-Nagel)

QIAprep spin miniprep kit (Qiagen)

RevertAid™ First Strand cDNA Synthesis Kits (Fermentas)

T & A Cloning vector kit (RBC)

2.1.4 Enzymes

Ampli*Taq* DNA polymerase (Fermentas)

*Bam*HI (Biolabs)

α-chymotrypsin, bovine pancreas (Sigma)

DyNAzyme_II DNA Polymerase (Finnzymes)

Elastase, porcine pancreas (Pacific Science)

Eco RI (Biolabs)

Enterokinase, light chain (Biolabs)

*Hin*dIII (Biolabs)

*Nco*I (Biolabs)

*Not*I (Biolabs)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

*Sal*I (Biolabs)

subtilisin Carlsberg, *Bacillus licheniformis* (Sigma)

T4 DNA ligase (Fermentas)

Trypsin, bovine pancreas (Sigma)

*Xho*I (Biolabs)

2.1.5   Substrate

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

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

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

2.1.6   Antibiotic

Ampicillin

Chloramphenicol

Kanamycin

Tetracycline

2.1.7   Bacterial strains

Bacillus megaterium

Bacillus subtilis

Escherichia coli JM109

Escherichia coli XL-1 Blue MRF'

Escherichia coli Rosetta(DE3)pLysS

Staphylococcus aureus

Vibrio harveyi 639

2.1.8 Software

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

Clustal X (Thompson, 1997)

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

GENETYX version 7.0 program (Software Development Inc.)

SECentral (Scientific & Educational Software)

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

SMART (http://smart.embl-

heidelberg.de/smart/set_mode.cgi.GENOMIC=1)

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

2.1.9 Vector

pET28b(+)

pVR500, a pET32a(+) derivative

2.2 Mining the *Penaeus monodon* EST database

The EST and contig pages in the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) were searched for the Kazal-type serine proteinase inhibitors. The nucleotide sequences of the contigs and singletons were analyzed for the open reading frames and the encoded amino acid sequences. ClustalX was used to align the sequences from different contigs and EST clones (Chenna et al., 2003). SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the

signal cleavage sites (Nielsen et al., 1997). The Gentyx version 7.0 was used to estimate the isoelectric point (pI) and molecular weight. The EST clones containing the entire open reading frames of the interesting contigs were re-sequenced to ensure the correctness of the sequence data.

2.3   General procedures

2.3.1   Quantitative method for DNA Determination

The concentration of DNA fragment was determined by measuring the OD at 260 nm and estimated in μg/ml using an equation: [DNA] (μg/ml) = OD_{260} × dilution factor × 50 for an OD at 260 nm corresponds to 50 μg/ml of DNA (Sambrook et al., 1989).

2.3.2   Primer design

PCR primer pairs were designed based on nucleotide sequences of the template DNA using the SECentral program (Scientific & Educational Software). Each primer in the pair should have about the same T_m values. They were checked for minimal self-priming and primer dimer formation. A housekeeping gene, β-actin was generally used as an internal control.

2.3.3   Plasmid DNA extraction using QIAprep® miniprep kit

The plasmid was isolated from the bacterial cell culture using a QIAprep® Miniprep kit. A recombinant plasmid was inoculated into 2 ml of Luria-Bertani (LB) medium containing appropriate antibiotic, usually 50 mg/ml of ampicillin and cultured overnight at 37 ºC. The culture was centrifuged at 800g for 3 minutes. The supernatant was removed. Bacterial cells were resuspended in 250 μl Buffer P1 containing RNase A. The 250 μl Buffer P2 was added and mix thoroughly by

inverting the tube 4–6 times to lyse the cells. The cell lysate was neutralized by adding 350 μl N3 buffer. After maximum speed centrifugation for 10 min, the supernatant containing the plasmid was applied to a QIAprep spin column by pipetting. The column was centrifuged for 30-60 s and the flow-through was discarded. The column was washed twice with 0.5 ml Buffer PB and 0.75 ml Buffer PE, respectively, and then centrifuged to remove residual ethanol from Buffer PE. Finally, the plasmid DNA was eluted by adding 30 μl EB buffer to the center of each column, incubating at room temperature for 1 min and centrifugation for 1 min. The flow-through containing the plasmid was then stored at -20 ºC until use.

2.3.4 Determination of protein concentration

The protein content was measured according to Bradford method (Bradford, 1976) using bovine serum albumin (Fluka) as a standard. This method is base on the binding of Coomassie brilliant blue G 250 dye to proteins converting the red dye color to blue. A sample solution of 100 μl was mixed with 1 ml Bradford working buffer and left for 2 min before the absorbance at 595 nm was measured. The Bradford working buffer (100 ml) was a mixture of 6 ml Bradford stock solution (350 g Coomassie blue G250, 100 ml 95% ethanol and 200 ml 85% phosphoric acid), 3 ml 95% ethanol, 6 ml 85% phosphoric acid and 85 ml distilled water.

2.4 Preparation of shrimp

Sub-adult *P. monodon*, approximately 3 month-old, 15 g of body weight, were purchased from local farms. The shrimp were acclimatized in aquaria at the ambient temperature (28 ± 4 ºC) and at the salinity of 15 ppt for at least 1 day before used in the experiments.

2.4.1 Detection of possible *V. harveyi* and WSSV infection

Five individual shrimp (1%) were sampling from the hatchery tank for possible *V. harveyi* and WSSV infection. The gill samples were briefly homogenized with a pestle in 0.2 ml of lysis buffer [2.5 N NaOH and 10% (w/v) SDS]. The homogenate was boiled for 10 min, incubated on ice for 3 min and centrifuged at 8,000g for 10 min at 4 ºC. Then, the supernatant was diluted 100-fold with water and used for PCR amplification.

For *V. harveyi*, the samples were amplified with a *gyr*B specific primer pair, FA2: 5' TCTAACTATCCACCGCGG 3' and RB3: 5' AGCAATGCCATCTTCACGTTC 3', gaving an amplicon of 363 bp. The PCR reaction consisted of 2 µl of DNA template, 1.6 µl of 2.5 mM dNTPs, 5 µl of each of the primers (2 µM), 0.1 µl of *Tag* polymerase (5 U/µl) (Fermentas), 2 µl of 10× PCR buffer, 2 µl of 2.5 mM MgCl₂ and 2.3 µl of distilled water. The amplification condition was 30 cycles of amplification consisting of denaturation at 94 ºC for 1 min, annealing at 63 ºC for 1 min, and extension at72 ºC for 2 min, followed by 1 cycle of 72 ºC for 7 min. Amplification was performed in a Perkin–Elmer Cycler (Thaithongnum et al., 2006).

For WSSV, a specific primer pair, FWSSV: 5' AGAGCCCGAATAGTGTTTCCTCAGC 3' and RWSSV: 5' AACACAGCTAACCTTTATGAG 3', were used for PCR detection. The reaction was performed in a 25 µl reaction volumn containing 1 µl of the sample, 0.5 µl of 10 mM dNTPs, 0.5 µl of each of the primers (10 mM), 0.2 µl of Tag polymerase (5 U/µl), 2.5 µl of 10× PCR buffer, 2.5 µl of 10 mM MgCl₂ and 17.3 µl of distilled water. The PCR

program was as follows: 90 ℃ for 2 min and 35 cycles of denaturation at 90 ℃ for 30

s, annealing at 60 ℃ for 30 s, and extension at72 ℃ for 30 s, followed by 1 cycle of

72 ℃ for 5 min. Amplification was performed in a DNA Engine (MI Research). The

PCR product of 8 μl was resolved by electrophoresis in 1.5% agarose gel.

2.4.2   Preparation of *V. harveyi* 639 for injection

V. harveyi 639 stock was streaked on a tryptic soy agar (TSA) plate

supplemented with 2% (w/v) NaCl and grown overnight at 30 ℃. A single colony was

inoculated into the tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl and

incubated at 30 ℃ with shaking at 250 rpm for 12-16 h at 30 ℃. The overnight

culture was diluted 1:100 into fresh TSB supplemented with 2% (w/v) NaCl. The

culture was grown at 30 ℃ with 250 rpm shaking until the A_{600} was 0.6 where the cell

density was 10^8 CFU/ml. The titer of this dilution was moniterd by a plate count

method in tryptic soy agar (TSA) supplemented with 2% (w/v) NaCl.

The *P. monodon* were divided into 5 groups of two individual. The first

group, the control group, was intramuscularly injected with 100 μl of sterile 0.85%

(w/v) NaCl. The second to fifth groups were intramuscularly injected with 100 μl of

10^5, 10^6, 10^7 and 10^8 CFU/ml *V. harveyi*. The mortality rates were observed for 24 h.

2.4.3   Preparation of *V. harveyi* 639 infected shrimp (modified from Roque et al., 1998)

The shrimp were separated into 2 groups, the unchallenged (normal) and

the challenged shrimp. The latter was experimentally infected with *Vibrio harveyi* by

intramuscularly injecting 100 μl of 10^6 CFU/ml diluted culture into the 4th abdominal

segment whereas the control was injected with 100 μl of 0.85% (w/v) NaCl solution.

At 0, 6, 12, 24 and 48 h post-injection, Haemolymph was collected and shrimp were tested for the infection by culturing the suspension of hepatopancreas on TCBS (thiosulfate-citrate-bile salts-sucrose agar) (Difco) plates supplemented with 2% (w/v) NaCl and incubated at 30 ºC overnight. Colonies of *V. harveyi* from the infected shrimps should show strong luminescence in the dark.

2.5   cDNA synthesis

2.5.1   Tissue collection and total RNA preparation

Gill, epipodite, antennal gland, heart, hepatopancreas, intestine, lymphoid, and eyestalk were isolated separately from an individual normal shrimp and immediately frozen in liquid nitrogen (-176 ºC). Haemocytes were prepared from Haemolymph. Haemolymph was collected from the ventral sinus of shrimp at 0, 6, 12, 24 and 48 h post-injection using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 µl of anticoagulant [10% (w/v) sodium citrate]. Haemolymph was immediately centrifuged at 8,000*g* for 10 min at 4 ºC to separate Haemocytes from the plasma. The tissue and haemocyte samples were briefly homogenized by a pestle in 1 ml of ice-cold Trizol reagent (Gibco BRL). The homogenate was stored at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. Then, 200 µl of chloroform was added, vigorously shaken for 15 s, incubated at room temperature for 2-5 min and centrifuge at 12,000*g* for 15 min at 4 ºC. The colorless upper aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube. RNA was precipitated with 500 µl of isopropanol. The mixture was left at room temperature for 5-10 min and centrifuged at 12,000*g* for 10 min at 4 ºC. The supernatant was removed. The RNA pellet was washed with 500 ml of 75%

ethanol. The RNA pellet was kept in 75% ethanol until use. When required, the samples were centrifuged at 12,000g for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was briefly air-dried for 5-10 min. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water.

2.5.2 Determination of the quantity and quality of RNA samples

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

The concentration of total RNA could be determined in ng/μl using the following formular: $[RNA] = A_{260} \times$ dilution factor $\times$ 40 for an OD at 260 nm corresponds to 40 ng/μl of RNA (Sambrook et al., 1989).

The relative purity of RNA samples was examined by measuring the ratio of $A_{260/280}$. The maximum absorption of protein is at 280 nm. The good quality RNA sample should have an $A_{260/280}$ ratio above 1.7.

The quality of the extracted RNA was analyzed by formaldehyde-agarose gel electrophoresis. A 1.0% (w/v) formaldehyde agarose gel was prepared in 1× MOPS buffer (0.2 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0). The gel slurry was boiled until completely solubilized and allowed to cool to 60 ºC. Formaldehyde (0.66 M final concentration) and ethidium bromide (0.2 μg) were added to the gel and poured into a gel caster.

About 10-20 μg of total RNA in 3.5 μl of DECP-treated H_2O, 5 μl of formamide, 1.5 μl of 10× MOPS and 2 μl of formaldehyde were mixed well and incubated at 70 ºC for 10 min. The mixture was immediately placed on ice. One-forth

volume of the gel-loading buffer [50% (v/v) glycerol, 1 mM EDTA, pH 8.0, 0.5% (w/v) bromophenol blue] was added to each sample. The sample was loaded to the 1.0% agarose gel containing formaldehyde. The RNA marker was used. Electrophoresis was carried out in 1× MOPS buffer at 100 volts until the bromophenol blue front was migrated to approximately ¾ of the gel length. The gel was washed 4-5 times of water before stained with ethidium bromide (0.5 μg/ml) for 1 h. The total RNA was visualized as fluorescent bands under a UV transilluminator.

2.5.3   DNase treatment of total RNA samples

The obtained total RNA was further treated with RQ1 RNase-free DNase (Promega) (1 unit/5 μg of total RNA) at 37 ºC for 30 min to remove the contaminating chromosomal DNA. Then, the RNA were purified by phenol/chloroform extraction following by ethanol precipitation. Briefly, the reaction was adjusted to 40 μl with DEPC-treated water, added 250 μl of Trizol reagent, vortexed for 10 s, added 200 μl of chloroform and vigorously shaken for 15 s. The resulting mixture was kept at room temperature for 2-5 min and centrifuged at 12,000g for 15 min at 4 ºC. The RNA in the upper phase was separated, precipitated with isopropanol and washed with 70% (v/v) ethanol. The RNA pellet was briefly air-dried and dissolved with an appropriate amount of DEPC-treated water. The concentration of DNA-free total RNA was determined.

2.5.4   First-strand cDNA synthesis

The first strand cDNA was synthesized from 1 μg of total RNA using an ImProm-II™ Reverse Transcription System kit (Promega). Total RNA was combined with 0.5 μg of oligo(dT)$_{15}$ primer and appropriate DECP-treated water in a volume of

8 μl, annealed at 70 ºC for 5 min and immediately placed on ice for 5 min. Then, 4 μl of 5× reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of dNTP mix (10 mM each), 20 units of ribonuclease inhibitor and 1 μl of ImProm-II reverse transcriptase were added and gently mixed. The reaction mixture was incubated at 25 ºC for 5 min and at 42 ºC for 60 min. Then, the reaction was incubated at 70 ºC for 15 min to terminate the reverse transcriptase activity. All cDNA samples were stored at -20 ºC until use.

Occasionally, the RevertAid™ First Strand cDNA Synthesis kit from Fermentas was used instead. Briefly, 1 μg of total RNA templates were mixed with 0.5 μg of the oligo(dT)$_{18}$ primer and appropriate DECP-treated water in a total volume of 12 μl and annealed at 65 ºC for 5 min. After on ice for 5 min, 4 μl of 5× reaction buffer, 1 μl of RiboLock™ RNase inhibitor (20 U/μl), 2 μl of dNTP mix (10 mM each) and 1 μl (200 U/μl) of RevertAid™ M-MuLV reverse transcriptase were added and gently mixed. The reaction mixture was incubated at 42 ºC for 60 min. The reaction was terminated at 70 ºC for 15 min.

2.6 Semi-quantification of Kazal-type serine proteinase inhibitor mRNA expression by RT-PCR

Semi-quantitative RT-PCR was used to examine tissue specific expression and expression pattern in response to *V. harveyi* infection of seven Kazal-type serine proteinase inhibitors mRNA. Total RNA from shrimp tissues was extracted and then subjected to cDNA synthesis as described above. The β-actin gene expression was used as the internal control. The PCR conditions were set up and optimized.

2.6.1 Determination of PCR conditions

Each PCR reactions were carried out in a total volume of 25 μl containing 10 mM Tris–HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, optimal concentration of $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, or 0.2 mM of each primer for β-actin, 1.5 unit of DyNAzyme_II DNA Polymerase (Finnzymes) and an optimal concentration of template cDNA or 3 μl of 10-fold diluted template cDNA for β-actin using a DNA Engine (MI Research). The reactions were predenatured at 94 °C for 2 min followed by optimal cycles or 25 cycles for β-actin of denaturation at 94 °C for 30 s, annealing at an optimal temperature or 52 °C for β-actin for 30 s and extension at 72 °C for 1 min. The final extension was at 72 °C for 5 min. The PCR products were analyzed using a TBE-2% agarose gel electrophoresis. The PCR parameters were optimized as follows: 1) The optimal $MgCl_2$ concentration between 0.5-3.0 mM $MgCl_2$ was examined using the standard PCR conditions. The concentration that gave the highest yield and specificity was chosen. 2) The PCR amplifications were carried out at different cycle numbers including 20, 24, 28, 32, 36 and 40. After amplifications, PCR products were run on 2% agarose gel. The number of cycles that amplified the PCR product in the exponential range and did not reach a plateau level was chosen. 3) The optimal amount of cDNA template were examined using the standard PCR condition. The concentration that gave the highest yield and specificity was chosen. 4) The optimal primer annealing temperature was adjusted to increase amplification specificity of each primer pair. Annealing temperatures that gave the best results was used.

The primer sequences, annealing temperatures and cycle numbers for RT-PCR are shown in Table 3.2. The PCR amplification was carried out in a DNA Engine (MI Research). The reactions were performed in triplicate. The PCR products were analyzed by electrophoresis on 2% agarose gels.

2.6.2 Agarose gel electrophoresis and quantitative analysis

The 2% (w/v) agarose gel was prepared using 1× TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). The slurry of agarose in 1× TBE buffer was melted in a microwave oven until completely dissolved. The solution was allowed to cool at room temperature to 60 ℃ before pouring into a casting tray with a well-forming comb. The gel was submerged in a chamber containing enough amount of 1× TBE buffer for covering the gel.

The PCR products were mixed with one-sixth volume of 6× loading dye (0.25% bromophenol blue and 25% Ficoll in water) before loading into the well. A DNA ladder (100 bp marker) was used as standard DNA markers. Electrophoresis was carried out in 1× TBE buffer at 100 volts for 25 min. The gel was stained in a 2.5 μg/ml ethidium bromide (EtBr) solution for 1 min and de-stained to remove excess EtBr by washing with distilled water for 15 min. The PCR product was visualized under a UV transilluminator and photographed.

The intensity of bands was measured and normalized relative to that of β-actin using the commercial image analysis software package (GeneSnap and GeneTools, SynGene). The data were then subjected to statistical analysis for comparison between groups. Significantly different expression levels were treated

using One Way Analysis of Variance (ANOVA) following by a post t hoc test (Duncan's new multiple range test). Significant differences were indicated at $p < 0.05$.

2.7   Expression analysis of SPI*Pm*5 using RT-PCR

Three-month-old subadult black tiger shrimp, *P. monodon*, of about 15–20 g weight, were obtained from a local farm and acclimatized in aquaria at an ambient temperature of about 28 ± 1 ºC and a salinity of 15 ppt for a few days before the experiments. The shrimp were divided into two groups, control and heat treated groups. Each group consisted of 3 subgroups of 3 individuals. The heat-treated group was immediately put and reared for 1 h in an aquarium with warm water of 33 ± 1 ºC. Then, the Haemolymph was collected from the shrimp ventral sinus into an anticoagulant solution of 10% (w/v) trisodium citrate dihydrate, pH 4.6. Haemocytes were pelleted by centrifugation, resuspended in TRI Reagent® (Molecular Research Center), homogenized and total RNA isolated. The equivalent amounts of total RNA preparations from individuals in each subgroup were pooled and treated with RNase-free DNase I (Promega) to remove contaminated genomic DNA. The concentration and integrity of total RNA were assessed by UV spectrophotometry and agarose gel electrophoresis. First-strand cDNAs were synthesized from 1 µg of total RNA samples using the First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's protocol. RT-PCR analysis was carried out using the SPI*Pm*5 specific primers: FSPIPm5, 5' TGGAACGGACGGCAAGACAT 3' and RSPIPm5, 5' GTCGTGACAGTCTTGAGTCC 3'. The β-actin gene was used as an internal control using the gene specific primers designed according to the shrimp actin cDNA sequence (GenBank accession no. DW042525): actinF, 5'

GCTTGCTGATCCACATCTGCT 3' and actinR, 5' ATCACCATCGGCAACGAGA

3'. Each PCR reactions were carried out in a total volume of 25 ml containing 10 mM

Tris–HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 2 mM $MgCl_2$ for

SPI*Pm*5 or 1 mM for β-actin, 0.2 mM of each dNTP, 0.4 mM of each primer for

SPI*Pm*5 or 0.2 mM of each primer for β-actin, 1.5 unit of DyNAzyme_II DNA

Polymerase (Finnzymes) and 3 ml of undiluted template cDNA for SPI*Pm*5 or 3 ml of

10-fold diluted template cDNA for β-actin. The reactions were predenatured at 94 ºC

for 2 min followed by 40 cycles for SPI*Pm*5 or 25 cycles for β-actin of denaturation at

94 ºC for 30 s, annealing at 60 ºC for SPI*Pm*5 or 52 ºC for β-actin for 30 s and

extension at 72 ºC for 1 min. The final extension was at 72 ºC for 5 min. The PCR

products were analyzed using a TBE-2% agarose gel electrophoresis.

2.8   Recombinant protein expression for SPI*Pm*4 and 5

2.8.1   Construction of the protein expression clones

To express the recombinant SPI, two primer pairs, FPm4: 5'
AAGGCCATGGGAAAGGGGGGGGATTCTCGACT   3'   and   RPm4:   5'
CCTTCTCGAGATATCCCGTCTTCCTGTCAA   3'   for   SPI*Pm*4;   FPm5:   5'
AAGGCCATGGGAAAAGGAGGCAAATTCAGACT   3'   and   RPm5:   5'
CCTTCTCGAGATATCCCTTCTTGATAGGCG 3' for SPI*Pm*5, were designed. The

primers were designed for the amplification of gene fragment encoding the mature

proteins without signal peptides. A pBlueScript SK plasmids containing SPI genes

were used as template. The included *Nco*I and *Xho*I sites (underlined), respectively, at

the 5' and 3' ends of the gene fragments were for cloning into the expression vector.

For convenience, the EST clones from the normalized haemocyte library, HC-N-N01-

4898-LF (GenBank accession no. GO269555) and HC-N-N01-2619-LF (GenBank accession no. GO269556) containing the complete open reading frames of SPI*Pm*4 and 5, respectively, were used as PCR templates. The SPI*Pm*4 and 5 gene fragments were PCR amplified in a final reaction volume of 50 μl containing 0.02 ng of plasmid template, 0.4 M of each primer, 0.2 mM of each dNTP and 3 units of *Pfu* polymerase (Promega). The PCR amplification was carried out at 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 1 min, and final extension at 72 ºC for 7 min. The amplified products were analyzed using 1.2% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). The purified DNA fragments were tailed with an adenine nucleotide for ligation into the T&A cloning vector (Real Biotech Corporation). The resulting T&A clones were isolated and subjected to nucleotide sequencing to confirm the insert sequences of SPI*Pm*4 and 5. The *Nco*I–*Xho*I fragments containing the SPI*Pm*4 and 5 were prepared from the T&A clones and subcloned into the *Nco*I–*Xho*I digested pET-28b(+) expression vector (Novagen). For some reasons, the SPI*Pm*4 was not expressed well; it was then subcloned into a pET-32a(+) derivative, pVR500, and expressed as fusion protein to the thioredoxin. The expression clones were named pSPI*Pm*4 and 5.

2.8.2 Purification of PCR product from agarose gel

The PCR product was purified from the agarose gel by NucleoSpin® Extract II kit (MACHEREY-NAGEL). The PCR band was excised from the agarose gel with a clean sharp scalpel. Extra agarose was removed to minimize the size of the gel slice. The weight of the gel slice was determined and transferred to a clean 1.5

microcentrifuge tube. For each 100 mg of agarose gel, 200 µl of NT buffer was added into the tube. The sample was incubated at 50 ºC with occasional vortexing until the gel pieces were dissolved (5-10 min). A NucleoSpin extract column was placed into a 2 ml collecting tube. The sample was loaded into the column and centrifuged at 10,000g for 1 min. The flow-through was discarded and the NucleoSpin® extract column was back into the collection tube. The membrane was washed with 600 µl of NT3 buffer and centrifuged at 10,000g for 1 min. The flow-through was discarded. The silica membrane was dried by centrifugation at 10,000g for 2 minute for completely removal of NT3 buffer. The column was placed into a clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 50 µl of elution buffer NE (5 mM Tris-Cl, pH 8.5) into the column, leaving at room temperature for 1 minute to increase the yield of eluted DNA and centrifugation. The flow-through containing the DNA fragment was, then, stored at -20 ºC until use.

2.8.3 A-tailing procedure for blunt-ended PCR fragments

Thermostable DNA polymerases with proofreading activity, such as *Pfu* DNA polymerase generate blunt-ended fragments during PCR amplification. Nevertheless, the PCR fragment can be modified using the A-tailing procedure for cloning into the T&A cloning vector. The purified DNA fragment was tailed with an adenine nucleotide. The 10 µl reaction consisted of 6 µl of purified PCR fragment generated by a proofreading polymerase, 1 µl of 10 mM Tris–HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 1 mM $MgCl_2$, 0.2 mM of dATP, 5 unit of DyNAzyme_II DNA polymerase (Finnzymes) and water. The reaction was incubated at 70 ºC for 30 min.

2.8.4   Cloning of DNA fragment into the T&A vector

The DNA fragment was ligated into the T&A cloning vector. The reaction was composed of 1 μl of each 1× Rapid A and B ligation buffers, 2 μl of T&A cloning vector (50 ng), proper amount of PCR product, 1 μl of T4 DNA ligase (3 Weiss units/μl) and water to a final volume of 10 μl. The reactions were mixed, briefly spun and incubated overnight at 4 ºC.

The recombinant plasmids were transformed into an *E. coli* XL1-Blue using CaCl₂ method. A 100 μl of competent cells were mixed with 10 μl of ligation mixture and placed on ice for 30 min. The mixture was immediately incubated at 42 ºC for 1 min and added 0.9 ml LB medium. The cell suspension was incubated at 37 ºC for 1 h. Then, appropriate amount of cell suspension was spread onto the LB agar plates. The plates were incubated overnight at 37 ºC.

The transformants were selected using blue-white screening; the agar plates contained 50 μg/ml ampicillin, 100 μl 100 mM IPTG and 20 μl of 50 mg/ml X-gal. The white colonies were grown in 2 ml LB medium containing 100 mg/ml ampicillin at 37 ºC overnight with shaking for plasmid DNA preparation. The recombinant plasmids were prepared using QIAprep® Miniprep kit (QIAGEN, Germany).

The recombinant plasmid containing the gene of interest was examined with restriction enzyme digestion using *Eco*RI and *Bam*HI. Approximately 0.2 μg DNA was digested in a 10 μl reaction containing 1× *Eco*RI buffer (50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100, pH 7.5), 5 U of *Bam*HI (Biolabs), 10 U of *Eco*RI (Biolabs) and 1 μg of BSA. The reaction was incubated at

37 ℃ overnight. The digested plasmid was analyzed by 1.2% agarose gel electrophoresis. The size of insert was determined as compared with a 100 bp DNA ladder (Fermentas). The recombinant plasmid was sequenced to ensure the correct DNA insert.

2.8.5 Preparation of pET-28b(+) and pVR500 expression clones

The expression vectors used in this experiment were pVR500 (Figure 2.1) and pET-28b(+) (Figure 2.2) for SPI*Pm*4 and SPI*Pm*5, respectively. The two expression vectors were digested with *Nco*I and *Xho*I. Approximately 0.2 μg plasmid DNA was digested in a 10 μl reaction containing 1× restriction endonuclease buffer, 5 U of *Nco*I (Biolabs) and 10 U of *Xho*I (Biolabs) and 1 μg/ml of BSA. The reaction was incubated at 37 ℃ overnight. The digested plasmid DNA was analyzed with 1.0% agarose gel electrophoresis. The linearized plasmids were purified by NucleoSpin® Extract II kits (MACHEREY-NAGEL). They were ligated with the DNA fragments containing the SPI*Pm*4 and SPI*Pm*5 gene fragments prepared from the T&A clones. The expression clones were named pSPI*Pm*4 and pSPI*Pm*5.

2.8.6 Recombinant protein expression

The expression plasmids, pSPI*Pm*4 and pSPI*Pm*5, were transformed into the expression host, *Escherichia coli* Rosetta(DE3)pLysS. The starter culture was prepared by inoculating a single colony from a freshly streaked plate into a 2 ml LB medium containing 100 μg/ml of ampicillin for SPI*Pm*4 or 70 μg/ml of kanamycin for SPI*Pm*5 and 34 μg/ml of chloramphenicol and incubating at 37 ℃ overnight with shaking at 250 rpm.

The starter was diluted 1:100 into two cultures, namely uninduced culture and induced culture, supplemented with antibiotics and incubated at 37 ℃ with shaking at 250 rpm until the OD_{600} reached 0.6. Protein expression was induced by the addition of 1 M isopropyl-β-D-thiogalactopyranoside to a final concentration 1 mM. The two cultures were incubated at 37 ℃ further with shaking for 0, 1, 2, 3 and 4 h, respectively. The 2 ml cultures were aliquot at each time point and the pellets collected by centrifugation at 10,000g for 10 min at 4 ℃. The cell pellets were stored at -80 ℃ for further analysis.
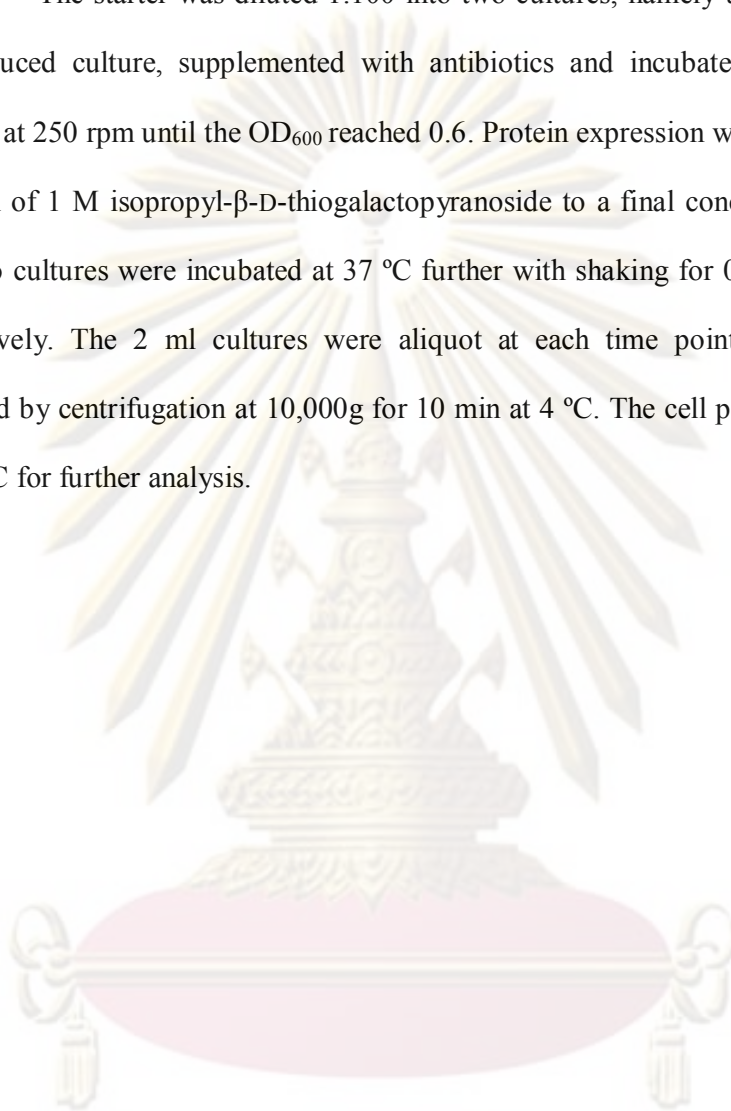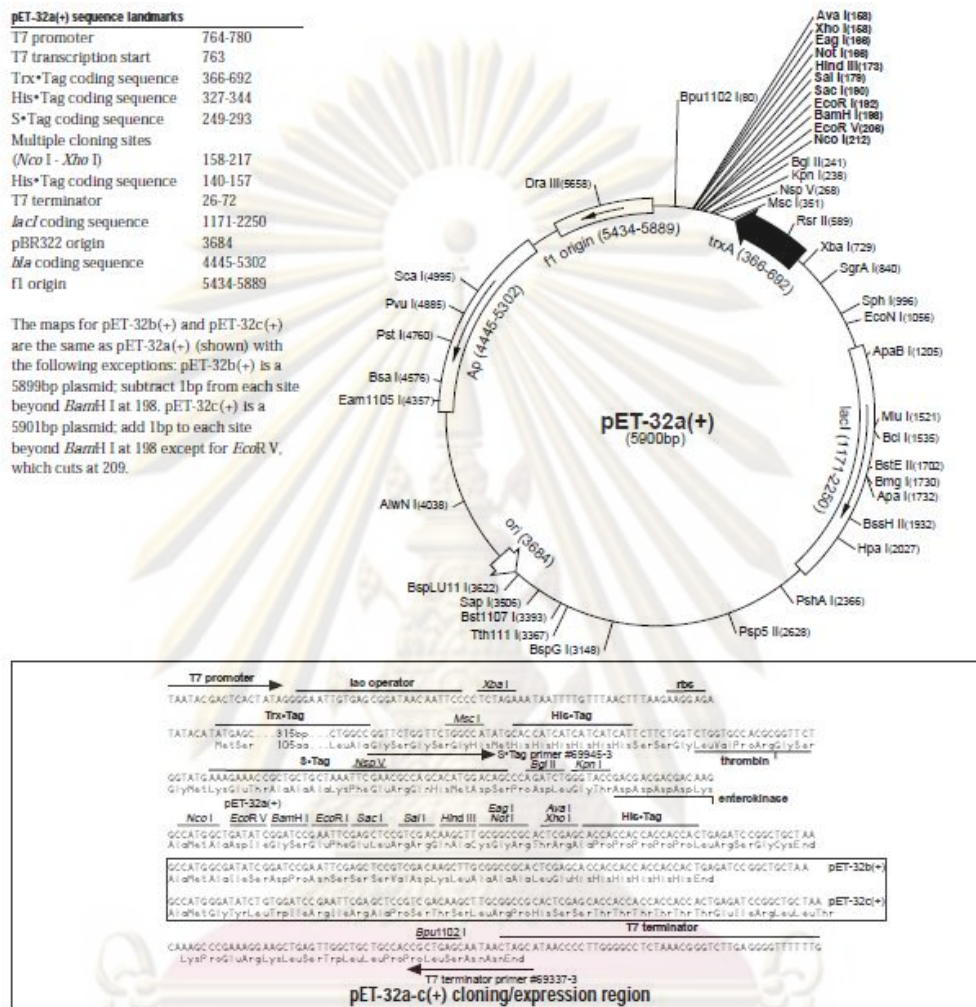
Figure 2.1 The pET-32a(+) vector map (Novagen®, Germany). The pVR500 was constructed by deleting the His_Tag and S_Tag between MscI and KpnI sites. The only His_Tag left was at the 30 side of the reading frame and used for the protein purification as described below. The pET-32a(+) was digested with MscI and KpnI, treated with T4 DNA polymerase to blunt the DNA ends and relegated.

pET-28a(+) sequence landmarks

T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites	
(*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.

Xho I(168)
Not I(166)
Eag I(166)
Hind III(173)
Sal I(179)
Sac I(190)
EcoR I(192)
BamH I(198)
Nhe I(231)
Nde I(238)
Nco I(296)

Bpu1102 I(80)

Dra III(5127)

f1 origin (4903-5358)

Xba I(335)
Bgl II(401)
SgrA I(442)
Sph I(598)

Pvu I(4426)
Sgf I(4426)

Sma I(4300)

Kan (3995-4807)

Cla I(4117)
Nru I(4083)

pET-28a(+)
(5369bp)

lacI (773-1852)

Mlu I(1123)
Bcl I(1137)
BstE II(1304)
Apa I(1334)
BssH II(1534)
EcoR V(1573)
Hpa I(1629)

Eco57 I(3772)

AlwN I(3640)

ori (3286)

PshA I(1968)

BssS I(3397)
BspLU11 I(3224)
Sap I(3108)
Bst1107 I(2995)
Tth111 I(2969)

Bgl I(2187)
Fsp I(2205)
Psp5 II(2230)

pET-28a-c(+) cloning/expression region

Figure 2.2   The pET-28b(+) vector map (Novagen®, Germany).

2.9   Purification of recombinant proteins

Cells were harvested at 3 h after IPTG induction by centrifugation at 8,000g for 20 minutes at 4 ºC. The cell pellet was collected and resuspended in 1× PBS buffer, pH 7.4 by pipetting up and down. The cell was disrupted by at least 4-5 rounds of freeze/thaw and then sonicated with a Bransonic 32 (BANDELIN SONOPULS, Germany) 4-5 time for 10 min each time. The cells lysate were centrifuged at 10,000g for 30 min at 4 ºC to remove cell debris. The supernatant was collected and purified under non-denaturing condition using nickel affinity chromatography (GE Healthcare).

The Ni-NTA agarose was packed into the PD-10 column and washed 3 times with distilled water and binding buffer (20 mM phosphate buffer, pH 7.4, containing 20 mM imidazole), respectively. The soluble fraction was loaded into the PD-10 column at room temperature. The flow through was collected by a gravity flow. The column was washed with binding buffer followed by the wash buffer (20 mM phosphate buffer pH 7.4 containing 20 mM imidazole) to remove unbound proteins. After washing, the protein was eluted with an elution buffer (20 mM phosphate buffer, pH 7.4, containing 500 mM imidazole). The presence and purity of the purified protein was evaluated by 18% SDS-PAGE. The imidazole was removed by dialysis for at least 10 h at 4 ºC against 50 mM phosphate buffer, pH 7.4 twice.

For the rSPI*Pm*4, the thioredoxin portion was removed by digestion with enterokinase. The enterokinase reaction was carried out in a final volume of 5 ml containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 2 mM $CaCl_2$, 0.01 mg/ml enterokinase (Promega) and approximately 0.6 mg/ml fusion protein. The reaction was incubated at 23 °C for 16 h. After digestion, the rSPI*Pm*4 was purified using

nickel-NTA column. The protein preparation was kept track and analyzed using 18% SDS-PAGE and western blot analysis.

2.10 Protein analysis

2.10.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A discontinuous system of SDS-PAGE was used. The gel solutions were prepared as shown in the Appendix B. After the glass plates and spacers were assembled, the components of the separation gel solution were mixed thoroughly and pipette into the gel plate setting. Then, a small amount of distilled water was careful layered over the top of the separation gel solution to ensure that a flat surface of gel be obtained. When the polymerization was complete, water was poured off. The stacking gel solution was prepared, mixed thoroughly and poured on top of the separating gel. A comb was placed in position with excess gel solution overflowing the front glass plate. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with distilled water to remove excess unpolymerized acrylamide.

Protein samples were prepared by resuspending the proteins in $1\times$ sample buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 2.88 mM 0.02% bromophenol blue, 2-mercaptoethanol). The samples were then boiled for 10 min and either held at room temperature or kept at 0 ºC until loaded into the gel.

After boiling, the protein samples and the prestained protein marker were loaded into the wells. Electrophoresis was conducted in $1\times$ running buffer [25 mM

Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS] at a constant current of 20 mA

until the tracking dye (bromophenol blue) reached the bottom of the separating gel.

The gel was placed in Coomassie blue staining solution [0.1% (w/v)

Coomassie brillianr blue R250, 10% (v/v) acetic acid, 45% (v/v) methanol] at room

temperature with gentle shaking for 1 h, immersed in destaining solution [10% (v/v)

acetic acid, 10% (v/v) methanol] and incubated at room temperature with agitation for

1-3 h. Destaining solution was replaced regularly to assist the removal of stain. The

gel was then placed between the two sheets of cellophane over the glass plate before

air-dried at room temperature.

2.10.2 Western blot detection of the His-tagged protein

After running the SDS-PAGE, the SDS-gel slab was removed from the

glass plates. The membrane, gel and filter paper were soaked in a transfer buffer (25

mM Tris base, 150 mM glycine and 20% methanol) for 30 min before they were

consequently laid on Trans-Blot[®] SD (Bio-Rad). The filter paper was placed on

platform, followed by the membrane, the gel and filter paper, respectively, as shown

in Figure 2.3.

1. Safety lid
2. Cathode assembly with latches
3. Filter paper
4. Gel
5. Membrane
6. Filter paper
7. Spring-loaded anode platform mounted
   On 4 guideposts
8. Power cables
9. Base
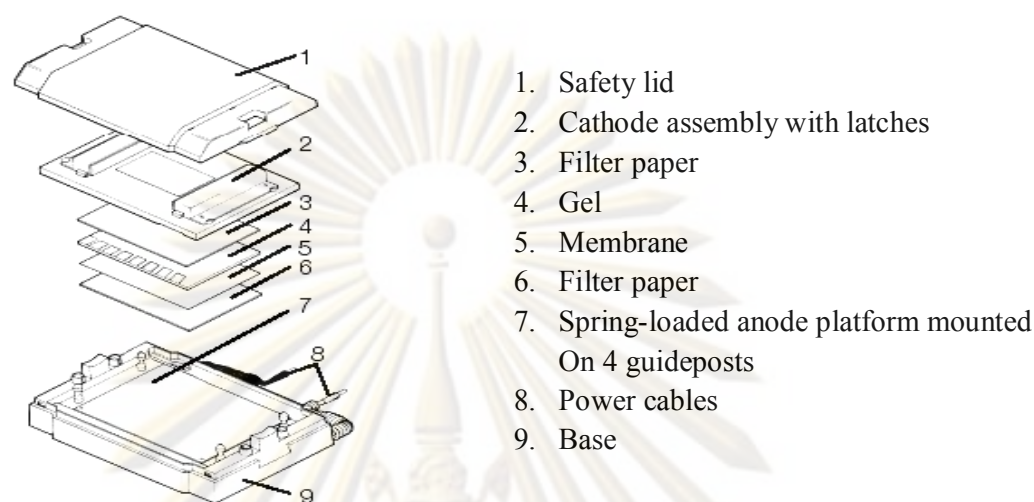
Figure 2.3   Exploded view of the Trans-Blot® SD (Bio-Rad).

Protein transfer was performed at constant 90 mA for 90 min from cathode towards anode. After transferring the proteins from the gel to the membrane, the orientation of the gel was marked on the membrane. Transfer the membrane to an appropriate container (petri dish). The membrane was incubated in blocking buffer (1× PBS buffer [10 mM phosphate buffer, 150 mM NaCl, pH 7.4, 0.05% (v/v) Tween™-20 and 5% (w/v) non-fat dry milk)] at room temperature for an overnight with gentle shaking. The membrane was washed 3 times for 10 minutes each in washing buffer (PBS-Tween buffer [1× PBS buffer (10 mM phosphate buffer, 150 mM NaCl, pH 7.4, 0.025% (v/v) Tween™-20) and incubated in an anti-His antibody solution, 1:3000 dilution in washing buffer with 1% (w/v) non-fat dry milk, at ambient temperature with gentle mixing for 3 h. Then, the membrane was washed 3 times for 10 minutes each in washing buffer and then incubated in a secondary antibody solution, 1:2500 dilutions in washing buffer with 1% (w/v) non-fat dry milk with agitation for 1 h. The membrane was washed 3 times for 10 minutes each in

washing buffer at room temperature. The bound antibody was detected by color development using NBT/BCIP (Fermentas) as substrate dissolving in 100 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl$_2$, pH 9.5.

2.11 Molecular mass determination of recombinant SPI by using MALDI-TOF mass spectrometry

The molecular masses of SPI*Pm*4 and 5 were accurately determined by MALDI-TOF mass spectrometry and used for the calculation in inhibitory activity and kinetic studies. It was performed in the commercial facility of the Proteomic Service Center, Bioservice Unit (BSU) (BIOTEC, Pathumthani, Thailand).

2.12 Proteinase inhibition assay

The proteinase inhibitory activities of rSPI*Pm*4 and rSPI*Pm*5 towards serine proteinase; subtilisin Carlsberg (Bacillus licheniformis,Sigma), trypsin (bovine pancreas, Sigma), α-chymotrypsin (bovine pancreas, Sigma) and elastase (porcine pancreas, Pacific Science) were assayed using a procedure of Hergenhahn et al. (1987) (Hergenhahn, 1987). The reaction mixture consisted of 50 mM Tris–HCl, pH 8.0; 290.67 mM of N-benzoyl-Phe-Val-Arg-*p*-nitroanilide as substrate for subtilisin and trypsin, 147.3 mM N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for chymotrypsin and 2215.18 mM of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide for elastase; appropriate concentrations of proteinases and proteinase inhibitors in a total volume of 100 ml. The concentration of subtilisin, trypsin, α-chymotrypsin and elastase were 0.04 for rSPI*Pm*4 and 0.037 for rSPI*Pm*5, 0.042, 0.04 and 0.077 mM, respectively. Two series of 2-fold diluted proteinase inhibitors were used; 3.88–0.01 mM for rSPI*Pm*4 and

2.98–0.01 mM for rSPI*Pm*5. Not all concentrations of proteinase inhibitors were used in the assays.

For each substrate, a reaction containing the highest amount of SPI preparation used in the assay but without added proteinase was prepared as a control to make certain that there were no contaminating proteinases from the bacterial host used for the preparation of SPIs. The reaction was incubated at 30 ºC for 15 min and then terminated by adding 50 μl of 50% acetic acid. The proteinase reaction product, *p*-nitroaniline, was measured at 405 nm using microplate reader (BMG Labtech). The percentages of remaining activity were calculated and plotted against the molar ratio of inhibitor to proteinase.

2.13   Kinetics of serine proteinase inhibition

Four sets of reactions for four different concentrations of proteinase inhibitor were carried out. Each set consisted of five concentrations of substrate in the presence of fixed amounts of serine proteinase and a concentration of proteinase inhibitor. For subtilisin inhibition, 0, 0.11, 0.22, 0.44 and 0.88 mM of N-benzoyl-Phe-Val-Arg-*p*-nitroanilide substrate were incubated with 3.58 nM of subtilisin for rSPI*Pm*4 or 53.74 nM of subtilisin for rSPI*Pm*5 and fixed amount of inhibitor. Four different concentrations of proteinase inhibitor: 0, 30.38, 60.75 and 121.50 nM for rSPI*Pm*4 and 0, 23.25, 46.50 and 93.00 nM for rSPI*Pm*5, were used. For elastase inhibition, 0, 0.18, 0.37, 0.74 and 1.48 mM of N-succinyl-Ala-Ala-Ala-*p*-nitroanilide substrate were incubated with 154.44 nM of elastase and a concentration of rSPI*Pm*5. Four different concentrations of rSPI*Pm*5: 0, 124.07, 248.14 and 496.28 nM, were used. The reactions were made a total volume of 100 ml in the presence of 50 mM Tris–

HCl, pH 8.0. The reactions were initiated by the addition of proteinase, incubated at 30 °C for 15 min and arrested with 50 ml of 50% acetic acid. Each reaction was performed in triplicate. The *p*-nitroaniline formed was measured at 405 nm using a microplate reader (BMG Labtech). The amount of *p*-nitroaniline was calculated using a millimolar extinction coefficient of 9.96. The activity was calculated as nmole of *p*-nitroaniline/min. The activities were plotted against the concentration of substrates as substrate saturation curve and a Lineweaver–Burk plot. The apparent K_Ms were calculated and plotted against the proteinase inhibitor concentrations. The latter was constructed for the calculation of inhibition constant (K_i).

2.14 Bacterial growth inhibition

Bacteriostatic activities of SPI*Pm*4 and 5 were assayed based on the procedure by Han et al. (2008) (Han et al., 2008)on Gram-positive bacteria, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, and Gram negative bacteria, *Vibrio harveyi* 639, *E. coli* JM109. For each bacterium, the assay was done in duplicate. An overnight culture of bacterium was diluted a hundred-fold, and grew in a shaking incubator in the presence of 0 and 20 mM of SPI*Pm*4 or 10 mM of SPI*Pm*5 at 30 °C for *B. megaterium* and *V. harveyi* 639 and 37 °C for *B. subtilis*, *S. aureus* and *E. coli* JM109. The bacterial growth was measured at 0, 4, 8, 12 and 16 h by monitoring the optical density at 595 nm.

CHAPTER III

RESULTS

3.1   Mining the *Penaeus monodon* EST database

From the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/) (Tassanakajon et al., 2006), we had searched for the Kazal-type proteinase inhibitor. From a total of 10,536 unique contigs and singletons derived from the clustering of 33,143 EST clones in the database, nine different Kazal-type serine proteinases were identified and named SPI*Pm*1 to 9 (Table 3.1 and Figure 3.1). Some SPIs were represented by more than one contig or singleton because there were variations in the cDNA sequences at the very ends of 3′-untranslated regions that were enough to be clustered as different contigs (data not shown). The number of Kazal domain in these SPIs varied from 2 to 7 and possibly more. The open reading frames of SPI*Pm*1, 2, 4, 5 and 6 were complete. That of SPI*Pm*3, a seven-domain SPI from hepatopancreas, was nearly complete as only a few amino acid residues were missing at the N-terminus. Those of other SPIs were awaited for completion.

Figure 3.1 shows the amino acid sequences of the nine Kazal inhibitors from *P. monodon*. The potential signal peptide cleavage sites, determined using the online SignalP 3.0 Server, are indicated in the sequence. Two cleavage sites with approximately equal possibility were identified in SPI*Pm*2.

In Table 3.1, the frequencies of EST members from mostly the standard libraries are shown to avoid the biased figures from the normalized libraries. It was found that the contig members were mostly from the haemocyte libraries, particularly,

the heat-treated haemocyte libraries. Other libraries were from hematopoietic tissue, hepatopancreas, ovary and lymphoid organ. Only one singleton, the SPI*Pm*9, was identified from the normalized libraries of lymphoid organ suggesting that the SPI*Pm*9 was rarely expressed. The SPI*Pm*2 (22 clones) is the most abundant SPI in the haemocytes followed by SPI*Pm*4 (6 clones) and 5 (6 clones). Other SPI clone members were fewer than two.
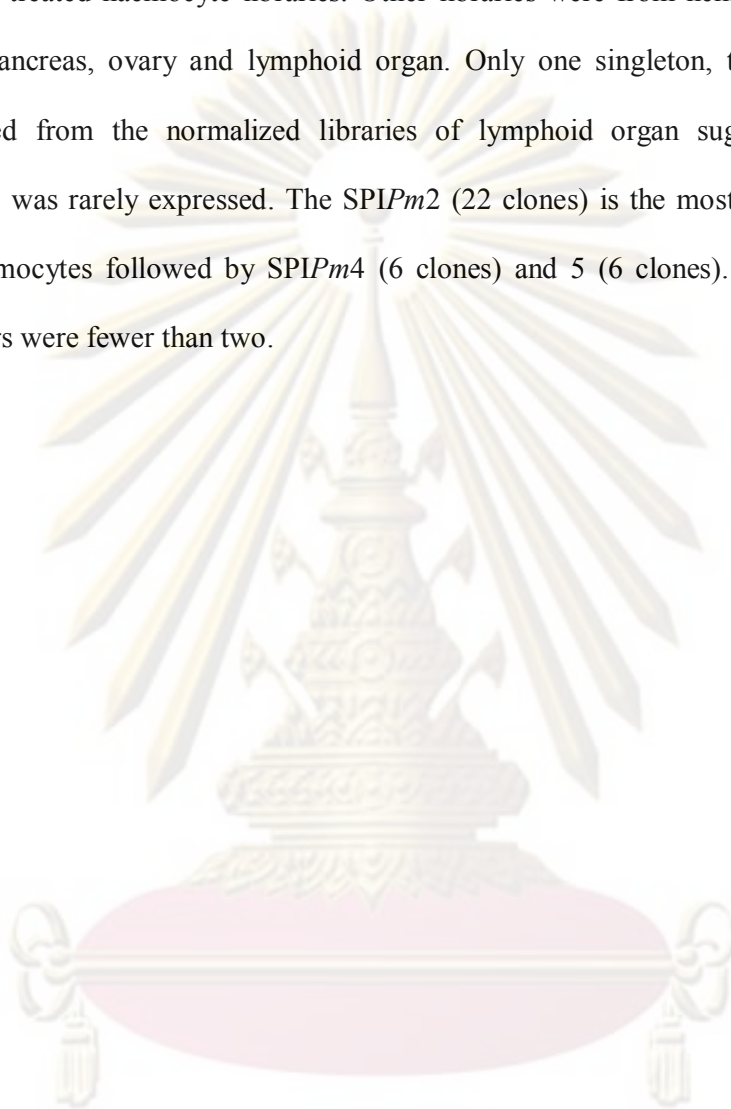
Table 3.1 The nine Kazal-type serine proteinase inhibitors identified from the *Penaeus monodon* EST database.

SPI	Contig or singleton	Representative clone (ORF) and GenBank accession number	Number of Kazal domain	Clone member*	Frequency in cDNA libraries**	
SPI*Pm*1	CT446 and CT447	HC-N-S01-0010-LF (complete ORF) GO269557	4	2	HC-N-S	2
SPI*Pm*2	CT165 and CT170	SH415 (complete ORF) BI018075	5	22	HC-N-S HC-V-S HC-H-S HPO-N-S	5 1 14 2
SPI*Pm*3	CT195	HPa-N-S01-0307-LF (incomplete ORF) EB409368	7	2	HPA-N-S	2
SPI*Pm*4	CT409 and CT410	HC-H-S01-0302-LF (complete ORF) DW677889	2	6	HC-N-S HC-H-S HPO-N-S	1 4 1
SPI*Pm*5	CT387 and CT391	HC-H-S01-0132-LF (complete ORF) DW677833	2	6	HC-N-S HC-H-S	1 5
SPI*Pm*6	CT980	HC-V-S01-0141-LF (complete ORF) GO269558	2	2	HC-N-S HC-V-S	1 1
SPI*Pm*7	CT2507 and SG6085	HC-H-S01-0190-LF (incomplete ORF) DW677851	≥6	1	HC-H-S	1
SPI*Pm*8	SG9720	OV-N-S01-0029-W (incomplete ORF) EB389687	≥3	1	OV-N-S	1
SPI*Pm*9	SG9029	LP-N-N01-0272-LF (incomplete ORF) EE662962	≥2	1 (normalized clone)	LP-N-N	1

. * Excluding the normalized clones unless otherwise stated.
** HC-N-S, HC-V-S and HC-H-S are standard (3rd S) normal (2nd N), standard viral infected (2nd V) and standard heat-treated (2nd H) hemocyte (HC) cDNA libraries, respectively. HPO-N-S, HPA-N-S and OV-N-S are standard (3rd S) normal (2nd N) hematopoietic tissue (HPO), hepatopancreas (HPA) and ovary (OV) cDNA libraries, respectively. The LP-N-N is normalized (3rd N) normal (2nd N) lymphoid organ (LP) cDNA library.

SPIPm1

MLLCKITLIHLLLQGFAVFNDANS◆DHD
CIGYCPEVYDPVCASNGWTYNNDCELQAMIKCQGWNITKTHDQACE
CLKACPTTFAPVCGSDNKTYLNEC-VFEVASCWDHSLDKASEGACGWGIH
CLQYCPEVYDPVCGSNGQTYTNECELQAAIQCRGLQIAKRHDQACE
CHATCPLIHDPVCGTDDRTYYNEC-FFTKASCWDRSILKKKNGPCDRKWKYLLEI

SPIPm2

MANKVALLTLLAVAVAVSG◆YGKG◆GKIRL
CAKH--CTTI-SPVCGSDGKTYDSRCHLENAA-CGGVSVTFHHAGPCPPPKR
CPGL--CPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSKKHDGRCG
CNPIVACPEIYAPVCGSDGKTYDNDCYFQAAV-CKNPDLKKVRDGNCD
CTPLIGCPKNYRPVCGSDGVTYNNDCFFKVAQ-CKNPALVKVSDTRCE
CNHV--CTEEYYPVCGSNGVTYSNICLLNNAA-CLDSSIYKVSDGICGRKTVPIKKGY

SPIPm3

........KTCWLLSLFLLASG◆QET
CDFVCPDHLDPVCGSDGITYPNLCVLELVDCLSDEDITLAHPGPCETKQES
CDILCSTDYDPVCGSDGVTYSNLCNLEVADCLSDEDITLAYEGECKEVKKGD
CDFPCPDNYDPVCGSNGVTYSNLCELERANCQSDQEITVAYDGECKG
CDFPCPDNYDPVCGSNGVTYSNLCELERANCQSDEEITVAYDGECKELKGD
CDFGCPENYDPVCGSNGVTYSNLCELERANCQSDEEITVAYPGECNS
CDFGCSGLWDPVCGSDGVTYSNLCQLEIANCLNGGGISLAYPGECEATDGS
CDIVCTANYDPVCGSDGNTYGNACDLEVADCMSDEDITLAHPGPC

SPIPm4

MANKVAFLTLLAMAVAASGYG◆KGGDSRL
CAQHCTTI-SPVCGSDGKTYDSRCHLQNAA-CGGVRVTFHHAGPCYPPKR
CPQACPFIYDPVCGTNGKTYSNSCELENDRTCNGAYVSKKHDGPCVDRKTGY

SPIPm5

MFYKTTLLILFAVAVSGYG◆KGGKFRL
CARFCPLREELPVCGSNGKTYQSRCHLDNEN-CRDESITFVHHGSCEPHQP
CPGVCPTVYD-PVCGTDGKTYSNVCDLVEAARCEGRAVSVDHDGACGRKTSPIKKGY

SPIPm6

MARFSLAFGLLAVALMASVEA◆RSPIPPGFRPGSN
CGAIACPAIYAPVCGSDGNTYPSRCNLQAARCSNPQLRVVSQGECPSQNN
CPTV-CPFNYDPVCGSNGKTYPNECALESDKCSVQGLTLSHRGEC

SPIPm7

...........DNKAYGNECKLLQEACRRPGLG-KKNDGPCVSSNN
CQRQ-CSSSYEPVCATDGVSYDNKCHLSIKSCENPSVQ-LRYEGPCAVPANPPS
PTSQPTSTGGSLQPASGSVNKVPQVGQSVLSLVTGAARPQNSAPAPAPITSPTARPASVGNAGGSTPR
CRDE-CQLGFRPVCGTDGKVYANECKLRVASCHDPTIQ-KKNDGVCEKD
CSIA-CPDDVEPVCGSNGVTYRNSCFYSIARCLDPDVT-KVANAPCANRRDTK
CDLA-CDKSINPVCGSDGRTYRNQCELEANACTKTSLI-KLYNGQCQEQQGAR
CDTV-CPNVVEEVCGDDGKTYRSHCDLIVLSCRANVRVNKAYDGRC

SPIPm8

MSRYHFVLVIFISVSAG◆HYLHTNNRK
CVATCPDLSDPVCGSNQVTYDNDCLLERAQCED-LSLHKVAEGPCE
CNSICNDEQASVCGSDGKTYESACNLQRAKCKDNPTLQQVAQGPCASSDA
CTRECGFALQPVCGSDGVTYRSECELEVAAC...

SPIPm9

...ILNIAICKAQARGVEIQKAYNGECDTATSTRTRPEQQQGEDYGAFAAQDDASD
CPEVCDDAFAPVCGTDSVTYDNLCSLKQSSCLTRAAGGGAPLVTRAFEGPCVAALPRGPI
CKEECVRTFLPVCGSDGETYNNICLLNLADCLNPFVTISLVKEGACTDGDEDSSADSD
EFVGLLSDTFPDPGPPPPPPGGSSSSDLTSSELTPHGGYLPPV

Figure 3.1 Amino acid sequences of the nine Kazal-type SPIs from the *Penaeus monodon* EST database. The conserved cysteine residues are shaded. Italicized and shaded residues are the reactive P1 residues. Diamonds indicate the potential signal peptide cleavage sites. Dotted lines indicate the incomplete ends of the amino acid sequences.

3.2   Detection of possible *V. harveyi* and WSSV infection

Five individual shrimp (1%) were sampling from the hatchery tank for possible *V. harveyi* and WSSV infection. Gills were collected and used for DNA extraction. Then, the sample was diluted 100-fold with distilled water and used as template for checking the *V. harveyi* and WSSV infection by PCR technique. The expected sizes of PCR products were 363 and 250 bp. The results showed that the 363 and 250 bp *V. harveyi* and WSSV specific bands could not be detected in shrimp gills at early time before injection (Figure 3.2).
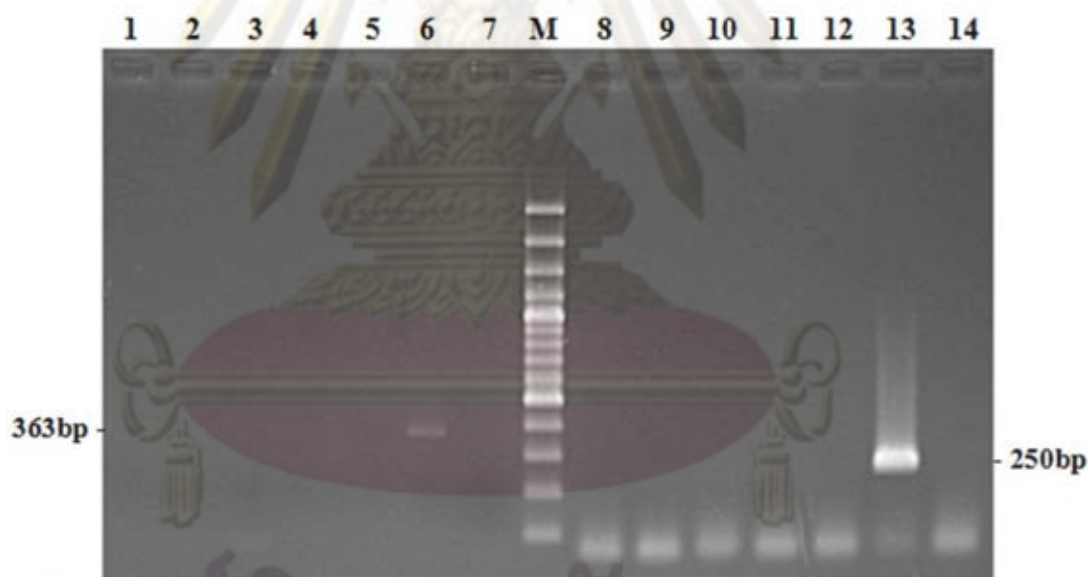
Figure 3.2   Ethidium bromide staining of the 250 and 363 bp PCR product amplified from the gill of DNA isolated from *V. harveyi* and WSSV infected individual shrimp on a 1.5% agarose gel. Lanes 1-7 for the detection of *V. harveyi* and lanes 8-14 for the detection of WSSV.

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

Lane 1-5: sample 1-5   Lane 8-12: sample 1-5

Lane 6: positive control   Lane 13: positive control

Lane 7: negative control   Lane 14: negative control

3.3   Tissue and total RNA preparation

Gill, epipodite, antennal gland, heart, haemocyte, hepatopancreas, intestine, lymphoid and eyestalk of shrimp were collected for total RNA isolation. Then, total RNA was treated with RQ1 RNase-free DNase to remove the genomic DNA. The A_{260}/A_{280} ratio of total RNA samples were 1.5 to 1.8 indicating the acceptable quality of total RNA. The quality of total RNA was analyzed using 1.2% formaldehyde-agarose gel electrophoresis. Each tissue revealed a predominant band of 18S rRNA (1.9 kb) as shown in Figure 3.3.
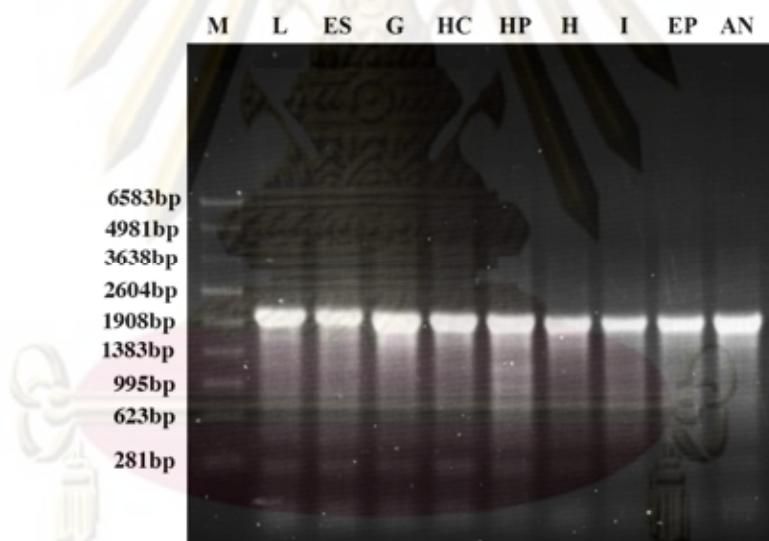
Figure 3.3   Total RNAs isolated from various tissues of *P. monodon* separated on a 1.2% formaldehyde agarose gel.

Lane M: RNA marker

Lane L: Total RNA from lymphoid   Lane H: Total RNA from heart

Lane ES: Total RNA from eyestalk   Lane I: Total RNA from intestine

Lane G: Total RNA from gill   Lane EP: Total RNA from epipodite

Lane HC: Total RNA from haemocytes   Lane AN: Total RNA from antennal gland

Lane HP: Total RNA from hepatopancreas

3.4 Tissues distribution

Tissue specific expression of the seven Kazal-type SPIs were then examined in various shrimp tissues by RT-PCR analysis. The Kazal-type SPI mRNAs was found in all analyzed tissues including gill, epipodite, antennal gland, heart, haemocyte, hepatopancreas, intestine, lymphoid gland and eye stalk. The SPI*Pm*1, 2, 4, 6 and 9 transcripts were shown to be the most abundant types found in normal shrimp haemocytes whereas SPI*Pm*7 was the most abundant in epipodite, gill as well as the haemocyte (Figure 3.4).

Figure 3.4 Expression of the six SPIs in various shrimp tissues. RT-PCR was performed using primer pairs, specific to SPI*Pm*1, 2, 4, 6, 7 and 9. The amplification was performed on a cDNA libraries obtained from individual shrimps. β- actin serves as an internal control.

Lane 1: Intestine
Lane 2: Heart
Lane 3: Hepatopancreas
Lane 4: Haemocytes
Lane 5: Gill

Lane 6: Eyestalk
Lane 7: Epipodite
Lane 8: Antennal gland
Lane 9: Lymphoid

3.5 Semi-quantification of Kazal-type serine proteinase inhibitor mRNA expression by RT-PCR

Expression levels of seven genes including SPI*Pm*1, 2, 4, 5, 6, 7 and 9 were examined by a semi-quantitative RT-PCR. This technique requires the optimization of several parameters used in PCR amplification to ensure a semi-quantitative analysis of the transcripts.

3.5.1 Primer design

Seven gene specific primer pairs were designed to amplify the SPI genes based on their cDNA sequences in the EST database using the SECentral program (Scientific & Educational Software). Because the ORFs of Kazal-type SPIs contain highly conserved domains, one primer either forward or reverse primers for each type of Kazal SPI was designed from the 3′ or 5′ untranslated region (3′ or 5′-UTR) to specifically amplify each type of SPI and the other one was designed from the ORF sequence to identify the gene. Seven Kazal-type (SPI*Pm*1, 2, 4, 5, 6, 7 and 9) were selected for RT-PCR (Table 3.2).

3.5.2 Determination of the optimal $MgCl_2$

Determination of the optimal concentrations for each primer pair was performed by varying the $MgCl_2$ concentrations (0.5-3.0 mM) in the standard PCR reaction. The PCR products were analyzed by agarose gel electrophoresis and the concentration of $MgCl_2$ that gave the highest yield of amplified product was chosen for used in the expression analysis (Table 3.2). The PCR products of SPI*Pm*5, 6 and 9 were showed to give the highest yield at 0.5, 1.5 and 2.0 mM, respectively, whereas the remaining genes gave the highest yields at 1.0 mM $MgCl_2$ (SPI*Pm*1, 2, 4 and 7).

3.5.3 Determination of cycling parameter

Cycling numbers were important. The appropriate numbers of amplification cycles before reaching a plateau amplification phase were determined. The amplification product as sharp DNA bands on an agarose gel could then be correctly quantified.

In this experiment, the numbers of cycles were determined in a range from 20 to 40 cycles. The number of cycles that gave the highest yield before the product reached a plateau phase was chosen. From Table 3.2, cycle numbers that gave the relatively abundance of PCR products for SPI*Pm*4 and β–actin were 25 cycles whereas SPI*Pm*1, 6 and 7 were 30 cycles. For SPI*Pm*2 and 9, they were 32 and 28 cycles, respectively. However, the mRNA transcript of SPI*Pm*5 might be rare and, thus, required the amplification of 40 cycles.

3.5.4 The optimal amount of cDNA template

The amount of cDNA template was optimized for PCR reaction. The detection of the amplified product should be at an exponential phase not the plateau amplification phase. The concentration that gave the highest yield and specificity was chosen. One μl of undiluted cDNA template for SPI*Pm*2 or 3 μl for SPI*Pm*5 were selected. For SPI*Pm*9, one μl of two-fold diluted cDNA template was used. The other remaining genes gave the highest yields at 3 μl of 10-fold diluted template cDNA (SPI*Pm*1, 4, 6, 7 and β-actin).

3.5.5 Determination of annealing temperature (T_m)

The optimal primer annealing temperature was adjusted to increase the amplification specificity of each primer pair. The annealing temperatures were

calculated for all primers. The annealing temperature of 55°C was chosen for SPI*Pm*9, 60°C for SPI*Pm*2, 4 and 5, and 65°C for SPI*Pm*1, 6 and 7. The amplification products of these genes were 215, 192, 311, 326, 233, 146, 150 and 317 bp for SPI*Pm*1, 2, 4, 5, 6, 7, 9 and β-actin, respectively. Non-specific amplification products were not observed for all amplification reactions.

The optimal conditions for RT-PCR of the Kazal-type genes are shown in Table 3.2.

Table 3.2   Optimal condition for RT-PCR of Kazal-type gene expression in *P. monodon*.

Target (cDNA)	Name	Sequence of primer (5′→3′)	Amount of template (μl)	MgCl$_2$ (mM)	Annealing temperature (°C)	PCR cycle	Product size (bp)
SPI*Pm*1	FPm1	AGACAAGTTGTTGCCGAGAG	3 (10 fold diluted)	1.0	65	30	215
	RPm1	ATAGCCTGTAGTTCGCAGTC					
SPI*Pm*2	FPm2	ATGCAACCACGTCTGTACTG	1 (undiluted)	1.0	60	32	192
	RPm2	CTGCAAGGTTCCACATCT					
SPI*Pm*4	FPm4	AGAGAACAGCAAGAGCACAG	3 (10 fold diluted)	1.0	60	25	311
	RPm4	ACCTGGTCAATTTTGAAAGC					
SPI*Pm*5	FPm5	TGGAACGGACGGCAAGACAT	3 (undiluted)	2.0	60	40	326
	RPm5	GTCGTGACAGTCTTGAGTCC					
SPI*Pm*6	FPm6	CCGCAATTAAGAGTCGTGAG	3 (10 fold diluted)	1.0	65	30	233
	RPm6	AGAAGACTGGAGGAGGAATC					
SPI*Pm*7	FPm7	GCCGGACGTACCGTAAT	3 (10 fold diluted)	1.0	65	30	146
	RPm7	TCCGCATACTTCCTCCACTA					
SPI*Pm*9	FPm9	TGTGAAGGAAGGAGCCTGTA	1 (2 fold diluted)	0.5	55	28	150
	RPm9	TCAGACGAGGTCAAGTCAGA					
β-actin	actinF	GCTTGCTGATCCACATCTGCT	3 (10 fold diluted)	1.0	52	25	317
	actinR	ATCACCATCGGCAACGAGA					

3.6 Expression analysis of Kazal-type serine proteinase inhibitor gene after *V. harveyi* 639 challenge by semi-quantitative RT-PCR

To investigate whether the expression of Kazal-type serine proteinase inhibitor is affected by *V. harveyi* challenge, the RT-PCR was performed using cDNA prepared from haemocytes from the normal shrimp (control) and *V. harveyi* infected shrimp. The shrimp were challenged with 10^5 CFU of *V. harveyi* per shrimp. The luminescence was used to confirm the *V. harveyi* infection. The *V. harveyi* could be detected at 12 h post-injection in the challenged shrimp. No luminescence was detected in normal shrimp (data not shown).

The total RNAs prepared from the haemocytes were used for the first-strand cDNA synthesis. The seven Kazal-type gene specific primer pairs were used to amplify the cDNA templates producing DNA fragments of 215, 192, 311, 326, 233, 146 and 150 bp for SPI*Pm*1, 2, 4, 5, 6, 7 and 9, respectively. The shrimp β-actin was used as an internal control. Distilled water was used as a negative control. The amplification products of the target gene and the control gene (β-actin) were run on the same 2% agarose gel. The ratio of band intensity of the target gene and the control gene was analyzed using Genetools analysis software (Syngene). The signal of interesting genes was normalized with that of β-actin.

The temporal mRNA expression patterns of the Kazal-type genes after *V. harveyi* challenge are shown in Figure 3.5. The results of RT-PCR and data analysis were shown in Table 3.3. No significant difference ($p<0.05$) in mRNA expression

level of SPI*Pm*1, 2, 6, 7 and 9 were observed after challenging shrimps with *V. harveyi*.

The expression level of SPI*Pm*4 could not be accurately determined because there were two to three specific amplified bands of very similar sizes. For unknown reasons, the cDNA samples of this experiment were not good for SPI*Pm*5.
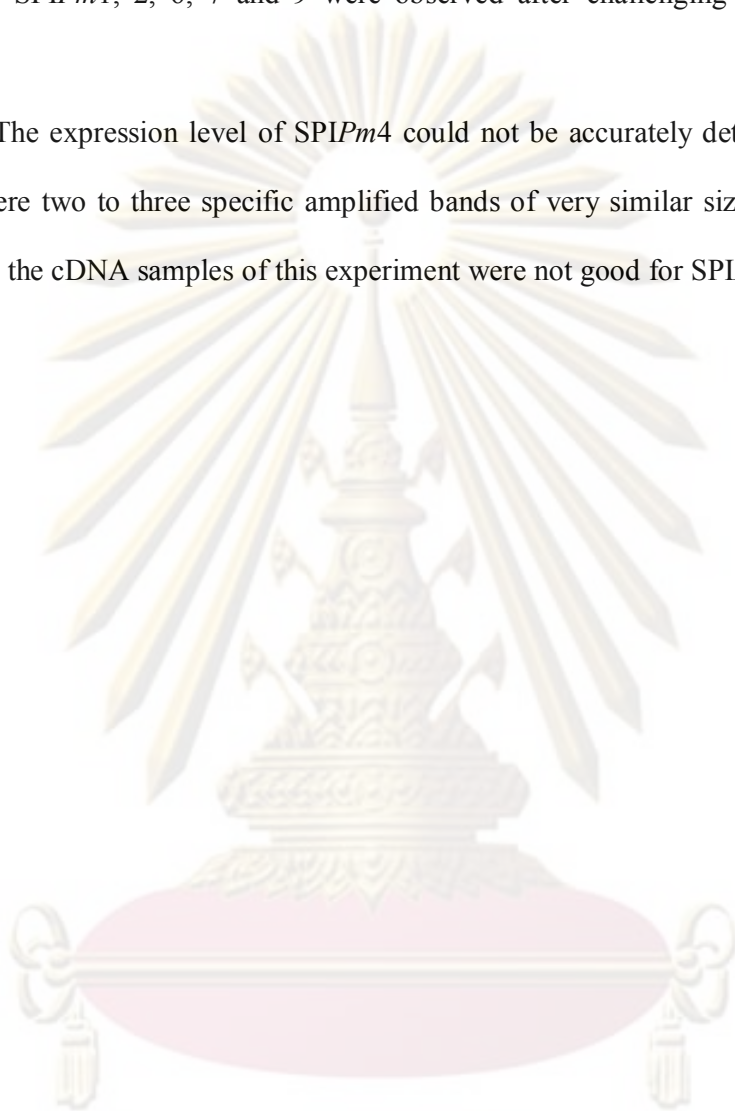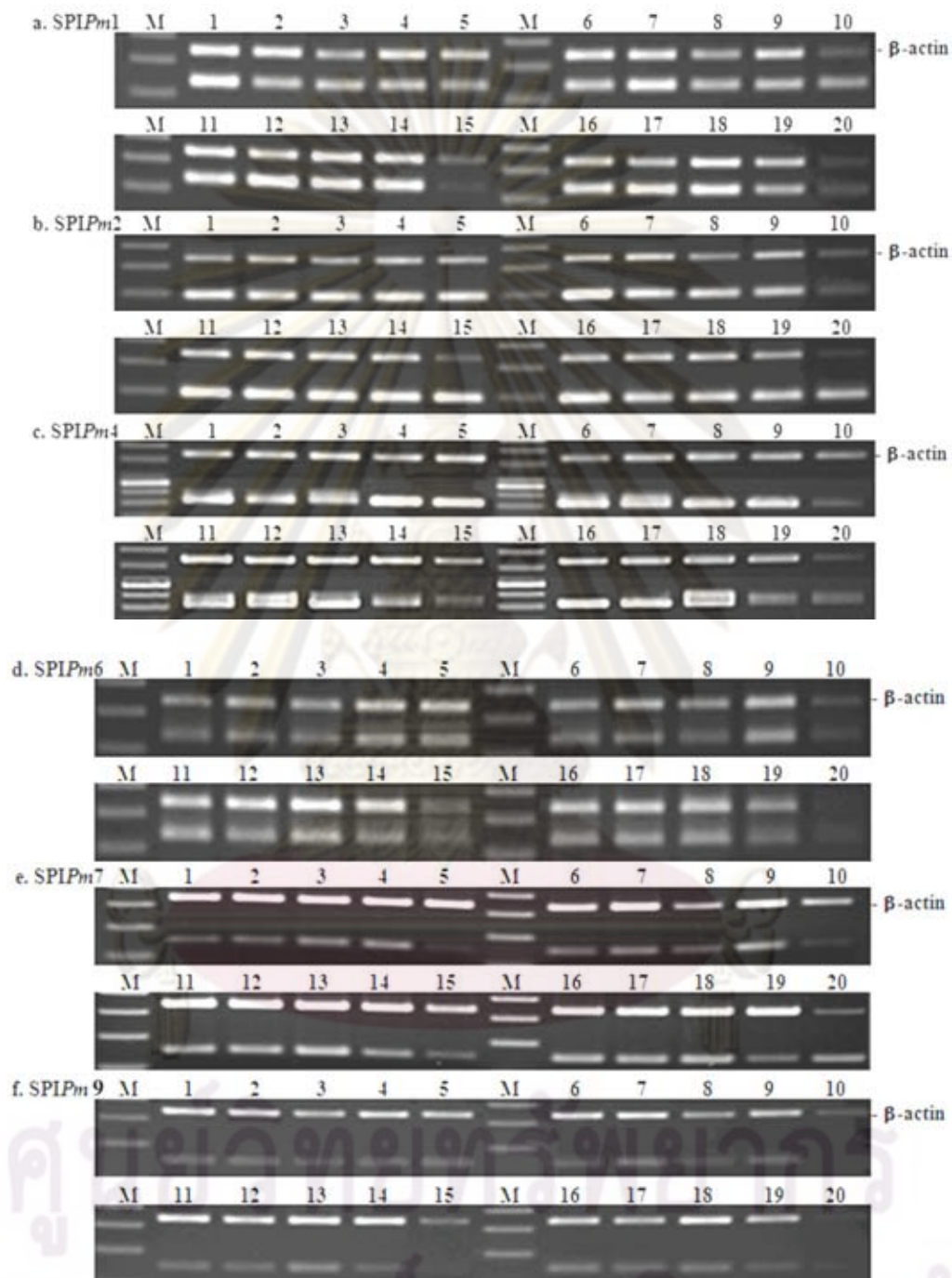
Figure 3.5 Expression analysis of Kazal-type genes, SPI*Pm*1, 2, 4, 6, 7 and 9 after 0.85% NaCl or *V. harveyi* 639 injection at 0, 6, 12, 24 and 48 h post-injection.

Lane 1-5: samples were injected with 0.85% NaCl at 0, 6, 12, 24 and 48 h
Lane 6-10: samples were challenged with *V. harveyi* 639 at 0, 6, 12, 24 and 48 h (group I)
Lane 11-15: samples were challenged with *V. harveyi* 639 at 0, 6, 12, 24 and 48 h (group II)
Lane 16-20: samples were challenged with *V. harveyi* 639 at 0, 6, 12, 24 and 48 h (group III)
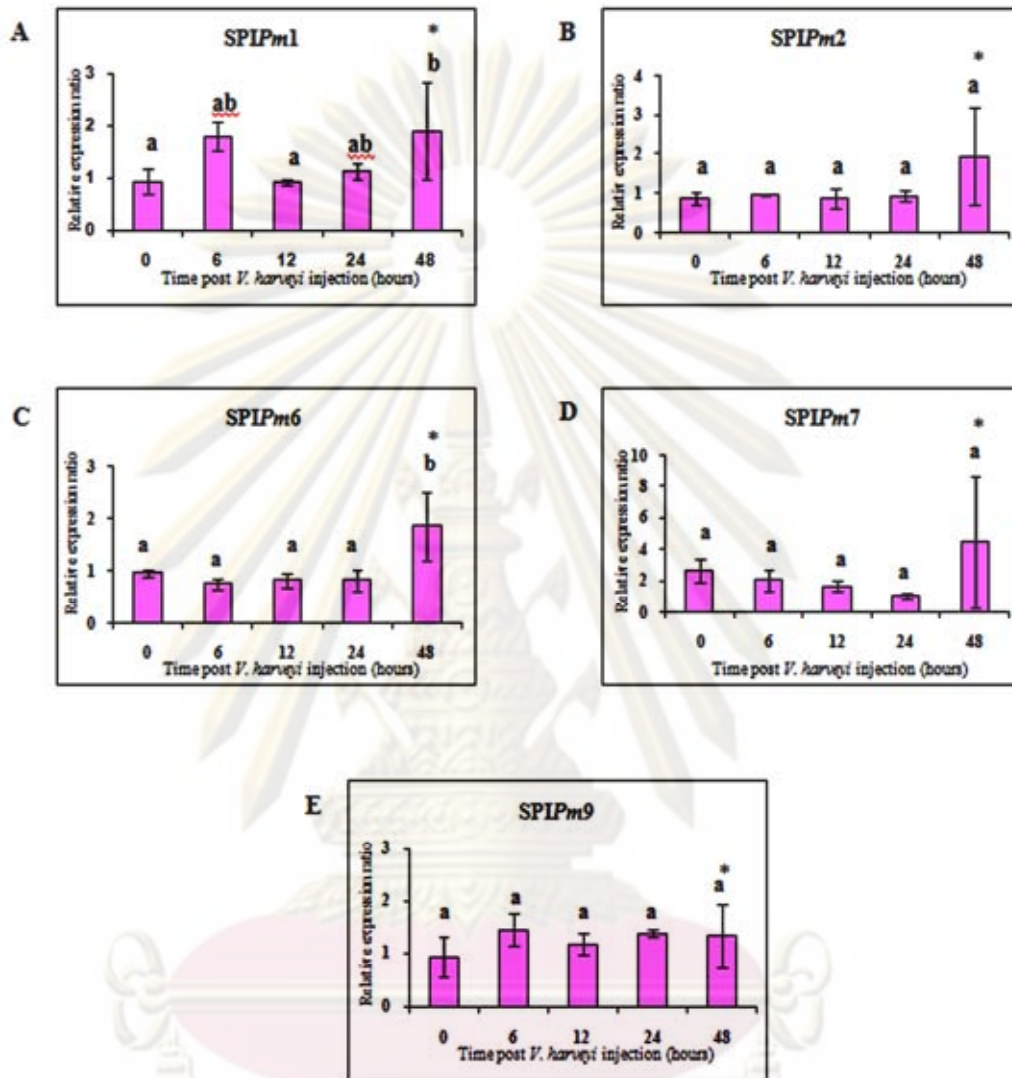
Figure 3.6 Relative expression levels of SPIs at different time intervals after injected with *V. harveyi* 639. Different letters indicate significant (*p*<0.05) difference in the mean expression level of the Kazal-type gene. ∗ Statistic error

Table 3.3   The summary of relative expression of the seven Kazal-type genes in *V. harveyi* 639-

infected shrimp.

SPI*Pm*1

I. Anova test of SPI*Pm*1 expression in *V. harveyi* challenged *P. monodon*

	Sum of squares	df	Mean square	F	Sig.
Between groups	2.678	4	0.669	3.251	0.059
Within groups	2.059	10	0.206		
Total	4.736	14			

II. Duncan test of SPI*Pm*1 exprssion in *V. harveyi* challenged *P. monodon*

Hours	N	Subset for alpha = 0.05	
		2	1
12	3	0.915	
0	3	0.934	
24	3	1.130	1.130
6	3	1.793	1.793
48	3		1.886
Sig.		0.051	0.080

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

SPI*Pm*2

I. Anova test of SPI*Pm*2 expression in *V. harveyi* challenged *P. monodon*

	Sum of squares	df	Mean square	F	Sig.
Between groups	2.503	4	0.626	1.884	0.19
Within groups	3.322	10	0.332		
Total	5.826	14			

II. Duncan test of SPI*Pm*2 exprssion in *V. harveyi* challenged *P. monodon*

Hours	N	Subset for alpha = 0.05
		1
12	3	0.885
0	3	0.887
24	3	0.937
6	3	0.975
48	3	1.94
Sig.		0.067

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

SPI*Pm*6

I. Anova test of SPI*Pm*6 expression in *V. harveyi* challenged *P. monodon*

	Sum of squares	df	Mean square	F	Sig.
Between groups	2.569	4	0.642	6.270	0.009
Within groups	1.024	10	0.102		
Total	3.594	14			

II. Duncan test of SPI*Pm*6 exprssion in *V. harveyi* challenged *P. monodon*

Hours	N	Subset for alpha = .05	
		2	1
6	3	0.742	
12	3	0.816	
24	3	0.823	
0	3	0.961	
48	3		1.855
Sig.		0.453	1.000

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

SPI*Pm*7

I. Anova test of SPI*Pm*7 expression in *V. harveyi* challenged *P. monodon*

	Sum of squares	df	Mean square	F	Sig.
Between groups	21.115	4	5.279	1.380	0.309
Within groups	38.253	10	3.825		
Total	59.368	14			

II. Duncan test of SPI*Pm*7 exprssion in *V. harveyi* challenged *P. monodon*

Hours	N	Subset for alpha = .05
		1
24	3	0.993
12	3	1.667
6	3	2.025
0	3	2.635
48	3	4.482
Sig.		0.072

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

SPI*Pm*9

I. Anova test of SPI*Pm*9 expression in *V. harveyi* challenged *P. monodon*

	Sum of squares	df	Mean square	F	Sig.
Between groups	0.540	4	0.135	1.024	0.441
Within groups	1.319	10	0.132		
Total	1.858	14			

II. Duncan test of SPI*Pm*9 exprssion in *V. harveyi* challenged *P. monodon*

Hours	N	Subset for alpha = .05
		1
0	3	0.925
12	3	1.1746
48	3	1.340
24	3	1.382
6	3	1.458
Sig.		0.129

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

3.7   Expression of SPI*Pm*5 in response to heat stress using semi-quantitative RT-PCR

Having shown that most of the EST members in the contigs, particularly SPI*Pm*2, 4 and 5, were from the heat-treated hemocyte cDNA libraries (Table 3.1), one might want to hypothesize that the expression of these SPIs were increased upon heat treatment. As a preliminary experiment on heat stress, we tested this hypothesis with SPI*Pm*5, one of the two SPIs in this study. The shrimp were heat-treated for 1 h at 5°C above normal rearing temperature. The cDNAs were prepared and tested for the increase in SPI*Pm*5 mRNA. Figure 3.7 revealed that the expression of SPI*Pm*5 gene was, indeed, up-regulated unambiguously upon heat treatment.

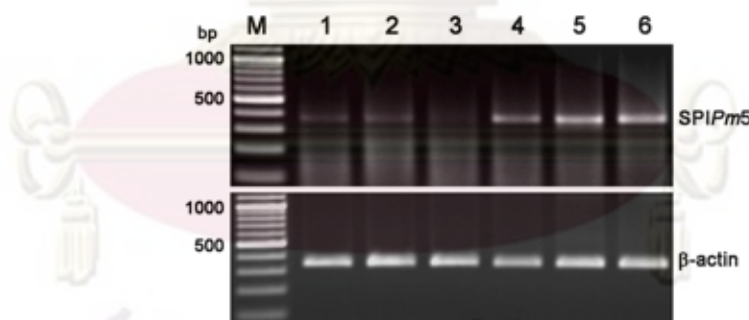**Figure 3.7**   RT-PCR analysis of SPI*Pm*5 expression in heat-treated shrimp. Six subgroups of 3 individuals were divided into 2 groups, the control (lanes 1-3) and heat-treated (lanes 4-6) groups. The total RNA samples from individuals in each subgroup were pooled for cDNA synthesis for the RT-PCR. Upper and lower panels are the RT-PCR of SPI*Pm*5 and β-actin, respectively. Lane M is the 100-bp ladder.

3.8   Recombinant expression of rSPI*Pm*4 and 5

3.8.1   Construction of the recombinant plasmid pSPIPm4 and 5

Two two-domain Kazal-type SPIs, SPI*Pm*4 and 5, were interesting for they were identified from the heat-treated haemocyte cDNA libraries. The SPI*Pm*4 and 5 cDNA clones, HC-N-N01-4898-LF and HC-N-N01-2619-LF contained the open reading frames of 387 and 399 bp that encoded polypeptides of 128 and 132 amino acids with putative signal peptides of 21 and 19 amino acid residues and mature proteins of 107 and 113 amino acid residues, respectively (Figure 3.8). To explore the activities of these two SPIs, the two SPI clones were used as PCR templates for the amplification of the SPI genes. The PCR primers were designed such that the PCR gene products contained the *Nco*I and *Xho*I restriction sites, respectively, at their 5′ and 3′ ends. The amplified products were analyzed using 1.2% agarose gel electrophoresis (Figure 3.9a), excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). The purified DNA fragments were tailed with an adenine nucleotide for ligation into the T&A cloning vector (Real Biotech Corporation). The recombinant plasmids were transformed into an *E. coli* XL1-Blue using CaCl$_2$ method. The transformants were selected using blue-white screening. The recombinant plasmid containing the gene of interest was examined with restriction enzyme digestion using *Eco*RI and *Bam*HI and 1.2% agarose gel electrophoresis (data not shown).

The resulting T&A clones were isolated and subjected to nucleotide sequencing to confirm the insert sequences of SPI*Pm*4 and 5. The *Nco*I–*Xho*I fragments containing the SPI*Pm*4 and 5 were prepared from the T&A clones and

subcloned into the *Nco*I–*Xho*I digested pET-28b(+) expression vector (Novagen). The recombinant clones were screened by plasmid extraction and verified by restriction enzyme (*Nco*1-*Xho*I) digestion as shown in Figure 3.9b.

For some reasons, the SPI*Pm*4 was not expressed well; it was then subcloned into a pET-32a(+) derivative, pVR500. The expression clones were named pSPI*Pm*4 and 5.

Figure 3.8 The open reading frames of SPI*Pm*4 (a) and 5 (b). The underlined amino acid sequences are signal peptides. The shaded and boxed amino acids are P1 amino acids. The shaded nucleotide sequences are the primer annealing sites for the PCR amplification of the SPI*Pm*4 and 5 gene fragments for the construction of protein expressing clones.
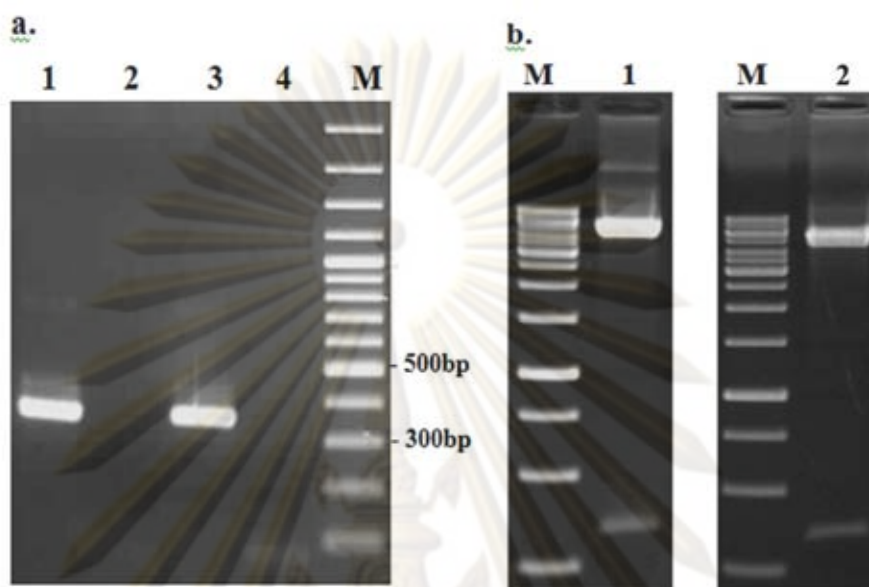
Figure 3.9 The amplified fragments of SPI*Pm*4 and SPI*Pm*5 (a) and the screening of the recombinant plasmid by digesting with *Nco*I and *Xho*I (b) on 1.2% agarose gel electrophoresis. For (a), laneM: GeneRuler™ 100 bp DNA ladder (Fermentas); lane1: the PCR product of SPI*Pm*4; lane2: negative control for SPI*Pm*4; lane3: the PCR product of SPI*Pm*5; lane4: negative control for SPI*Pm*5. For (b), laneM: GeneRuler™ 1 kb DNA ladder (Fermentas); lane1: pSPI*Pm*4, lane2: pSPI*Pm*5.

3.8.2 Over-expression of the SPI*Pm*4 and SPI*Pm*5 in the *E. coli*

The SPI*Pm*4 and 5 were over-expressed in an *E. coli* system using pVR500 [a pET-32a(+) derivative] and pET-28b(+), respectively, as expression vectors. The two expression clones, pSPI*Pm*4 and 5, were transformed into an *E. coli* Rosetta(DE3)pLysS. A single colony was cultured in LB medium containing 100 mg/ml ampicillin for SPI*Pm*4 or 70 mg/ml kanamycin for SPI*Pm*5 and 34 mg/ml chloramphenicol at 37 °C until the OD_{600} reached 0.6. Isopropyl-b-D-thiogalactoside (IPTG) was added to the final concentration of 1 mM to induce the expression. The

cells were harvested at 0, 1, 2, 3 and 4 h for SPI*Pm*4 and SPI*Pm*5 after IPTG induction and then analyzed by 18% SDS-PAGE.

The results showed that the protein expression was continuously increased after 3 h of IPTG induction (Figure 3.10). This condition was, therefore, employed for a large-scale expression of the recombinant SPI*Pm*4 and 5.
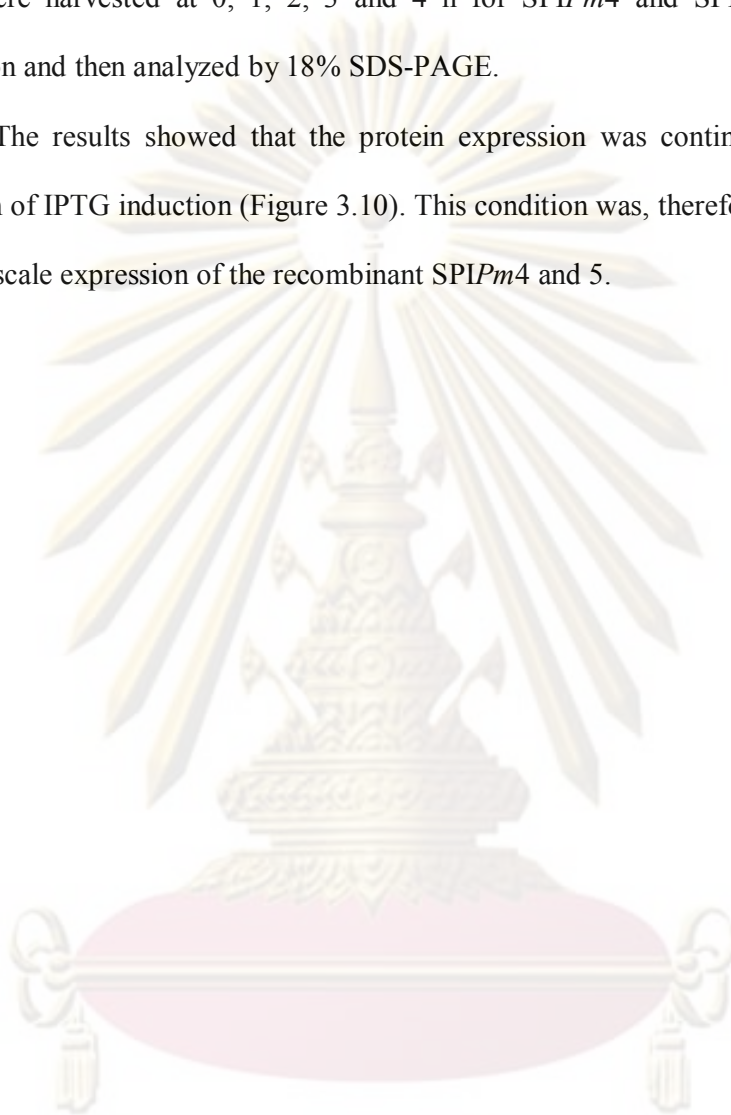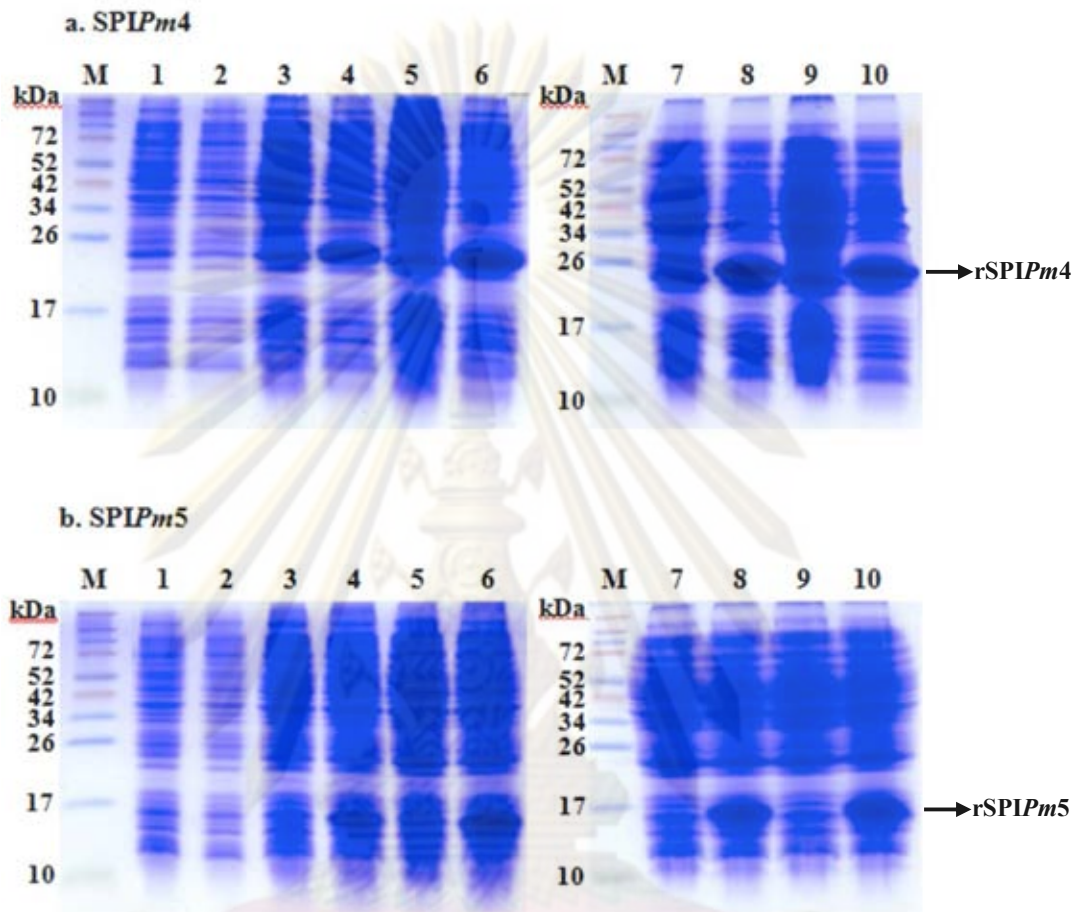
Figure 3.10 SDS-PAGE analysis of rSPI*Pm*4 (a) and rSPI*Pm*5 (b) expressed in *E. coli* Rosetta(DE3)pLysS at various times of induction. For rSPI*Pm*4 (a), lanes 1, 3, 5, 7 and 9 are the lysate of cells carrying pSPI*Pm*4 from uninduced cells at 0, 1, 2, 3 and 4 h, respectively. Lanes 2, 4, 6, 8 and 10 are the lysate of cells carrying pSPI*Pm*4 from induced cells at 0, 1, 2, 3 and 4 h, respectively. For rSPI*Pm*5 (b), lanes 1, 3, 5, 7 and 9 are the lysate of cells carrying pSPI*Pm*4 from uninduced cells at 0, 1, 2, 3 and 4 h, respectively. Lanes 2, 4, 6, 8 and 10 are the lysate of cells carrying pSPI*Pm*4 from induced cells at 0, 1, 2, 3 and 4 h, respectively. Lanes M are prestained protein marker (Fermentas).

3.9   Purification of recombinant protein

The recombinant proteins were efficiently purified by two purification steps for SPI*Pm*4 and a single purification step for SPI*Pm*5 using a nickel-NTA affinity column under nondenaturing condition. The rSPI*Pm*4 was expressed as a soluble fusion protein of about 25 kDa. From our calculation, the size of the cleaved thioredoxin and rSPI*Pm*4 were approximately equal. The rSPI*Pm*4 was purified with nickel-NTA column. Then, the thioredoxin was removed by enterokinase digestion. The digested protein was purified again by nickel-NTA column and analyzed for the identity and purity on 18% SDS-PAGE (Figure 3.11a). Small amount of SPI dimer could be seen as usual for cysteine-rich proteins.

The rSPI*Pm*5 was expressed about equally as soluble protein and inclusion bodies. The soluble rSPI*Pm*5 fraction was purified with nickel-NTA column and analyzed for its identity and purity on 18% SDS-PAGE (Figure 3.11b).
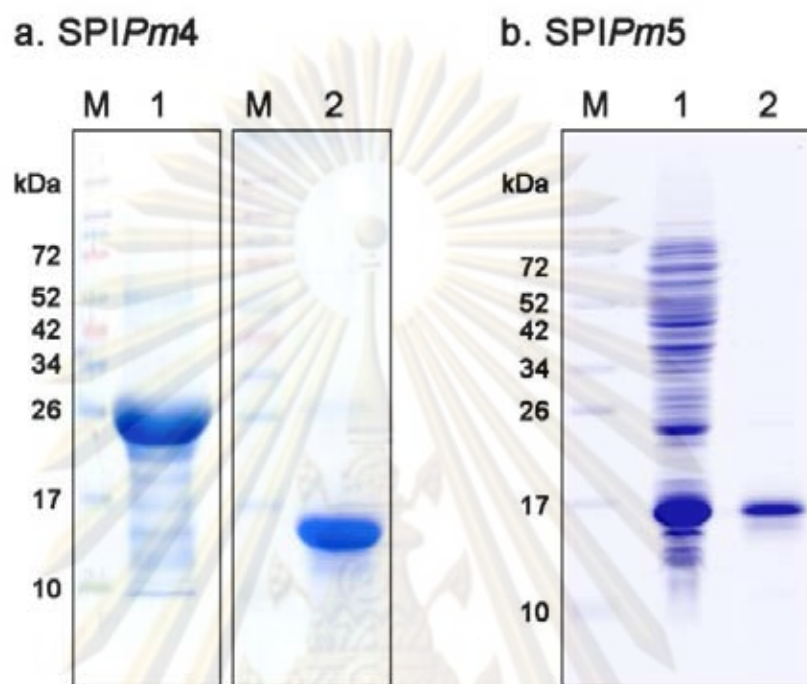
Figure 3.11 Recombinant expression of rSPI*Pm*4 and 5. The cell cultures were grown for 3 h after IPTG induction and harvested. The rSPIs were purified. For SPI*Pm*4 (a), the fusion protein was purified through nickel-NTA column (lane 1). After digestion with enterokinase, the SPI*Pm*4 was purified again with nickel-NTA column (lane 2). For SPI*Pm*5 (b), the cell lysate (lane 1) was purified through a nickel-NTA column giving a considerable pure SPI*Pm*5 preparation (lane 2). The rSPIs were kept track of using 18% SDS-PAGE. Lanes M are protein size marker.

3.10 Molecular mass determination of recombinant SPI by using

MALDI-TOF Mass Spectrometry

The molecular masses of rSPI*Pm*4 and 5 were determined using MALDI-TOP mass spectrometer to be 12.862 and 13.433 kDa, respectively (Figure 3.12). The calculated figures for rSPI*Pm*4 and 5 were 12.871 and 13.575 kDa, respectively. Without the start amino acid, the SPI*Pm*5 was calculated to be 13.444. It was possible that the rSPI*Pm*5 might have its start amino acid removed as had been shown for proteins produced in *E. coli*.
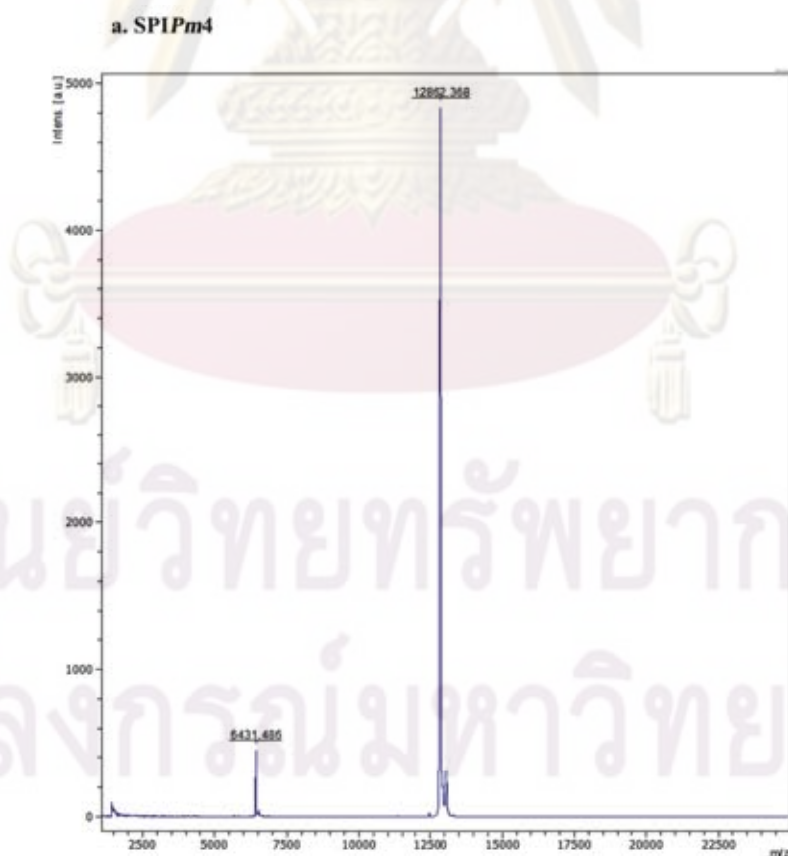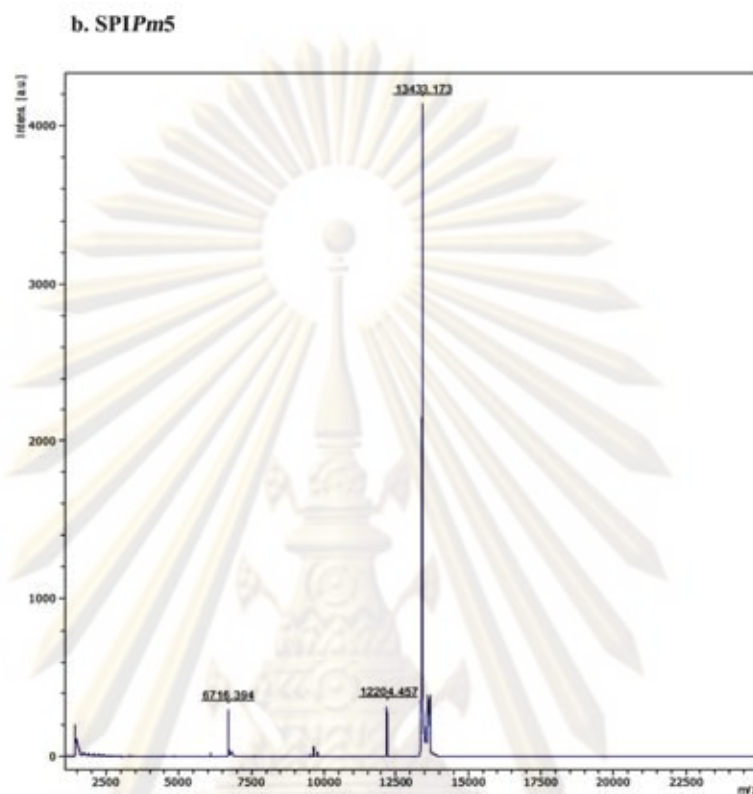
a. SPI*Pm*4

Figure 3.12 The MALDI-TOF spectra for recombinant SPI, (a) SPI*Pm*4 and (b) SPI*Pm*5.

3.11 Serine proteinase inhibitory assay

The inhibitor activity of recombinant serine proteinase inhibitor towards serine proteinase was tested by incubation of recombinant serine proteinase inhibitor and serine proteinase. The residual serine proteinase activity was determined by addition of chromogenic substrate which can be cleaved by the serine proteinase and released *p*-nitroaniline. Absorbance of chromophore p-nitroaniline produced was measured at 405 nm. Therefore, inhibition of serine proteinase hydrolysis of chromogenic substrate can be measured by following the change in absorbance 405 nm. The

purified recombinant proteins from nickel-NTA agarose column were tested for serine proteinase inhibitory activity. The recombinant inhibitors were assayed for their inhibitory activities against trypsin, chymotrypsin, elastase and subtilisin A (Figure 3.13). The mole ratios up to 50 of inhibitor against proteinase were used. For a strong inhibitor, the inhibition was readily seen at the mole ratios less than 10. The SPI*Pm*4 and 5 strongly inhibited subtilisin. SPI*Pm*5 also strongly inhibited elastase. The SPI*Pm*4 hardly inhibited trypsin and elastase. The SPI*Pm*5 did not inhibit trypsin and chymotrypsin. The inhibition against subtilisin of SPI*Pm*4 and 5 as well as that against elastase of SPI*Pm*5 were studied further for their kinetics.
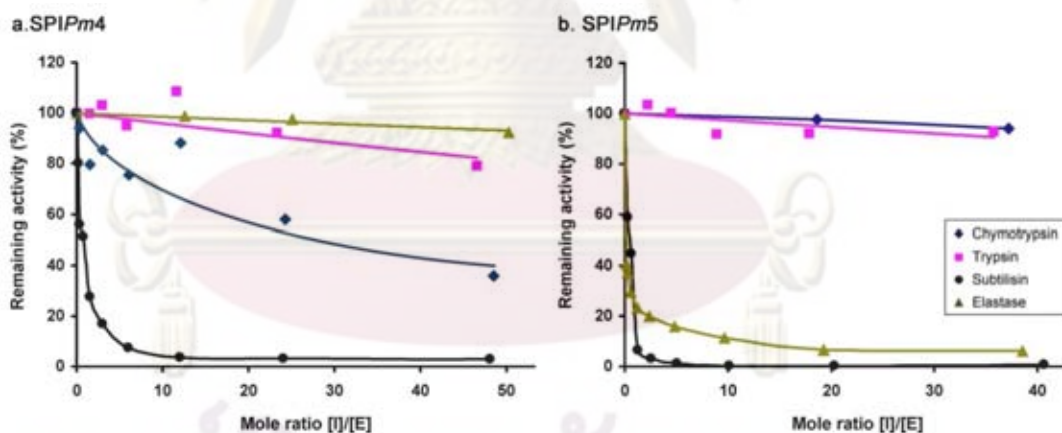
Figure 3.13 Proteinase inhibition assay of the recombinant SPI*Pm*4 (a) and 5 (b). The inhibitor was incubated at various ratios with chymotrypsin (◆), trypsin (■), subtilisin (●) and elastase (▲) in the reactions containing chromogenic substrates. The reduction in proteinase activity was determined.

3.12   Kinetic studies

3.12.1 Determination of subtilisin inhibition constant

The inhibition against subtilisin of SPI*Pm*4 and 5 were studied further for their kinetics. To gain more insight on the inhibition of subtilisin by the serine proteinase inhibitor, the inhibition reactions were performed by varying the substrate concentrations with fixed amount of proteinase in the presence of different inhibitor concentrations. The inhibition constant was determined by measuring its inhibitory effect on enzymatic hydrolysis of N-benzoyl-Phe-Val-Arg-*p*-nitroanilide substrate at 30°C for 15 min as described in section 2.13. The substrate saturation curve of subtilisin inhibition from SPI*Pm*4 and 5 were plotted as shown in Figure 3.14a and b. The Lineweaver-Burk plots or the inverted substrate saturation curves were constructed (Figure 3.15a and b). As expected, the inhibition was essentially competitive. The following Michaelis-Menten equation was applied to determine the dissociation constant of the subtilisin-inhibitor complex, K_i.

$$V = \frac{V\text{max} \cdot [S]}{K_{M,\,app} + [S]}$$

$$V = \frac{V\text{max} \cdot [S]}{K_M \left(1 + \dfrac{[I]}{K_i}\right) + [S]}$$

$$K_{M,\,app} = K_M \left(1 + \frac{[I]}{K_i}\right)$$

The inhibition constant was determined from the re-plot of the apparent $K_{M,app}$ values against the inhibitor concentration [I] at which they were obtained Figure 3.16a

and b. By extrapolating the plot to where the $K_{M,app}$ equaled zero, the value of K_i was obtained. The V_{max}s, K_Ms and K_is were calculated and summarized in Figure 3.15d. The inhibition constants (K_is) were in the range of nanomolar while the K_Ms were in the range of millimolar. The inhibition was, indeed, quite strong.

3.12.2 Determination of elastase inhibition constant

Using a similar experiment, the inhibition against elastase of SPI*Pm*5 was studied for its kinetics. The substrate was N-succinyl-Ala-Ala-Ala-p-nitroanilide. The substrate saturation curve of elastase inhibition was plotted as shown in Figure3.14c. The Lineweaver-Burk plots was constructed (Figure 3.15c). Again the inhibition was of competitive type. The inhibition constant was determined Figure 3.16c. The V_{max}, K_M and K_i were calculated and summarized in Figure 3.15d. The inhibition constants (K_i) was in the range of nanomolar while the K_M were in the range of millimolar. The inhibition was, similarly, quite strong.
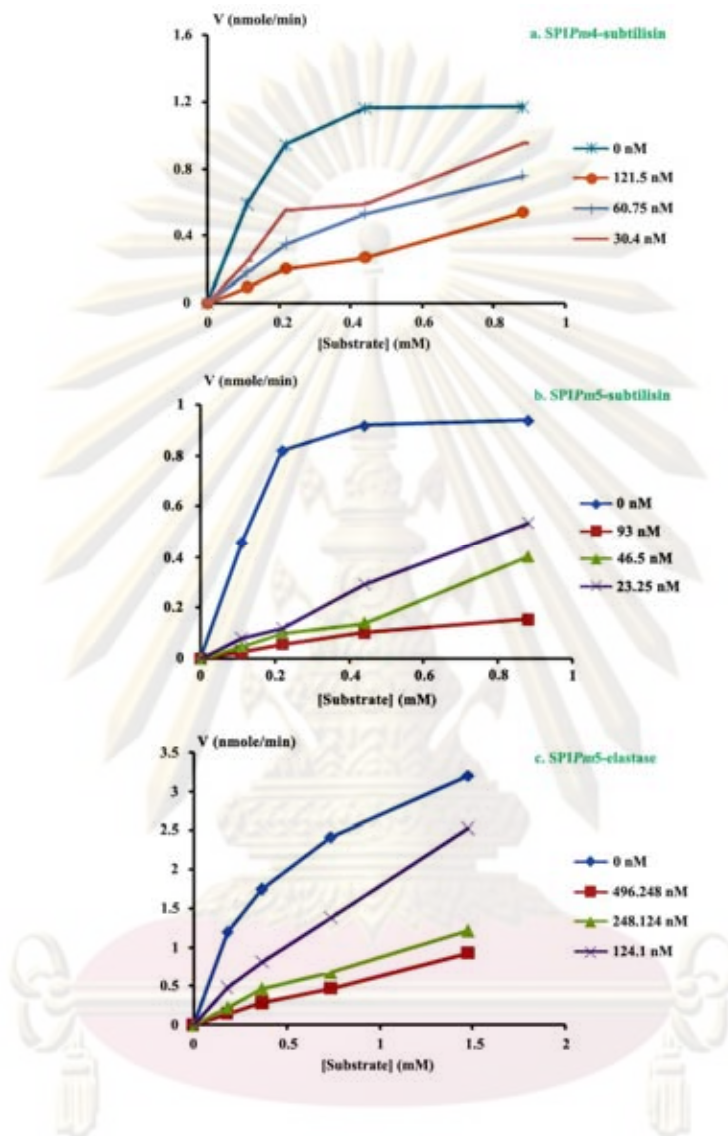
Figure 3.14 Substrate saturation curve of subtilisin and elastase with and without SPI. The substrate saturation curves of subtilisin inhibition are (a) SPI*Pm*4 and (b) SPI*Pm*5. The substrate saturation curve of elastase inhibition by SPI*Pm*5 is (c).
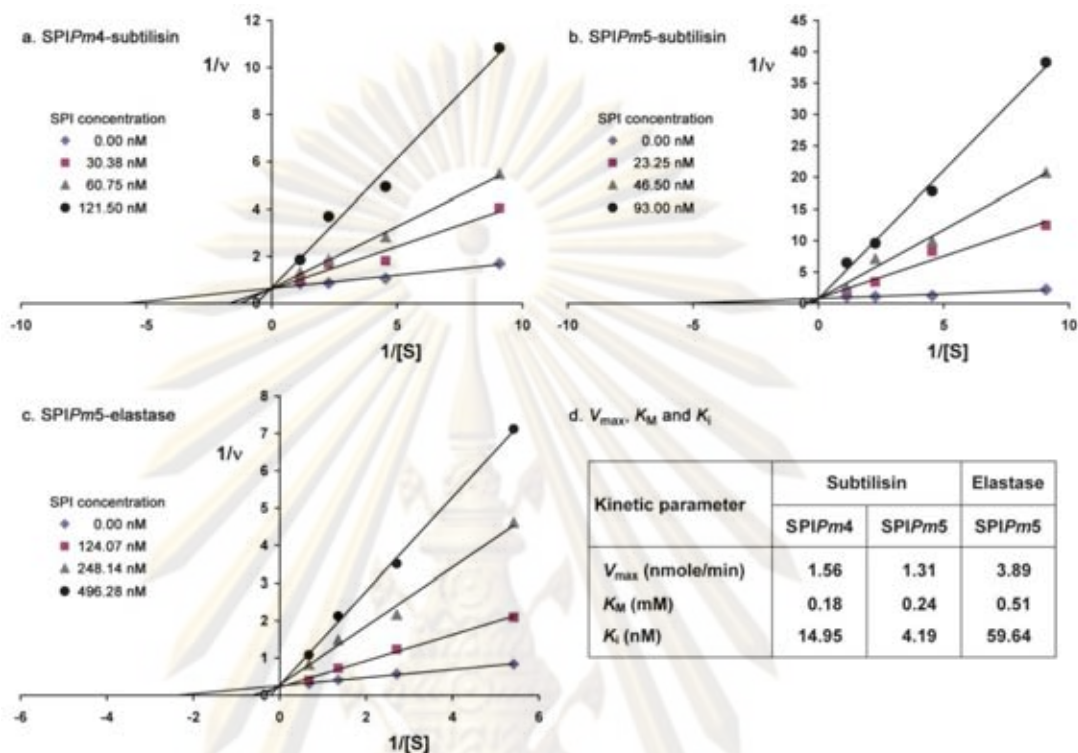
Figure 3.15 Lineweaver-Burg plots of the inhibitory reactions between SPIs and proteinases. The inhibitory activity of SPI*Pm*4 and 5 were kinetically studied against subtilisin (a and b, respectively). The SPI*Pm*5 was also studied against elastase (c). The proteinases were assayed at various substrate concentrations in the presence of various concentrations of inhibitors as indicated in the plots. The calculated V_{max}s, K_Ms and K_is are shown (d).
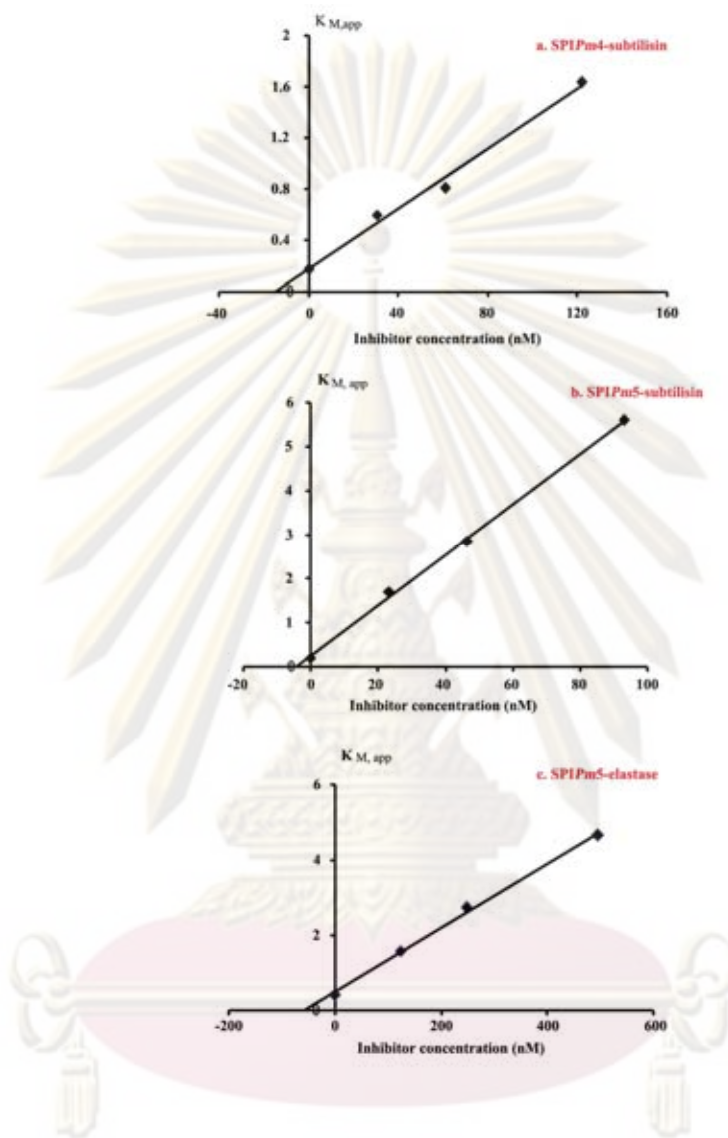
Figure 3.16 The plot of $K_{M, app}$ of subtilisin (a and b for SPI*Pm*4 and 5) and elastase (c for SPI*Pm*5) inhibition against the concentrations of inhibitors for the determination of K_i.

3.13 Bacterial growth inhibition

It had been a thought that a potent subtilisin inhibitor might have negative effect on bacterial growth particularly that of *B. subtilis* as those observed with a serine proteinase inhibitor from frog eggs, ranaserpin, and a Kazal inhibitor from *P. monodon*, SPI*Pm*2 (Donpudsa et al., 2008; Han et al., 2008). The growth inhibition activity of the SPI*Pm*4 and 5 were tested. By incubating the diluted culture of bacteria with considerably higher concentrations of the inhibitors than the SPI*Pm*2, 20 mM SPI*Pm*4 or 10 mM SPI*Pm*5, and monitoring the growth of bacteria up to 16 h, it was found that both SPIs had no bacteriostatic effect on the growth of Gram-positive bacteria: *B. subtilis*, *B. megaterium*, *S. aureus*, and Gram-negative bacteria: *V. harveyi* 639, *E. coli* JM109 (Figure 3.17 for only *B. subtilis*).
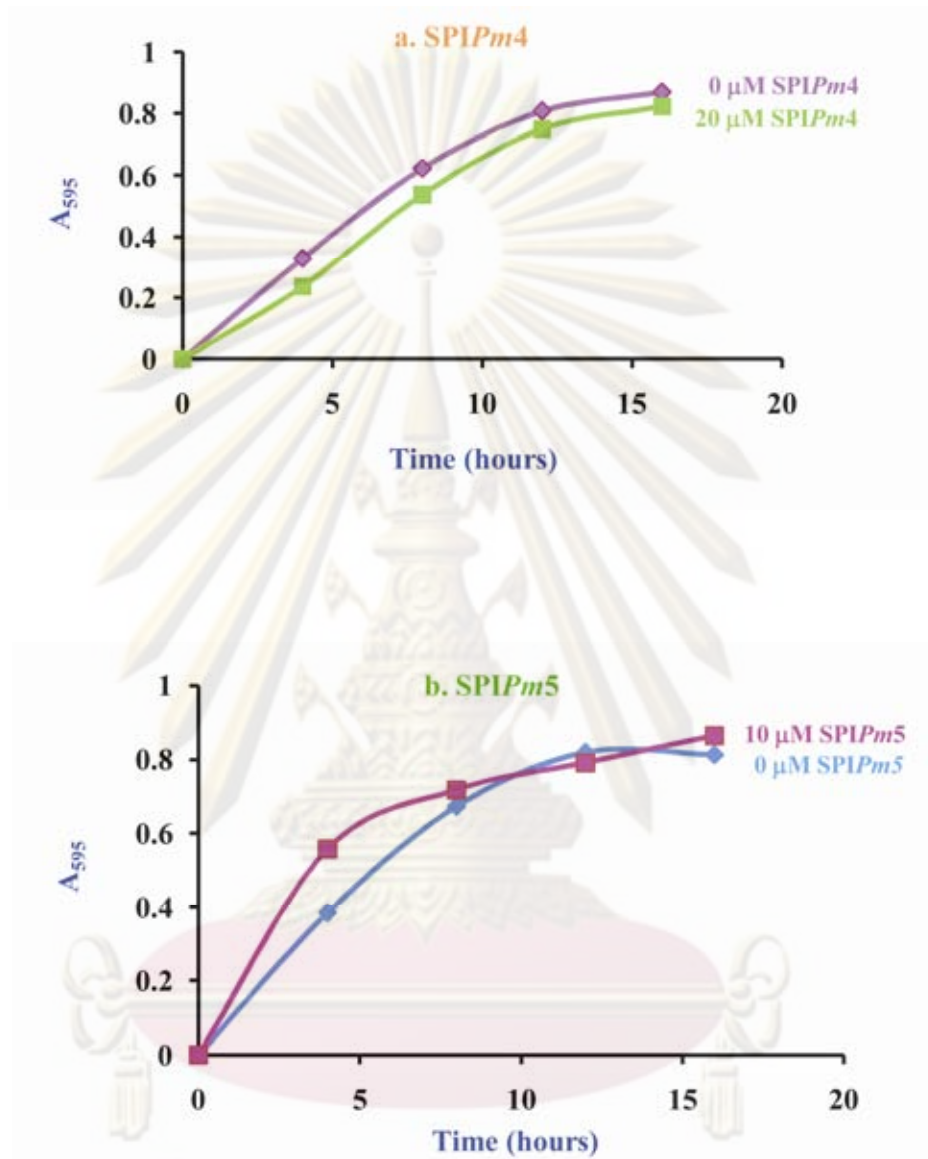
Figure 3.17 Bacteriostatic activity of SPI*Pm*4 (a) and 5 (b) on *B. subtilis*.

CHAPTER IV

DISCUSSION

The establishment of *Penaeus monodon* EST database and the clustering of the EST data had enabled us to identify a number of the Kazal-type serine proteinase inhibitors in the shrimp. We found in the *P. monodon* that there were at least 9 types of Kazal SPIs. Most of the EST clone members of the SPIs were derived from the haemocyte libraries indicating that the haemocyte was the main site of SPI biosynthesis. Since the blood circulation was the major site of immune defense system in shrimp, the notion that Kazal SPIs were involved in the host defense innate immune response was also supported. This was in line with a recent finding that an SPI from *F. chinensis* was up-regulated in the haemocytes in response to white spot syndrome virus (WSSV) infection (Kong et al., 2009).

By using amino acid and nucleotide sequence comparison, we found no indication of any relationships due to alternate splicing of the pre-mRNA among these inhibitors. Nevertheless, the Kazal domains might evolve from the common ancestor for they were very much similar with certain degree of homology both in the amino acid and nucleotide sequences. These SPIs were multi-domain inhibitors with multiple Kazal domains of 2–7 domains. The important reactive P1 amino acids were quite variable.

Among the *P. monodon* Kazal SPIs, the SPI*Pm*2, a five-domain Kazal inhibitor, has been extensively studied as it is the most abundant Kazal inhibitor in the shrimp (Jarasrassamee et al., 2005; Somprasong et al., 2006). Amino acid sequence

comparison indicated that the four-domain Kazal inhibitor from *Litopenaeus vannamei* was a four-domain orthologous variant of the five-domain SPI*Pm*2 (Jimenez-Vega et al., 2005).

Vibrio harveyi is a Gram-negative luminescent marine bacterium found both as a free-living state (Makemson et al., 1986; Ramesh et al., 1989; Lavilla-Pitogo et al., 1990) and a commensal organism in the enteric contents of marine animals (Reichelt et al., 1973; Hoyt et al., 1982). It has been recognized as a primary pathogen of many commercially cultured invertebrate species, such as the black tiger shrimp. *V. harveyi* can cause up to 100% mortality of larvae in the hatchery stage of penaeid culture (Lavilla-Pitogo et al., 1990; Chantanachookin et al., 1993). Studying the susceptibility of shrimp to *Vibrio* infection, the medial lethal dose, LD_{50} figures are used as a general indicator of acute toxicity (Wang et al., 1981). In this study, *Vibrio* infection was performed by intramuscular injection of 10^5 CFU of *V. harveyi* 639 at the second abdominal segment of *P. monodon* (Ponprateep et al., 2009).

Semi-quantitative RT-PCR was carried out to analyze the tissue distribution of seven Kazal-type SPIs. The result revealed that the Kazal-type SPIs mRNAs were highly expressed in the haemocytes, while low expressions could be found in the intestine, hepatopencreas, eyestalk, epipodite, antennal gland and lymphoid of normal healthy shrimp (Figure 3.4). This was consistent with the previous reports that the haemocytes of insects and other arthropods contained relatively high concentrations of serine proteinase inhibitors (Kanost, 1999). The recent studies also reported that the Kazal-type SPI was found mainly in the haemocytes (Li et al.; Li et al., 2009; Soonthornchai et al., 2009; Wang et al., 2009a; b).

Interestingly, only the SPI*Pm*7 were expressed in all tissues tested but highly particularly in epipodite and gill. We speculated that the SPIs in these tissues might originate from the haemocytes. Since the epipodite and gill are, respectively, involved in ion-homeostasis and respiration, they are exposed directly to the environment and threatened by the pathogens more severely and frequently. The Kazal SPIs are, therefore, essential and required as the initial defense against the pathogens from the surrounding water.

It would be extremely interesting to find out whether the seven Kazal-type SPIs were involved in the immune response against pathogenic infection. In the present study, the temporal expression of the Kazal-type mRNAs in the haemocyte of *P. monodon* challenged by bacteria was examined by RT-PCR analysis. A time-dependent pattern of Kazal-type expression was observed in the haemocytes after *V. harveyi* 639 challenge. The mRNA expression level of SPI*Pm*1 (Figure 3.6a) was significantly increased at 6 hpi and returned to normal level at 12 hpi. For the remaining genes: SPI*Pm*2, 6, 7 and 9, there were hardly any differences in mRNA expression level compared with the control group injected with normal saline. The expression profiles of these SPI genes were almost similar with that of a four-Kazal-domain from *L. vannamei* (Jimenez-Vega et al., 2005).

In this study, the SPI*Pm*4 and 5 were chosen. They were identified in heat-treated or heat-stressed shrimps. They are two-domain Kazal type inhibitors with the P1 residues Ile and Phe in SPI*Pm*4 and Leu and Thr in SPI*Pm*5. A recently isolated SPI from *F. chinensis* is found to be a SPI*Pm*5 ortholog with different P1 amino acids in the second domain, Thr in SPI*Pm*5 and Ala in SPI from *F. chinensis* (Kong et al., 2009).

Being identified from the heat-treated haemocyte cDNA libraries, we had tested whether it was also true that the mRNA of such SPIs were increased upon heat treatment using RT-PCR. The experiment was done upon the chosen SPI*Pm*5 and found that it is up-regulated after the shrimp were heat-treated. This finding raised the possibility that the SPIs were also involved in stress responses. Further study has been conducted on this issue.

For the proteinase inhibition study, the expression clones for the two SPI genes were engineered. The recombinant proteins were produced and tested for their inhibitory activities against four proteinases: subtilisin, chymotrypsin, trypsin and elastase. It was found that the SPI*Pm*4 strongly inhibited subtilisin and weakly inhibited chymotrypsin. The SPI*Pm*5 strongly inhibited subtilisin and elastase. It is well known that SPIs with P1 Lys and Arg are apt to inhibit trypsin. Those with P1 amino acid having bulky hydrophobic side chains, for example Tyr, Phe and Leu, inhibit chymotrypsin (Laskowski et al., 1980). It is possible too that the Kazal domain in certain sequence contexts may be inactive or moderately active like that observed in particular domains of SPI from the crayfish *Pacifastacus leniusculus*, SPI*Pm*2 from *P. monodon* and the MRPINK from *M. rosenbergii* (Johansson et al., 1994; Donpudsa et al., 2008; Li et al., 2009).

In this study, the P1 Phe in SPI*Pm*4 might contribute to the weak inhibition of chymotrypsin. The strong inhibition of subtilisin was somewhat puzzling. The two domains might contribute to the strong inhibition of subtilisin. This was possible because the P1 specificity against subtilisin was somewhat broader. For example, the P1 Ala and Glu in SPI*Pm*2 (Donpudsa et al., 2008), Thr in GmSPI 2 from *Galleria mellonella* L. (Nirmala et al., 2001), Leu in greglin from the desert locust

Schistocerca gregaria (Brillard-Bourdet et al., 2006), Asp in EPI1 from the oomycete *P. infestans* (Tian et al., 2004) and even Arg in CmPI-II from the marine snail *Cenchritis muricatus* (Gonzalez et al., 2007) were P1 amino acids of subtilisin inhibitors. For the SPI*Pm*5 whose P1 amino acids were Leu and Thr, strong inhibition of subtilisin and elastase was observed. It was tempting to conclude that the Thr was responsible for subtilisin inhibition and Leu for elastase inhibition. The clue was from the study of a four-domain Kazal SPI TgPI-1 from an obligate intracellular parasite of human *T. gondii* (Morris et al., 2002). The inhibitor with its P1 Arg, Arg, Leu and Leu was able to inhibit trypsin, chymotrypsin, pancreatic elastase, and neutrophil elastase. Nevertheless, the actual inhibitory activities of the Kazal domains remained to be elucidated.

Most Kazal inhibitors are potent inhibitors with the inhibition constants, K_is, in the range of nanomolar. The potent inhibitory activities of SPI*Pm*4 against subtilisin and SPI*Pm*5 against subtilisin and elastase led us to study their kinetics. As anticipated, the inhibition constants of SPI*Pm*4 and 5 against subtilisin were 14.95 and 4.19 nM. That of SPI*Pm*5 against elastase was 59.64 nM. The inhibition constants were higher compared with 0.52 nM against subtilisin and 3.27 nM against elastase of SPI*Pm*2 (Somprasong et al., 2006). In SPI*Pm*2, there are 3 Kazal domains responsible for the inhibition of subtilisin (Donpudsa et al., 2008). It is, therefore, not surprising that the inhibition constant is lower. In fact, the inhibition constants against subtilisin of SPI*Pm*4 and 5 were comparable to those individual Kazal domains of SPI*Pm*2. Nevertheless, the inhibition against elastase of SPI*Pm*5 was 20-fold less active than that of a single responsible domain in SPI*Pm*2.

Since the SPI*Pm*4 and 5 potently inhibited subtilisin, it was thought that they might possess bacteriostatic activity against some bacteria, particularly *B. subtilis*, like the SPI*Pm*2 (Donpudsa et al., 2008). It was found, however, that they did not inhibit the growth of bacteria both Gram-positive and Gram-negative. Thus, the notion that subtilisin inhibitors inhibit the growth of *B. subtilis* did not always hold true (Han et al., 2008).
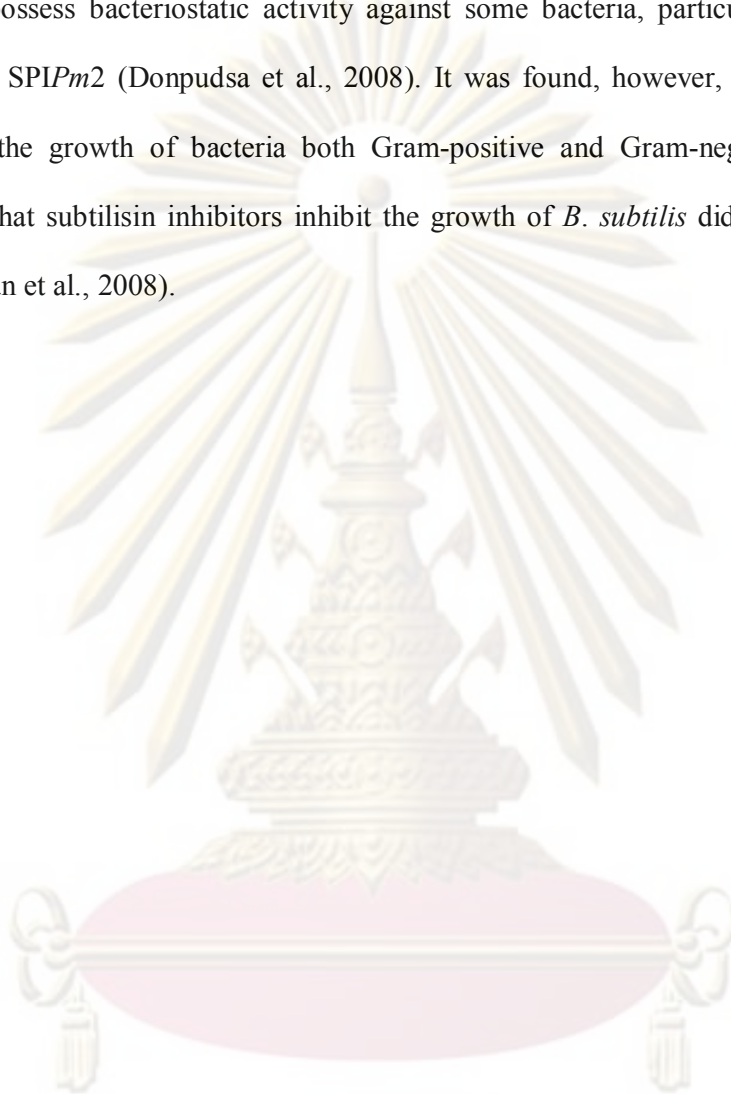
CHAPTER V

CONCLUSIONS

1. There are nine different Kazal-type SPIs, namely SPI*Pm*1-9, identified from the cDNA libraries of hemocyte, hepatopancreas, hematopoietic tissue, ovary and lymphoid in the *Penaeus monodon* EST database (http://pmonodon.biotech.or.th/).

2. The semi-quantitative RT-PCR of seven Kazal-type SPI genes (SPI*Pm*1, SPI*Pm*2, SPI*Pm*4, SPI*Pm*5, SPI*Pm*6, SPI*Pm*7 and SPI*Pm*9) after *V. harveyi* challenge showed that the expression of SPI*Pm*1 was significantly increased for 1.92 fold at 6 hpi and returned to normal level at 12 hpi. Other SPI genes: SPI*Pm*2, 6, 7 and 9 showed hardly any differences in mRNA expression level.

3. RT-PCR study of the SPI*Pm*5 gene revealed that it was up-regulated in response to heat treatment suggesting the involvement of SPIs in stress responses.

4. The molecular masses of SPI*Pm*4 and 5 were determined using MALDI-TOF mass spectrometry to be 12.862 and 13.575 kDa, respectively.

5. The SPI*Pm*4 exhibited potent inhibitory activity against subtilisin and weakly inhibited chymotrypsin but not trypsin and elastase.

6. The SPI*Pm*5 exhibited potent inhibitory activity against subtilisin and elastase, but not trypsin and chymotrypsin.

7. The inhibition was a competitive type with inhibition constants (Ki) of 14.95 nM for SPI*Pm*4 against subtilisin, 4.19 and 59.64 nM for SPI*Pm*5 against subtilisin and elastase, respectively.

8. They had no bacteriostatic effect against Gram-positive bacteria: *Bacillus subtilis*, *B. megaterium*, *Staphylococcus aureus*, and Gram-negative bacteria: *V. harveyi* 639, *E. coli* JM109.

REFERENCES

Aeschlimann D, and Pualsson, M. (1994). "Transglutaminases: protein crosslinking enzynes in tissues and body fluids". *Thromb. Haemost* **71**:402-415.

Aono H, Mori K. (1996). "Interaction between hemocytes and plasma is necessary for hemolymph coagulation in the spiny lobster, *Panulirus japonicus*". *Comparative Biochemistry and Physiology - A Physiology* **113**(3):301-305.

Ariki S, Koori K, Osaki T, Motoyama K, Inamori KI, Kawabata SI. (2004). "A serine protease zymogen functions as a pattern-recognition receptor for lipopolysaccharides". *Proceedings of the National Academy of Sciences of the United States of America* **101**(4):953-958.

Aspán A, and Soderhall, K. (1991). "Purification of prophenoloxidase from crayfish blood-cells, and its activation by an endogenous serine proteinase". *Insect Biochem* **21**:363-373.

Aspán A, Huang T., Cerenius, L., Soderhall, K. (1995). "cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation". *Proc. Natl. Acad. Sci.* **92**:939-943.

Barrett AJ, Rawlings ND. (1995). "Families and Clans of Serine Peptidases". *Archives of Biochemistry and Biophysics* **318**(2):247-250.

Batchelor AK, Boutilier K, Miller SS, Labb H, Bowman L, Hu M et al. (2000). "The seed coat-specific expression of a subtilisin-like gene, SCS1, from soybean". *Planta* **211**(4):484-492.

Baticados MCL, Lavilla-Pitogo, C. R., Cruz-Lacierda, E. R., de la pena, L. D., and Sunaz, N. A. (1990). "Studies on the chemical control of luminous bacteria *Vibrio*

harveyi and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water". *Dis. Aquat. Org.* **9**:133-139.

Bode W, Huber R. (2000). "Structural basis of the endoproteinase-protein inhibitor interaction". *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1477**(1-2):241-252.

Bode Wea. (1992). "Natural protein proteinase inhibitors and their interaction with proteinases". *European Journal of Biochemistry* **204**(2):433-451.

Bradford MM. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding". *Anal Biochem* **72**:248-254.

Brillard-Bourdet M, Hamdaoui A, Hajjar E, Boudier C, Reuter N, Ehret-Sabatier L et al. (2006). "A novel locust (*Schistocerca gregaria*) serine protease inhibitor with a high affinity for neutrophil elastase". *Biochem J* **400**(3):467-476.

Campos ITN, Amino R, Sampaio CAM, Auerswald EA, Friedrich T, Lemaire HG et al. (2002). "Infestin, a thrombin inhibitor presents in Triatoma infestans midgut, a Chagas' disease vector: Gene cloning, expression and characterization of the inhibitor". *Insect Biochemistry and Molecular Biology* **32**(9):991-997.

Chantanachookin C, Boonyaratpalin S, Kasornchandra J, Direkbusarakom S, Ekpanithanpong U, Supamataya K et al. (1993). "Histology and ultrastructure reveal a new granulosis-like virus in *Penaeus monodon* affected by yellow-head disease". *Diseases of Aquatic Organisms* **17**(2):145-157.

Chen MY, Hu KY, Huang CC, Song YL. (2005). "More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation". *Developmental and Comparative Immunology* **29**(12):1003-1016.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG et al. (2003). "Multiple sequence alignment with the Clustal series of programs". *Nucleic Acids Research* **31**(13):3497-3500.

Chou H-Y, Huang C-Y, Wang C-H, Chiang H-C, Lo C-F. (1995). "Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan". *Diseases of Aquatic Organisms* **23**(3):165-173.

Christeller JT. (2005). "Evolutionary mechanisms acting on proteinase inhibitor variability". *FEBS Journal* **272**(22):5710-5722.

Cowley JA, Dimmock CM, Spann KM, Walker PJ. (2000). "Detection of Australian gill-associated virus (GAV) and lymphoid organ virus (LOV) of *Penaeus monodon* by RT-nested PCR". *Dis Aquat Organ* **39**(3):159-167.

Cowley JA, Dimmock CM, Wongteerasupaya C, Boonsaeng V, Panyim S, Walker PJ. (1999). "Yellow head virus from Thailand and gill-associated virus from Australia are closely related but distinct prawn viruses". *Diseases of Aquatic Organisms* **36**(2):153-157.

Cruciani RA, Barker JL, Zasloff M, Chen HC, Colamonici O. (1991). "Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation". *Proc Natl Acad Sci U S A* **88**(9):3792-3796.

Destoumieux-Garzon D, Saulnier D, Garnier J, Jouffrey C, Bulet P, Bachere E. (2001). "Crustacean immunity. Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge". *J Biol Chem* **276**(50):47070-47077.

Destoumieux D, Bulet P, Loew D, Van Dorsselaer A, Rodriguez J, Bachere E. (1997). "Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda)". *J Biol Chem* **272**(45):28398-28406.

Dhar AK, Roux MM, Klimpel KR. (2002). "Quantitative assay for measuring the Taura syndrome virus and yellow head virus load in shrimp by real-time RT-PCR using SYBR Green chemistry". *Journal of Virological Methods* **104**(1):69-82.

Donpudsa S, Tassanakajon A, Rimphanitchayakit V. (2009). "Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp Penaeus monodon". *Developmental & Comparative Immunology* **33**(4):481-488.

Dunn DL, Simmons RL. (1982). "Fibrin in peritonitis. III. The mechanism of bacterial trapping by polymerizing fibrin". *Surgery* **92**(3):513-519.

Flegel TW. (1997). "Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand". *World Journal of Microbiology & Biotechnology* **13**(4):433-442.

Gonzalez Y, Pons T, Gil J, Besada V, Alonso-del-Rivero M, Tanaka AS et al. (2007). "Characterization and comparative 3D modeling of CmPI-II, a novel 'non-classical' Kazal-type inhibitor from the marine snail *Cenchritis muricatus* (Mollusca)". *Biol Chem* **388**(11):1183-1194.

Greenberg CS, Birckbichler PJ, Rice RH. (1991). "Transglutaminases: Multifunctional cross-linking enzymes that stabilize tissues". *Faseb Journal* **5**(15):3071-3077.

Gross PS, Bartlett TC, Browdy CL, Chapman RW, Warr GW. (2001). "Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, Litopenaeus vannamei, and the Atlantic White Shrimp, L. setiferus". *Developmental & Comparative Immunology* **25**(7):565-577.

Hamilton JMU, Simpson DJ, Hyman SC, Ndimba BK, Slabas AR. (2003). "Ara12 subtilisin-like protease from Arabidopsis thaliana: Purification, substrate specificity and tissue localization". *Biochemical Journal* **370**(1):57-67.

Han Y, Yu H, Yang X, Rees HH, Liu J, Lai R. (2008). "A serine proteinase inhibitor from frog eggs with bacteriostatic activity". *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **149**(1):58-62.

Hancock RE, Diamond G. (2000). "The role of cationic antimicrobial peptides in innate host defences". *Trends Microbiol* **8**(9):402-410.

Hergenhahn H, Aspan, A., and Söderhäll, K. (1987). "Purification and characterization of a high-M_r proteinase inhbitor of pro-phenol oxidase activation from crayfish plasma". *Biochem. J.* **248**:223-228.

Hoebe K, Janssen E, Beutler B. (2004). "The interface between innate and adaptive immunity". *Nature Immunology* **5**(10):971-974.

Hoffmann JA, Kafatos FC, Janeway Jr CA, Ezekowitz RAB. (1999). "Phylogenetic perspectives in innate immunity". *Science* **284**(5418):1313-1318.

Hoyt PR, Sizemore RK. (1982). "Competitive dominance by a bacteriocin-producing *Vibrio harveyi* strain". *Applied and Environmental Microbiology* **44**(3):653-658.

Huang CC, Sritunyalucksana K, Soderhall K, Song YL, Yeh MS, Huang CJ et al. (2004). "Molecular cloning and characterization of tiger shrimp (*Penaeus monodon*) transglutaminase". *Dev Comp Immunol* **28**(4):279-294.

Hung SH, Hedstrom L. (1998). "Converting trypsin to elastase: Substitution of the S1 site and adjacent loops reconstitutes esterase specificity but not amidase activity". *Protein Engineering* **11**(8):669-673.

Inouye K, Miwa, S., Oseko, N., Nakano, H., Kimura, T., Momoyama, K., and Hiraoka, M. (1994). "Mass mortalitied of cultured Kuruma shrimp *Penaeus japonicus* in Japan in 1993: electron micriscopic evidence of the causative virus". *Fish Pathol.* **29**:149-158.

Iwanaga S, Bok LL. (2005). "Recent advances in the innate immunity of invertebrate animals". *Journal of Biochemistry and Molecular Biology* **38**(2):128-150.

Janeway Jr CA. (1998). "Approching the asymptole evolution and revolution in immunology Colo. Spring Habour Symp.". *Quant. Biol.* **54**:1-13.

Jarasrassamee B, Supungul P, Panyim S, Klinbunga S, Rimphanichayakit V, Tassanakajon A. (2005). "Recombinant expression and characterization of five-domain Kazal-type serine proteinase inhibitor of black tiger shrimp (*Penaeus monodon*)". *Marine Biotechnology* **7**(1):46-52.

Jimenez-Vega F, Vargas-Albores F. (2005). "A four-Kazal domain protein in *Litopenaeus vannamei* hemocytes". *Developmental and Comparative Immunology* **29**(5):385-391.

Jiravanichpaisal P, Lee BL, Soderhall K. (2006). "Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization". *Immunobiology* **211**(4):213-236.

Jitrapakdee S, Unajak S, Sittidilokratna N, Hodgson RA, Cowley JA, Walker PJ et al. (2003). "Identification and analysis of gp116 and gp64 structural glycoproteins of yellow head nidovirus of *Penaeus monodon* shrimp". *J Gen Virol* **84**(Pt 4):863-873.

Kanchanaphum P, Wongteerasupaya C, Sitidilokratana N, Boonsaeng V, Panyim S, Tassanakajon A et al. (1998). "Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp *Penaeus monodon*". *Diseases of Aquatic Organisms* **34**(1):1-7.

Kanost MR. (1999). "Serine proteinase inhibitors in arthropod immunity". *Developmental and Comparative Immunology* **23**(4-5):291-301.

Karunasasagas I, Otta, S. K., and Sagar, I. K. (1997). "Histopathological and bacteriological study of white spot syndrome of *Penaeus monodon* along the west cost of India". *Aquaculture* **153**:9-13.

Kim Y-A, 2006. Haematopoiesis, Kazal Inhibitors and Crustins in a Crustacean, Uppsala, 1-48 pp.

Kobayashi T, Kobayashi E, Sato S, Hotta Y, Miyajima N, Tanaka A et al. (1994). "Characterization of cDNAs Induced in Meiotic Prophase in Lily Microsporocytes". *DNA Res* **1**(1):15-26.

Kopácek P, Hall M, Soderhall K. (1993). "Characterization of a clotting protein, isolated from plasma of the freshwater crayfish *Pacifastacus leniusculus*". *Eur J Biochem* **213**(1):591-597.

Kurth T, Ullmann D, Jakubke HD, Hedstrom L. (1997). "Converting trypsin to chymotrypsin: Structural determinants of S1' specificity". *Biochemistry* **36**(33):10098-10104.

Laskowski Jr M, Qasim MA. (2000). "What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes?". *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology* **1477**(1-2):324-337.

Laskowski M, Kato I. (1988). "Protein inhibitors of proteinases". *Annu Rev Riochem* **49**:593-626.

Lavilla-Pitogo CR, Baticados MCL, Cruz-Lacierda ER, de la Pena LD. (1990). "Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines". *Aquaculture* **91**(1-2):1-13.

Li Y, Qian YQ, Ma WM, Yang WJ. (2009). "Inhibition mechanism and the effects of structure on activity of Male Reproduction-Related Peptidase Inhibitor Kazal-

Type (MRPINK) of *Macrobrachium rosenbergii*". *Marine Biotechnology* **11**(2):252-259.

Lightner DV, 1983. Diseases of cultured penaeid shrimp. In: McVey, I.J. (Ed.), Handbook of Mariculture, Boca Raton.

Lightner DV, 1988. *Vibrio* disease of penaeid shrimp. In: In C. J. Sinderman, D.V.L. (Ed.), Disease Dianosis and Control in North American Marine Aquaculture. Elsevier, Amsterdam, pp. 42-47.

Lightner DV. (1996). "A Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp". *World aquaculture society*.

Lo CF, Ho CH, Peng SE, Chen CH, Hsu HC, Chiu YL et al. (1996). "White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods". *Disease of Aquatic Organisms* **27**(3):215-225.

Lu W, Apostol I, Qasim MA, Warne N, Wynn R, Zhang WL et al. (1997). "Binding of amino acid side-chains to S1 cavities of serine proteinases". *Journal of Molecular Biology* **266**(2):441-461.

Lu Y, Tapay LM, Loh PC. (1996). "Development of a nitrocellulose-enzyme immunoassay for the detection of yellow-head virus from penaeid shrimp". *Journal of Fish Diseases* **19**(1):9-13.

Martin GG, Omori JEHS, Chong C, Hoodbhoy T, McKrell N. (1991). "Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans". *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **100**(3):517-522.

Mayo MA. (2002). "A summary of taxonomic changes recently approved by ICTV". *Archives of Virology* **147**(8):1655-1656.

Mende K, Lange U, Nowak G. (2004). "Three recombinant serine proteinase inhibitors expressed from the coding region of the thrombin inhibitor dipetalogastin". *Insect Biochemistry and Molecular Biology* **34**(9):971-979.

Millar DA, and Ratcliffe, N. A., 1994. Invertebrated. In: Turner, I.R.J. (Ed.), Immunology: A Comparative Approach, Chichester: Wiley, pp. 29-68.

Morris MT, Coppin A, Tomavo S, Carruthers VB. (2002). "Functional analysis of Toxoplasma gondii protease inhibitor 1". *Journal of Biological Chemistry* **277**(47):45259-45266.

Murakami T, Niwa M, Tokunaga F, Miyata T, Iwanaga S. (1991). "Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes". *Chemotherapy* **37**(5):327-334.

Nadala ECB, Loh PC. (2000). "Dot-blot nitrocellulose enzyme immunoassays for the detection of white-spot virus and yellow-head virus of penaeid shrimp". *Journal of Virological Methods* **84**(2):175-179.

Nash GL, 1990. *Penaeus monodon* grow-out diseases. In: Singh, I.M.B.N.H.d.S.T. (Ed.), Technical and Economic Aspects of Shrimp Farming, Kuala Lumpur, Malaysia.

Nelsen NS, Li Z, Warner AL, Matthews BF, Knap HT. (2004). "Genomic Polymorphism Identifies a Subtilisin-Like Protease near the Rhg4 Locus in Soybean". *Crop Science* **44**(1):265-273.

Neurath H. (1985). "Proteolytic enzymes, past and present". *Federation Proceedings* **44**(14):2907-2913.

Neurath H. (1989). "Proteolytic processing and physiological regulation". *Trends in Biochemical Sciences* **14**(7):268-271.

Neurath H, Walsh KA. (1976). "Role of proteolytic enzymes in biological regulation (A review)". *Proceedings of the National Academy of Sciences of the United States of America* **73**(11):3825-3832.

Neuteboom LW, Ng JMY, Kuyper M, Clijdesdale OR, Hooykaas PJJ, Van Der Zaal BJ. (1999). "Isolation and characterization of cDNA clones corresponding with mRNAs that accumulate during auxin-induced lateral root formation". *Plant Molecular Biology* **39**(2):273-287.

Nielsen H, Engelbrecht J, Brunak S, Von Heijne G. (1997). "Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites". *Protein Engineering* **10**(1):1-6.

Nirmala X, Kodrik D, Zurovec M, Sehnal F. (2001). "Insect silk contains both a Kunitz-type and a unique Kazal-type proteinase inhibitor". *Eur J Biochem* **268**(7):2064-2073.

O'Brien D, and McVey, J, 1993. Blood coagulation, inflammation, and defence. In: Sim, I.E. (Ed.), The natueal immune system, humoral factors, New York: IRL Press, pp. 257-280.

Okumura T, Nagai F, Yamamoto S, Oomura H, Inouye K, Ito M et al. (2005). "Detection of white spot syndrome virus (WSSV) from hemolymph of Penaeid shrimps Penaeus japonicus by reverse passive latex agglutination assay using high-density latex particles". *Journal of Virological Methods* **124**(1-2):143-148.

Othman R, Nuraziyan A. "Fruit-specific expression of papaya subtilase gene". *Journal of Plant Physiology* **In Press, Corrected Proof.**

Patat SA, Carnegie RB, Kingsbury C, Gross PS, Chapman R, Schey KL. (2004). "Antimicrobial activity of histones from hemocytes of the Pacific white shrimp". *Eur J Biochem* **271**(23-24):4825-4833.

Perazzolo LM, Barracco MA. "The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors". *Developmental & Comparative Immunology* **21**(5):385-395.

Pham TN, Hayashi K, Takano R, Itoh M, Eguchi M, Shibata H et al. (1996). "A new family of serine protease inhibitors (Bombyx family) as established from the unique topological relation between the positions of bisulfide bridges and reactive site". *Journal of Biochemistry* **119**(3):428-434.

Phillips MA, Fletterick RJ. (1992). "Proteases". *Current Opinion in Structural Biology* **2**(5):713-720.

Ponprateep S, Somboonwiwat K, Tassanakajon A. (2009). "Recombinant anti-lipopolysaccharide factor isoform 3 and the prevention of vibriosis in the black tiger shrimp, *Penaeus monodon*". *Aquaculture* **289**(3-4):219-224.

Ramesh A, Loganathan BG, Venugopalan VK. (1989). "Seasonal distributions of luminous bacteria in the sediments of a tropical estuary". *Journal of General and Applied Microbiology* **35**(5):363-368.

Rawlings ND, Tolle DP, Barrett AJ. (2004). "Evolutionary families of peptidase inhibitors". *Biochem J* **378**(Pt 3):705-716.

Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. (2008). "MEROPS: the peptidase database". *Nucleic Acids Res* **36**:D320-325.

Ribeiro A, Akkermans AD, van Kammen A, Bisseling T, Pawlowski K. (1995). "A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development". *Plant Cell* **7**(6):785-794.

Salzet M. (2001). "Vertebrate innate immunity resembles a mosaic of invertebrate immune responses". *Trends in Immunology* **22**(6):285-288.

Sambrook J, Fritsch EF, Maniatis T, 1989. Molecular cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Shiao SH, Higgs S, Adelman Z, Christensen BM, Liu SH, Chen CC. (2001). "Effect of prophenoloxidase expression knockout on the melanization of microfilariae in the mosquito *Armigeres subalbatus*". *Insect Mol Biol* **10**(4):315-321.

Simonet G, Claeys I, Broeck JV. (2002a). "Structural and functional properties of a novel serine protease inhibiting peptide family in arthropods". *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **132**(1):247-255.

Sithigorngul P, Chauychuwong P, Sithigorngul W, Longyant S, Chaivisuthangkura P, Menasveta P. (2000). "Development of a monoclonal antibody specific to yellow head virus (YHV) from *Penaeus monodon*". *Diseases of Aquatic Organisms* **42**(1):27-34.

Sithigorngul P, Rukpratanporn S, Longyant S, Chaivisuthangkura P, Sithigorngul W, Menasveta P. (2002). "Monoclonal antibodies specific to yellow-head virus (YHV) of *Penaeus monodon*". *Diseases of Aquatic Organisms* **49**(1):71-76.

Sittidilokratna N, Hodgson RAJ, Cowley JA, Jitrapakdee S, Boonsaeng V, Panyim S et al. (2002). "Complete ORF1b-gene sequence indicates yellow head virus is an invertebrate nidovirus". *Diseases of Aquatic Organisms* **50**(2):87-93.

Slowinska M, Olczak M, Wojtczak M, Glogowski J, Jankowski J, Watorek W et al. (2008). "Isolation, characterization and cDNA sequencing of a Kazal family proteinase inhibitor from seminal plasma of turkey (*Meleagris gallopavo*)". *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **150**(2):207-215.

Söderhäll K, Ajaxon R. (1982). "Effect of quinones and melanin on mycelial growth of Aphanomyces spp. and extracellular protease of *Aphanomyces astaci*, a parasite on crayfish". *Journal of Invertebrate Pathology* **39**(1):105-109.

Söderhäll K, Cerenius L. (1998). "Role of the prophenoloxidase-activating system in invertebrate immunity". *Curr Opin Immunol* **10**(1):23-28.

Somprasong N, Rimphanitchayakit V, Tassanakajon A. (2006). "A five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon* and its inhibitory activities". *Developmental and Comparative Immunology* **30**(11):998-1008.

Soonthornchai W, Rungrassamee W, Karoonuthaisiri N, Jarayabhand P, Klinbunga S, Soderhall K et al. (2009). "Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*". *Dev Comp Immunol* **7**:7.

Soowannayan C, Flegel TW, Sithigorngul P, Slater J, Hyatt A, Cramerri S et al. (2003). "Detection and differentiation of yellow head complex viruses using monoclonal antibodies". *Dis Aquat Organ* **57**(3):193-200.

Spann KM, McCulloch RJ, Cowley JA, East IJ, Walker PJ. (2003). "Detection of gill-associated virus (GAV) by in situ hybridization during acute and chronic infections of *Penaeus monodon* and *P. esculentus*". *Dis Aquat Organ* **56**(1):1-10.

Sritunyalucksana K, Soderhall K. (2000). "The proPO and clotting system in crustaceans". *Aquaculture* **191**(1-3):53-69.

Supamattaya K, Hoffmann RW, Boonyaratpalin S, Kanchanaphum P. (1998). "Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Portunus pelagicus*, mud crab Scylla setrata and krill Acetes sp". *Diseases of Aquatic Organisms* **32**(2):79-85.

Supungul P, Klinbunga S, Pichyangkura R, Hirono I, Aoki T, Tassanakajon A. (2004). "Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach". *Dis Aquat Organ* **61**(1-2):123-135.

Takahashi Y, Itami, T., Maeda, M., Suzuki, N., Kasorchandra, J., Supamattaya, K., Khongpradit, R., Boonyaratpalin, S., Kondo, M., Kawai, K., Kusuda, R., Hirono, I., and Aoki, T. (1996). "Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PJ) DNA in *Penaeus japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricius". *J. Fish. Dis.* **19**:399-403.

Tang KF, Lightner DV. (1999). "A yellow head virus gene probe: nucleotide sequence and application for in situ hybridization". *Dis Aquat Organ* **35**(3):165-173.

Tang KF, Spann KM, Owens L, Lightner DV. (2002). "In situ detection of Australian gill-associated virus with a yellow head virus gene probe". *Aquaculture* **205**(1-2):1-5.

Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, Jitrapakdee S et al. (2006). "*Penaeus monodon* gene discovery project: The generation of an EST collection and establishment of a database". *Gene* **384**(1-2):104-112.

Taylor AA, Morsch A, Rzepczyk A, Hasenkampf CA, Daniel Riggs C. (1997). "Maturation and secretion of a serine proteinase is associated with events of late microsporogenesis". *Plant Journal* **12**(6):1261-1271.

Thaithongnum S, Ratanama P, Weeradechapol K, Sukhoom A, Vuddhakul V. (2006). "Detection of *V. harveyi* in shrimp postlarvae and hatchery tank water by the Most Probable Number technique with PCR". *Aquaculture* **261**(1):1-9.

Theopold U, Schmidt O, Soderhall K, Dushay MS. (2004). "Coagulation in arthropods: defence, wound closure and healing". *Trends in Immunology* **25**(6):289-294.

Tian M, Benedetti B, Kamoun S. (2005). "A second Kazal-like protease inhibitor from Phytophthora infestans inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato". *Plant Physiology* **138**(3):1785-1793.

Tian M, Huitema E, Da Cunha L, Torto-Alalibo T, Kamoun S. (2004). "A Kazal-like extracellular serine protease inhibitor from Phytophthora infestans targets the tomato pathogenesis-related protease P69B". *Journal of Biological Chemistry* **279**(25):26370-26377.

Tornero P, Conejero V, Vera P. (1996). "Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants: Similarity of functional domains to subtilisin-like endoproteases". *Proc Natl Acad Sci U S A* **93**(13):6332-6337.

Van de Locht A, Lamba D, Bauer M, Huber R, Friedrich T, Kroger B et al. (1995). "Two heads are better than one: Crystal structure of the insect derived double domain Kazal inhibitor rhodniin in complex with thrombin". *EMBO Journal* **14**(21):5149-5157.

Vandenberghe J, Li Y, Verdonck L, Li J, Sorgeloos P, Xu HS et al. (1998). "*Vibrios* associated with *Penaeus chinensis* (Crustacea : Decapoda) larvae in Chinese shrimp hatcheries". *Aquaculture* **169**(1-2):121-132.

Wang G, Finch MD, Trevan D, Hellmann K. (1981). "Reduction of daunomycin toxicity by razoxane". *British Journal of Cancer* **43**(6):871-877.

Wang R, Lee SY, Cerenius L, Soderhall K. (2001). "Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*". *Eur J Biochem* **268**(4):895-902.

Wang Y-C, Lo C-F, Chang P-S, Kou G-H. (1998). "Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan". *Aquaculture* **164**(1-4):221-231.

Wang P-H, Gu Z-H, Huang X-D, Liu B-D, Deng X-x, Ai H-S et al. (2009a). "An immune deficiency homolog from the white shrimp, *Litopenaeus vannamei*, activates antimicrobial peptide genes". *Molecular Immunology* **46**(8-9):1897-1904.

Wang ZH, Zhao XF, Wang JX. (2009). "Characterization, kinetics, and possible function of Kazal-type proteinase inhibitors of Chinese white shrimp, *Fenneropenaeus chinensis*". *Fish Shellfish Immunol* **26**(6):885-897.

Whaley K, Lemercier, C., 1993. The complement system. In: Sim, I.E. (Ed.), The natural immune system, humoral factors, New York: IRL Press, pp. 121-150.

Witteveldt J, Vermeesch AMG, Langenhof M, De Lang A, Vlak JM, Van Hulten MCW. (2005). "Nucleocapsid protein VP15 is the basic DNA binding protein of white spot syndrome virus of shrimp". *Archives of Virology* **150**(6):1121-1133.

Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V et al. (1995). "A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn Penaeus monodon". *Diseases of Aquatic Organisms* **21**(1):69-77.

Wongteerasupaya C, Tongchuea W, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B et al. (1997). "Detection of yellow-head virus (YHV) of *Penaeus monodon* by RT-PCR amplification". *Diseases of Aquatic Organisms* **31**(3):181-186.

Wu W, Wang, L., and Zhang, X. (2005). "Identification of white spot syndrome virus (WSSV) envelope proteins involved in shrimp onfection". *Virology* **332**:578-583.

Yamagata H, Masuzawa T, Nagaoka Y, Ohnishi T, Iwasaki T. (1994). "Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor". *Journal of Biological Chemistry* **269**(52):32725-32731.

Yano A, Suzuki K, Shinshi H. (1999). "A signaling pathway, independent of the oxidative burst, that leads to hypersensitive cell death in cultured tobacco cells includes a serine protease". *Plant Journal* **18**(1):105-109.

Yeh MS, Chen YL, Tsai IH. (1998). "The hemolymph clottable proteins of tiger shrimp, *Penaeus monodon*, and related species". *Comp Biochem Physiol B Biochem Mol Biol* **121**(2):169-176.

Yoganandhan K, Narayanan RB, Sahul Hameed AS. (2003). "Larvae and early post-larvae of *Penaeus monodon* (Fabricius) experimentally infected with white spot syndrome virus (WSSV) show no significant mortality". *Journal of Fish Diseases* **26**(7):385-391.

Zhang X, Huang C, Qin Q. (2004). "Antiviral properties of hemocyanin isolated from shrimp Penaeus monodon". *Antiviral Research* **61**(2):93-99.

Zhang X, Huang, C., and Qin, Q. (2004). "Antiviral properties of haemocyanin isolated from shrimp *Penaeus monodon*". *Antiviral Res.* **61**:93-99.

Zheng Q-L, Chen J, Nie Z-M, Lv Z-B, Wang D, Zhang Y-Z. (2007). "Expression, purification and characterization of a three-domain Kazal-type inhibitor from silkworm pupae (Bombyx mori)". *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **146**(2):234-240.

Appendices

Appendix A

A-tailing procedure for blunt-ended PCR fragment

1. Start with 6 µl of purified PCR fragment generated by a proofreading polymerase, *Pfu* DNA polymerase.

2. Add 1 µl DyNAzyme_II DNA polymerase 10×Reaction Buffer with $MgCl_2$.

3. Add dATP to final concentration of 0.2 mM.

4. Add 5 units of DyNAzyme_II DNA polymerase.

5. Add deionizes water to a final reaction volumn of 10 µl.

6. Incubate at $70^{\circ}C$ for 30 minutes.

7. Use 1-2 µl in a ligation reaction with T&A vector.

Appendix B

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. **Preparation for polyacrylamide gel electrophoresis**

- **30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, 100 ml**

acrylamide	29.2	g
bis-acrylamide	0,8	g

  Adjust volume to 100 ml with distilled water.

- **1.5 M Tris-HCl pH 8.8**

  Tris (hydroxymethyl)-aminomethen 18.17 g

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

- **2.0 M Tris-HCl (pH 8.8)**

  Tris (hydroxymethyl)-aminomethen 24.2 g

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

- **0.5 M Tris-HCl pH 6.8**

  Tris (hydroxymethyl)-aminomethen 6.06 g

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

- **1.0 M Tris-HCl pH 6.8**

  Tris (hydroxymethyl)-aminomethen 12.1 g

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

2.   **SDS-PAGE**

- **18% Separating gel**

H_2O	1.013	ml
30% (w/v) Acrylamide solution	4.8	ml
1.5 M Tris (pH 8.8)	2.3	ml
10% SDS	0.08	ml
10% Ammonium persulfate	0.11	ml
TEMED	10	μl

- **5.0% Stacking gel**

H_2O	2.7	ml
30% (w/v) Acrylamide solution	0.67	ml
1.0 M Tris (pH 6.8)	0.5	ml
10% SDS	0.04	ml
10% Ammonium persulfate	0.04	ml
TEMED	5	μl

- **5x Sample buffer**

1 M Tris-HCl pH 6.8	0.6	ml
50% (w/v) Glycerol	5.0	ml
10% SDS	2.0	ml
2-mercaptoethanol	0.5	ml
1% Bromophenol blue	1.0	ml
Distilled water	0.9	ml

One part of sample buffer was added to four parts of sample. The mixture
was heated 5 min. in boiling water before loading to the gel.

3. Electrophoresis buffer, 1 litre

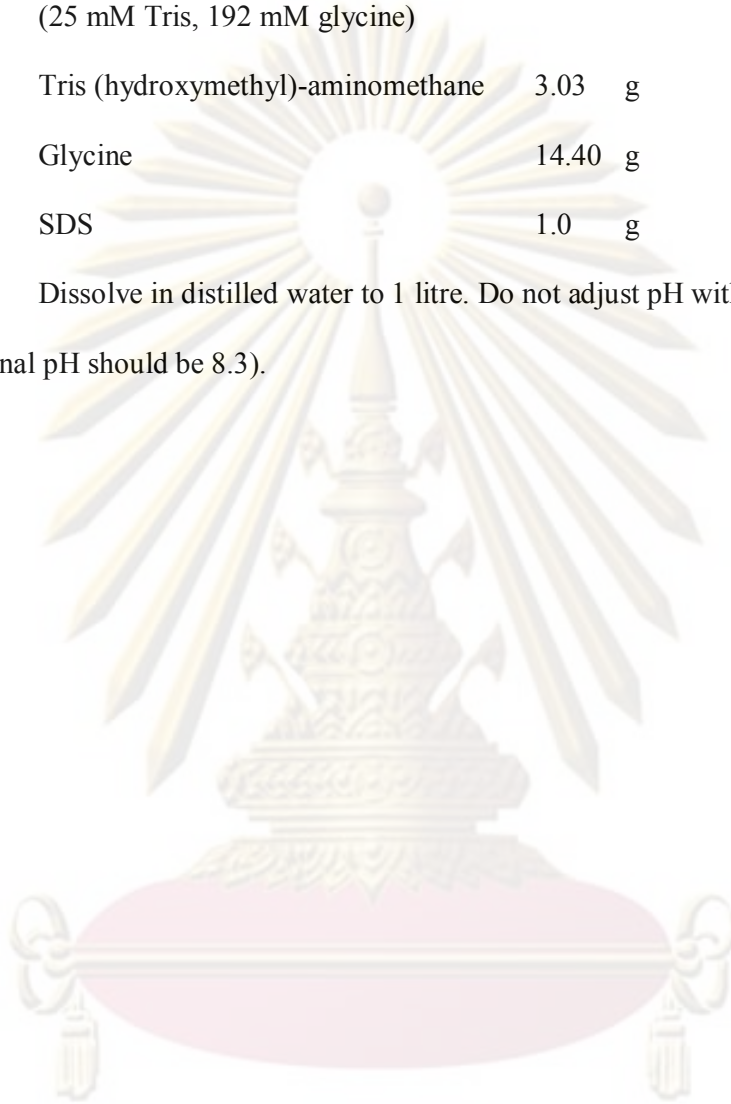(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.40	g
SDS	1.0	g

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

(final pH should be 8.3).

Appendix C

Publications

1. Visetnan S, Donpusa S, Supungul P, Tassanakajon A, Rimphanitchayakit V. Kazal-type serine proteinase inhibitors from the black tiger shrimp *Penaeus monodon* and the inhibitory activities of SPI*Pm*4 and 5. Fish & Shellfish Immunology 2009; 27:266-274.

Kazal-type serine proteinase inhibitors from the black tiger shrimp *Penaeus monodon* and the inhibitory activities of SPIPm4 and 5

Suwattana Visetnan [a], Suchao Donpudsa [a], Premruethai Supungul [a,b], Anchalee Tassanakajon [a], Vichien Rimphanitchayakit [a,*]

[a] Shrimp Molecular Biology and Genomics Laboratory, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani 12120, Thailand

ARTICLE INFO

ABSTRACT

Serine proteinase inhibitors (SPIs) play important roles in physiological and immunological processes involving proteinases in all multicellular organisms. In black tiger shrimp *Penaeus monodon*, nine different Kazal-type SPIs, namely SPIPm1-9, were identified from the cDNA libraries of hemocyte, hepatopancreas, hematopoietic tissue, ovary and lymphoid organ. They are multi-domain SPIs containing 2–7 and possibly more Kazal domains. Two interesting cDNA clones, SPIPm4 and SPIPm5 coding for two-domain Kazal-type SPIs, were identified from the heat-treated hemocyte cDNA libraries. The SPIPm4 and SPIPm5 consist of open reading frames of 387 and 399 bp coding for polypeptides of 128 and 132 amino acids with putative signal peptides of 21 and 19 amino acid residues and mature SPIs of 107 and 113 amino acid residues, respectively. Recombinant expression in an *Escherichia coli* expression system yielded recombinant proteins, rSPIPm4 and rSPIPm5, with molecular masses of 12.862 and 13.433 kDa, respectively. The inhibitory activities of SPIPm4 and SPIPm5 were tested against trypsin, chymotrypsin, subtilisin and elastase. The SPIPm4 exhibited potent inhibitory activity against subtilisin and weakly against chymotrypsin whereas the SPIPm5 strongly inhibited subtilisin and elastase. The inhibition was a competitive type with inhibition constants (K_i) of 14.95 nM for SPIPm4 against subtilisin, 4.19 and 59.64 nM, respectively, for SPIPm5 against subtilisin and elastase. They had no bacteriostatic effect against Gram-positive bacteria: *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, and Gram-negative bacteria: *Vibrio harveyi* 639, *E. coli* JM109. Gene expression study revealed that the SPIPm5 gene was up-regulated in response to heat treatment suggesting the involvement of SPIs in stress responses.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

In multicellular organisms, serine proteinase inhibitors (SPIs) are essential factors involving in controlling the various proteinase-mediated biological processes, such as the complement system, blood coagulation, melanization, apoptosis, etc. [1,2]. Not only do they control the extent of deleterious protease digestion in such processes, they potentially fight as part of the humoral defence of the innate immune system against the invading pathogens [3]. For example, a subtilisin inhibitor, BmSPI, from *Bombyx mori* might function as an inhibitor to the microbial proteases and protected the silkworm pupae from infection by pathogens [4].

Some microbial pathogens and parasites use the SPIs to counter-defense the host protective proteinases. For example, the oomycete *Phytophthora infestans*, a cause of disease in potato and tomato, produces an extracellular protease inhibitor to counter-defence the plant defensive proteinases [5,6]. The obligate intracellular parasite of human *Toxoplasma gondii* produces a serine protease inhibitor to protect itself from the digestive enzymes during its residency in small intestine [7]. Some other SPIs are involved in reproductive processes. A male reproduction-related SPI is isolated from *Macrobrachium rosenbergii* with inhibitory activity on sperm gelatinolytic activity [8]. Another reproductive SPI was from the turkey male reproductive tract [9]. For hematophagous insects such as *Dipetalogaster maximus* and *Triatoma infestans*, they secrete potent thrombin inhibitors dipetalogastin and infestin, respectively, to prevent blood clotting during blood meal [10,11].

Since SPIs are widely distributed and more are to be found and characterized, to facilitate the studies in this field, they were classified on the basis of amino acid sequence similarities into at least 63 families (http://merops.sanger.ac.uk/) [12,13]. One of the well known SPIs is the Kazal-type SPIs which are grouped into family I1. The Kazal inhibitors are usually multi-domain proteins containing more than one Kazal domain. Each domain of 50–60 amino acid

* Corresponding author. Tel.: +66 2 2185436; fax: +66 2 2185418.
E-mail address: kpvr2@yahoo.com (V. Rimphanitchayakit).

residues contains six well-conserved cysteine residues capable of forming three intra-domain disulphide bridges resulting in a characteristic three-dimensional structure [14]. Each domain binds tightly and competitively via its reactive site loop to the active site of cognate proteinase rendering the proteinase inactive. Structural studies reveal that there are several contact positions responsible for the interactions between Kazal domains and the proteinases [15,16]. However, the inhibitory specificity is determined mainly by the P1 amino acid residue resided at the second amino acid residue after the second cysteine residue of the domain.

Recently, two Kazal SPIs are identified from scallop and shrimp. They were believed to be involved in host defence mechanism for they were up-regulated upon microbial infection. The novel 12-domain Kazal-type SPI from the Zhikong scallop *Chlamys farreri* SPI was isolated. Its expression in hemocytes was up-regulated upon *Vibrio anguillarum* challenge [17]. The two-domain Kazal-type SPI from the oriental white shrimp *Fenneropenaeus chinensis* was characterized. Its expression was up-regulated in the white spot syndrome virus (WSSV) infected shrimp [18].

For the black tiger shrimp *Penaeus monodon*, a few SPIs had been reported before the *P. monodon* EST database was established, particularly the SPIPm2 [19,20]. The EST approach was later used to identify more genes from the shrimp. A total of 40,001 EST clones were collected and sequenced leading to the establishment of the EST database (http://pmonodon.biotec.or.th/home.jsp) [21]. Upon clustering of the EST clones to remove the redundancy, 10,536 unique genes were obtained. In this study, we searched the cDNA clustering databases for the Kazal-type SPIs. Analyses of the nucleotide sequences of contigs and their EST clone members enabled us to classify the Kazal inhibitors in the black tiger shrimp. Two interesting SPIs, SPIPm4 and 5, were over-expressed and their inhibitory activities towards proteinases studied. The expression of SPIPm5 gene in response to heat stress was also tested.

2. Materials and methods

2.1. Searching the P. monodon EST database

The contig pages in the *P. monodon* EST database (http://pmonodon.biotec.or.th/home.jsp) was searched for the Kazal-type serine proteinase inhibitors. The nucleotide sequences of the obtained contigs and singletons were analyzed for the open reading frames and the encoded amino acid sequences. The EST clones containing the entire open reading frames of the interesting contigs were re-sequenced to ensure the correctness of the sequence data. SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal cleavage sites [22]. ClustalX was used to align the sequences from different contigs and EST clones [23].

2.2. Expression analysis of SPIPm5 using RT-PCR

Three-month-old subadult black tiger shrimp, *P. monodon*, of about 15–20 g weight, were obtained from a local farm and acclimatized in aquaria at an ambient temperature of about 28 ± 1 °C and a salinity of 15 ppt for a few days before the experiments. The shrimp were divided into two groups, control and heat-treated groups. Each group consisted of 3 subgroups of 3 individuals. The heat-treated group was immediately put and reared for 1 h in an aquarium with warm water of 33 ± 1 °C. Then, the hemolymph was collected from the shrimp ventral sinus into an anticoagulant solution of 10% (w/v) trisodium citrate dihydrate, pH 4.6. Hemocytes were pelleted by centrifugation, resuspended in TRI REAGENT® (Molecular Research Center), homogenized and total RNA isolated. The equivalent amounts of total RNA preparations from individuals in each subgroup were pooled and treated with RNase-free DNase I (Promega) to remove contaminated genomic DNA. The concentration and integrity of total RNA were assessed by UV spectrophotometry and agarose gel electrophoresis. First-strand cDNAs were synthesized from 1 µg of total RNA samples using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol.

RT-PCR analysis was carried out using the SPIPm5 specific primers: FSPIPm5, 5′ TGGAACGGACGGCAAGACAT 3′ and RSPIPm5, 5′ GTCGTGACAGTCTTGAGTCC 3′. The β-actin gene was used as an internal control using the gene specific primers designed according to the shrimp actin cDNA sequence (GenBank accession no. DW042525): actinF, 5′ GCTTGCTGATCCACATCTGCT 3′ and actinR, 5′ ATCACCATCGGCAACGAGA 3′. Each PCR reactions were carried out in a total volume of 25 µl containing 10 mM Tris–HCl, pH 8.8, 50 mM (NH₄)₂SO₄, 0.1% (v/v) Triton X-100, 2 mM MgCl₂ for SPIPm5 or 1 mM for β-actin, 0.2 mM of each dNTP, 0.4 µM of each primer for SPIPm5 or 0.2 µM of each primer for β-actin, 1.5 unit of DyNAzyme™ II DNA Polymerase (Finnzymes) and 3 µl of undiluted template cDNA for SPIPm5 or 3 µl of 10-fold diluted template cDNA for β-actin. The reactions were predenatured at 94 °C for 2 min followed by 40 cycles for SPIPm5 or 25 cycles for β-actin of denaturation at 94 °C for 30 s, annealing at 60 °C for SPIPm5 or 52 °C for β-actin for 30 s and extension at 72 °C for 1 min. The final extension was at 72 °C for 5 min. The PCR products were analyzed using a TBE-2% agarose gel electrophoresis.

2.3. Construction of the protein expression clones

To express the recombinant SPI, two primer pairs, FPm4: 5′ AA GGGCCATGGGAAAGGGGGGGGATTCTCGACT 3′ and RPm4: 5′ CCTT CTCGAGATATCCCGTCTTCCTGTCAA 3′ for SPIPm4; FPm5: 5′ AAGG CCATGGGAAAAGGAGGCAAATTCAGACT 3′ and RPm5: 5′ CCTTCTCG AGATATCCCTTCTTGATAGGCG 3′ for SPIPm5, were designed. The primers were designed for the amplification of gene fragment encoding the mature proteins without signal peptides (Fig. 1). The included NcoI and XhoI sites (underlined), respectively, at the 5′ and 3′ ends of the gene fragments were for cloning into the expression vector. For convenience, the EST clones from the normalized hemocyte library, HC-N-N01-4898-LF (GenBank accession no. GO269555) and HC-N-N01-2619-LF (GenBank accession no. GO269556) containing the complete open reading frames of SPIPm4 and 5, respectively, were used as PCR templates.

The SPIPm4 and 5 gene fragments were PCR amplified in a final reaction volume of 50 µl containing 0.02 ng of plasmid template, 0.4 M of each primer, 0.2 mM of each dNTP and 3 units of *Pfu* polymerase (Promega). The PCR amplification was carried out at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 7 min. The amplified products were analyzed using 1.2% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). The purified DNA fragments were tailed with an adenine nucleotide for ligation into the T&A cloning vector (Real Biotech Corporation). The resulting T&A clones were isolated and subjected to nucleotide sequencing to confirm the insert sequences of SPIPm4 and 5.

The NcoI–XhoI fragments containing the SPIPm4 and 5 were prepared from the T&A clones and subcloned into the NcoI–XhoI digested pET-28b(+) expression vector (Novagen). For some reasons, the SPIPm4 was not expressed well; it was then subcloned into a pET-32a(+) derivative, pVR500 [24], and expressed as fusion protein to the thioredoxin. The expression clones were named pSPIPm4 and 5.

2.4. Preparation of the recombinant proteins

The expression clones, pSPIPm4 and 5, were transformed into an *Escherichia coli* Rosetta(DE3)pLysS. A single colony was cultured in
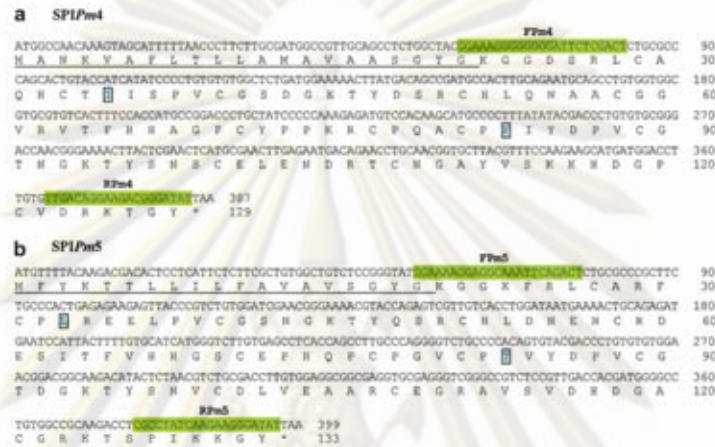
Fig. 1. The open reading frames of SPI*Pm*4 (a) and 5 (b). The underlined amino acid sequences are putative signal peptides. The shaded and boxed amino acids are P1 amino acids. The shaded nucleotide sequences are the primer annealing sites for the PCR amplification of the SPI*Pm*4 and 5 gene fragments for the construction of protein expressing clones.

LB medium containing 100 μg ml^{-1} ampicillin for SPI*Pm*4 or 70 μg ml^{-1} kanamycin for SPI*Pm*5 and 34 μg ml^{-1} chloramphenicol at 37 °C until the OD$_{600}$ reached 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to the final concentration of 1 mM to induce the expression. The culture was continued. Cells were harvested at 3 h after IPTG induction for both rSPIs.

The recombinant SPI*Pm*4 (rSPI*Pm*4) was produced soluble as thioredoxin fusion protein. The fusion protein was purified using nickel-NTA column. The thioredoxin portion was removed by digestion with enterokinase. The enterokinase reaction was carried out in a final volume of 5 ml containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 2 mM CaCl$_2$, 0.01 μg ml^{-1} enterokinase (Promega) and approximately 0.6 mg ml^{-1} fusion protein. The reaction was incubated at 23 °C for 16 h. The cleaved SPI*Pm*4 containing a His-tag at its C-terminus was purified using nickel-NTA column.

The recombinant SPI*Pm*5 (rSPI*Pm*5) was produced partly soluble and partly inclusion bodies. The soluble fraction was purified using nickel-NTA columns.

The protein preparation was kept track and analyzed using SDS-PAGE and western blot analysis. For unknown reason, the rSPI*Pm*4 was not reacted well with the Bradford reagent. Therefore, the concentration of rSPI*Pm*4 preparation was determined using ExPASy ProtParam (http://au.expasy.org/tools/protparam.html) [25]. That of rSPI*Pm*5 preparation was determined using the Bradford method [26].

The molecular masses of SPI*Pm*4 and 5 were accurately determined by MALDI-TOF mass spectrometry and used for the calculation in inhibitory activity and kinetic studies. It was performed in the commercial facility of the Proteomic Service Center, Bioservice Unit (BSU) (BIOTEC, Pathumthani, Thailand).

2.5. Proteinase inhibition assay

The proteinase inhibitory activities of rSPI*Pm*4 and rSPI*Pm*5 towards serine proteinase; subtilisin Carlsberg (*Bacillus licheniformis*, Sigma), trypsin (bovine pancreas, Sigma), α-chymotrypsin

(bovine pancreas, Sigma) and elastase (porcine pancreas, Pacific Science) were assayed using a procedure of Hergenhahn et al. [27].

The reaction mixture consisted of 50 mM Tris–HCl, pH 8.0; 290.67 μM of N-benzoyl-Phe-Val-Arg-p-nitroanilide as substrate for subtilisin and trypsin, 147.3 μM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin and 2215.18 μM of N-succinyl-Ala-Ala-Ala-p-nitroanilide for elastase; appropriate concentrations of proteinases and proteinase inhibitors in a total volume of 100 μl. The concentration of subtilisin, trypsin, α-chymotrypsin and elastase were 0.04 for rSPI*Pm*4 and 0.037 for rSPI*Pm*5, 0.042, 0.04 and 0.077 μM, respectively. Two series of 2-fold diluted proteinase inhibitors were used; 3.88–0.01 μM for rSPI*Pm*4 and 2.98–0.01 μM for rSPI*Pm*5. Not all concentrations of proteinase inhibitors were used in the assays (see Fig. 5). For each substrate, a reaction containing the highest amount of SPI preparation used in the assay but without added proteinase was prepared as a control to make certain that there were no contaminating proteinases from the bacterial host used for the preparation of SPIs. The reaction was incubated at 30 °C for 15 min and then terminated by adding 50 μl of 50% acetic acid. The proteinase reaction product, p-nitroaniline, was measured at 405 nm using microplate reader (BMG Labtech). The percentages of remaining activity were calculated and plotted against the molar ratio of inhibitor to proteinase.

2.6. Kinetics of serine proteinase inhibition

Four sets of reactions for four different concentrations of proteinase inhibitor were carried out. Each set consisted of five concentrations of substrate in the presence of fixed amounts of serine proteinase and a concentration of proteinase inhibitor. For subtilisin inhibition, 0, 0.11, 0.22, 0.44 and 0.88 mM of N-benzoyl-Phe-Val-Arg-p-nitroanilide substrate were incubated with 3.58 nM of subtilisin for rSPI*Pm*4 or 53.74 nM of subtilisin for rSPI*Pm*5 and fixed amount of inhibitor. Four different concentrations of proteinase inhibitor: 0, 30.38, 60.75 and 121.50 nM for rSPI*Pm*4 and 0, 23.25, 46.50 and 93.00 nM for rSPI*Pm*5, were used. For elastase

inhibition, 0, 0.18, 0.37, 0.74 and 1.48 mM of N-succinyl-Ala-Ala-Ala-p-nitroanilide substrate were incubated with 154.44 nM of elastase and a concentration of rSPIPm5. Four different concentrations of rSPIPm5: 0, 124.07, 248.14 and 496.28 nM, were used.

The reactions were made a total volume of 100 μl in the presence of 50 mM Tris–HCl, pH 8.0. The reactions were initiated by the addition of proteinase, incubated at 30 °C for 15 min and arrested with 50 μl of 50% acetic acid. Each reaction was performed in triplicate. The p-nitroaniline formed was measured at 405 nm using a microplate reader (BMG Labtech). The amount of p-nitroaniline was calculated using a millimolar extinction coefficient of 9.96. The activity was calculated as nmole of p-nitroaniline/min. The activities were plotted against the concentration of substrates as substrate saturation curve and a Lineweaver–Burk plot. The apparent K_{MS} were calculated and plotted against the proteinase inhibitor concentrations. The latter was constructed for the calculation of inhibition constant (K_i).

2.7. Bacterial growth inhibition

Bacteriostatic activities of SPIPm4 and 5 were assayed based on the procedure by Han et al. [28] on Gram-positive bacteria, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, and Gram-negative bacteria, *Vibrio harveyi* 639, *E. coli* JM109. For each bacterium, the assay was done in duplicate. An overnight culture of bacterium was diluted a hundred-fold, and grew in a shaking incubator in the presence of 0 and 20 μM of SPIPm4 or 10 μM of SPIPm5 at 30 °C for *B. megaterium* and *V. harveyi* 639 and 37 °C for *B. subtilis*, *S. aureus* and *E. coli* JM109. The bacterial growth was measured at 0, 4, 8, 12 and 16 h by monitoring the optical density at 595 nm.

3. Results

3.1. Mining of the P. monodon EST database

From the *P. monodon* EST database (http://pmonodon.biotec.or.th/) [21], we had searched for the Kazal-typed proteinase inhibitor. From a total of 10,536 unique contigs and singletons derived from the clustering of 33,143 EST clones in the database, nine different Kazal-type serine proteinases were identified and named SPIPm1 to 9 (Table 1 and Fig. 2). Some SPIs were represented by more than one contig or singleton because there were variations in the cDNA sequences at the very ends of 3'-untranslated regions that were enough to be clustered as different contigs (data not shown). The number of Kazal domain in these SPIs varied from 2 to 7 and possibly more. The open reading frames of SPIPm1, 2, 4, 5 and 6 were complete. That of SPIPm3, a seven-domain SPI from hepatopancreas, was nearly complete as only a few amino acid residues were missing at the N-terminus. Those of other SPIs were awaited for completion.

Fig. 2 shows the amino acid sequences of the nine Kazal inhibitors from *P. monodon*. The potential signal peptide cleavage sites, determined using the online SignalP 3.0 Server, are indicated in the sequence. Two cleavage sites with approximately equal possibility were identified in SPIPm2.

In Table 1, the frequencies of EST members from mostly the standard libraries are shown to avoid the biased figures from the normalized libraries. It was found that the contig members were mostly from the hemocyte libraries, particularly the heat-treated hemocyte libraries. Other libraries were from hematopoietic tissue, hepatopancreas, ovary and lymphoid organ. Only one singleton, the SPIPm9, was identified from the normalized libraries of lymphoid organ suggesting that the SPIPm9 was rarely expressed. The SPIPm2 (22 clones) is the most abundant SPI in the hemocytes followed by

SPIPm4 (6 clones) and 5 (6 clones). Other SPI clone members were fewer than two.

3.2. Expression of SPIPm5 in response to heat stress using RT-PCR

Having shown that most of the EST members in the contigs, particularly SPIPm2, 4 and 5, were from the heat-treated hemocyte cDNA libraries (Table 1), one might want to hypothesize that the expression of these SPIs were increased upon heat treatment. As a preliminary experiment on heat stress, we tested this hypothesis with SPIPm5, one of the two SPIs in this study. The shrimp were heat-treated for 1 h at 5 °C above normal rearing temperature. The cDNAs were prepared and tested for the increase in SPIPm5 mRNA. Fig. 3 revealed that the expression of SPIPm5 gene was, indeed, up-regulated unambiguously upon heat treatment.

3.3. Recombinant expression of rSPIPm4 and 5

Two two-domain Kazal-type SPIs, SPIPm4 and 5, were interesting for they were identified from the heat-treated hemocyte cDNA libraries. The SPIPm4 and 5 cDNA clones, HC-N-N01-4898-LF and HC-N-N01-2619-LF contained the open reading frames of 387 and 399 bp that encoded polypeptides of 128 and 132 amino acids with putative signal peptides of 21 and 19 amino acid residues and mature proteins of 107 and 113 amino acid residues, respectively (Fig. 1). To explore further the activities of these two SPIs, the two SPI clones were used as PCR templates for the amplification of the SPI genes. The PCR primers were designed such that the PCR gene products contained the NcoI and XhoI restriction sites, respectively, at their 5' and 3' ends. By digesting with NcoI and XhoI restriction enzymes, the SPI gene fragments were readily cloned into the expression vectors.

The SPIPm4 and 5 were over-expressed in an *E. coli* system using pVR500 [a pET-32a(+) derivative] and pET-28b(+), respectively, as expression vectors. The two expression clones were called pSPIPm4 and 5. They were transformed into an *E. coli* Rosetta(DE3)pLysS. The transformants were cultured and induced with IPTG for the over-expression of the SPIs. The rSPIPm4 was expressed as a soluble fusion protein of about 25 kDa. The thioredoxin was removed by enterokinase digestion. From our calculation, the size of the cleaved thioredoxin and rSPIPm4 were approximately equal. Since only the rSPIPm4 contained the His-tag at its C-terminus, it was purified with the nickel-NTA column (Fig. 4a). Small amount of SPI dimer could be seen as usual for cysteine-rich proteins. The rSPIPm5 was expressed about equally as soluble protein and inclusion bodies. The soluble rSPIPm5 fraction was adequate for use in this study and was purified using nickel-NTA column (Fig. 4b). Western blot analysis was used to confirm the identity of the SPIs for they were the only proteins that contained the His-tag (data not shown).

The molecular masses of rSPIPm4 and 5 were determined using MALDI-TOP mass spectrometer to be 12.862 and 13.433 kDa, respectively (data not shown). The calculated figures for rSPIPm4 and 5 were 12.871 and 13.575 kDa, respectively. Without the start amino acid, the SPIPm5 was calculated to be 13.444. It was possible that the rSPIPm5 might have its start amino acid removed as had been shown for proteins produced in *E. coli* [29].

3.4. Proteinase inhibition and kinetics

The recombinant inhibitors were assayed for their inhibitory activities against trypsin, chymotrypsin, elastase and subtilisin A (Fig. 5). The mole ratios up to 50 of inhibitor against proteinase were used. For a strong inhibitor, the inhibition was readily seen at the mole ratios less than 10. The SPIPm4 and 5 strongly inhibited subtilisin. The SPIPm4 weakly inhibited chymotrypsin while the

Table 1

The nine Kazal-type serine proteinase inhibitors identified from the *Penaeus monodon* EST database.

SPI	Contig or singleton	Representative clone (ORF) and GenBank accession number	Number of Kazal domain	Clone member[a]	Frequency in cDNA libraries[b]	
SPIPm1	CT446, CT447	HC-N-S01-0010-LF (complete ORF) GO269557	4	2	HC-N-S	2
SPIPm2	CT165, CT170	SH415 (complete ORF) BI018075	5	22	HC-N-S	5
					HC-V-S	1
					HC-H-S	14
					HPO-N-S	2
SPIPm3	CT195	HPa-N-S01-0307-LF (incomplete ORF) EB409368	7	2	HPA-N-S	2
SPIPm4	CT409, CT410	HC-H-S01-0302-LF (complete ORF) DW677889	2	6	HC-N-S	1
					HC-H-S	4
					HPO-N-S	1
SPIPm5	CT387, CT391	LFHC-H-S01-0132-LF (complete ORF) DW677833	2	6	HC-N-S	1
					HC-H-S	5
SPIPm6	CT980	HC-V-S01-0141-LF (complete ORF) GO269558	2	2	HC-N-S	1
					HC-V-S	1
SPIPm7	CT2507, SG6085	HC-H-S01-0190-LF (incomplete ORF) DW677851	≥6	1	HC-H-S	1
SPIPm8	SG9720	OV-N-S01-0029-W (incomplete ORF) EB389687	≥3	1	OV-N-S	1
SPIPm9	SG9029	LP-N-N01-0272-LF (incomplete ORF) EE662962	≥2	1 (normalized clone)	LP-N-N	1

[a] Excluding the normalized clones unless otherwise stated.
[b] HC-N-S, HC-V-S and HC-H-S are standard (3rd S) normal (2nd N), standard viral infected (2nd V) and standard heat-treated (2nd H) hemocyte (HC) cDNA libraries, respectively. HPO-N-S, HPA-N-S and OV-N-S are standard (3rd S) normal (2nd N) hematopoietic tissue (HPO), hepatopancreas (HPA) and ovary (OV) cDNA libraries, respectively. The LP-N-N is normalized (3rd N) normal (2nd N) lymphoid organ (LP) cDNA library.

SPIPm5 also strongly inhibited elastase. The SPIPm4 hardly inhibited trypsin and elastase. The SPIPm5 did not inhibit trypsin and chymotrypsin.

The inhibition against subtilisin of SPIPm4 and 5 as well as that against elastase of SPIPm5 were studied further for their kinetics. By varying the substrate concentrations with fixed amount of proteinase in the presence of different inhibitor concentrations, Lineweaver–Burg plots of several inverted substrate saturation curves were constructed. As expected, the inhibition was essentially competitive. The V_{max}s, K_Ms and K_is were calculated and summarized in Fig. 6. The inhibition constants (K_is) were in the range of nanomolar while the K_Ms were in the range of millimolar. The inhibition was, indeed, quite strong.

3.5. Bacterial growth inhibition

It had been a thought that a potent subtilisin inhibitor might have negative effect on bacterial growth particularly that of *B. subtilis* as those observed with a serine proteinase inhibitor from frog eggs, ranaserpin, and a Kazal inhibitor from *P. monodon*, SPIPm2 [24,28]. The growth inhibition activity of the SPIPm4 and 5 were tested. By incubating the diluted culture of bacteria with considerably higher concentrations of the inhibitors than the SPIPm2, 20 mM SPIPm4 or 10 mM SPIPm5, and monitoring the growth of bacteria up to 16 h, it was found that both SPIs had no bacteriostatic effect on the growth of Gram-positive bacteria: *B. subtilis*, *B. megaterium*, *S. aureus*, and Gram-negative bacteria: *V. harveyi* 639, *E. coli* JM109 (data not shown).

4. Discussion

The establishment of *P. monodon* EST database and the clustering of the EST data had enabled us to identify a number of the Kazal-type serine proteinase inhibitors in the shrimp. We found in the *P. monodon* that there were at least 9 types of Kazal SPIs. Most of the EST clone members of the SPIs were derived from the hemocyte libraries indicating that the hemocyte was the main site of SPI biosynthesis. Since the blood circulation is the major site of immune defence system in shrimp, the notion that Kazal SPIs are involved in host defence innate immune response is also supported. This is in line with a recent finding that an SPI from *F. chinensis* is up-regulated in the hemocytes in response to white spot syndrome virus (WSSV) infection [18].

By using amino acid and nucleotide sequence comparison, we found no indication of any relationships due to alternate splicing of the pre-mRNA among these inhibitors. Nevertheless, the Kazal domains may evolve from the common ancestor for they are very much similar with certain degree of homology both in the amino acid and nucleotide sequences. These SPIs are multi-domain inhibitors with multiple Kazal domains of 2–7 domains. The important reactive P1 amino acids are quite variable.

Among the *P. monodon* Kazal SPIs, the SPIPm2, a five-domain Kazal inhibitor, has been extensively studied as it is the most abundant Kazal inhibitor in the shrimp [20,30]. Amino acid sequence comparison indicates that the four-domain Kazal inhibitor from *Litopenaeus vannamei* is a four-domain orthologous variant of the five-domain SPIPm2 [31]. The SPIPm2 strongly inhibits subtilisin and elastase. Domain 2 with its P1 amino acid Ala contributes solely to the inhibition of elastase. Moreover, domain 2 along with domains 3 (P1 Glu) and 5 (P1 Glu) contribute to the inhibition of subtilisin. Domain 1 with P1 Thr is inactive. Domain 4 with P1 Lys contributes weakly to the inhibition of subtilisin and trypsin [24].

Like the SPIPm2, the less abundant SPIPm4 and 5 were identified in heat-treated or heat-stressed shrimps. It is likely that they have important function in host physiological system involving stresses. They are two-domain Kazal type inhibitors with the P1 residues Ile and Phe in SPIPm4 and Leu and Thr in SPIPm5. A recently isolated SPI from *F. chinensis* is found to be a SPIPm5 ortholog with different P1 amino acids in the second domain, Thr in SPIPm5 and Ala in SPI from *F. chinensis* [18]. The SPIPm4 and 5 were chosen in this study.

Being identified from the heat-treated hemocyte cDNA libraries, we had tested whether it was also true that the mRNA of such SPIs were increased upon heat treatment using RT-PCR. The experiment was done upon the chosen SPIPm5 and found that it is up-regulated after the shrimp were heat-treated. This finding raised the possibility that the SPIs were also involved in stress responses. Further study has been conducted on this issue.

SPIPm1
```
MLLCKITLIHLLLQGFAVFNDANS◆DHD
CIGYCPEVYDPVCASNGWTYNNDCELQAMIKCQGWNITKTHDQACE
CLKACPTFAPVCGSDNKTYLNEC-VFEVASCWDHSLDKASEGACGWGIH
CLQYCPEVYDPVCGSNGQTYTNECELQAAIQCRGLQIAKRHDQACE
CHATCPLIHDPVCGTDDRTYYNEC-FFTKASCWDRSILKKKNGPCDRKWKYLLEI
```

SPIPm2
```
MANKVALLTLLAVAVAVSG◆YGKG◆GKIRL
CAKH--CTTI-SPVCGSDKTYDSRCHLENAA-CGGVSVTFHHAGPCPPPKR
CPGL--CPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSKKHDGRCG
CNPIVACPEIYAPVCGSDGKTYDNDCYFQAAV-CKNPDLKKVRDGNCD
CTPLIGCPKNYRPVCGSDGVTYNNDCFFKVAQ-CKNPALVKVSDTRCE
CNHV--CTEEYYPVCGSNGVTYSNICLLNNAA-CLDSSIYKVSDGICGRKTVPIKKGY
```

SPIPm3
```
    ...KTCWLLSLFLLASG◆QET
CDFVCPDHLDPVCGSDGITYPNLCVLELVDCLSDEDITLAHPGPCETKQES
CDILCSTDYDPVCGSDGVTYSNLCNLEVADCLSDEDITLAYEGECKEVKKGD
CDFPCPDNYDPVCGSNGVTYSNLCELERANCQSDQEITVAYDGECKG
CDFPCPDNYDPVCGSNGVTYSNLCELERANCQSDEEITVAYDGECKELKGD
CDFGCPENYDPVCGSNGVTYSNLCELERANCQSDEEITVAYPGECNS
CDFGCSGLWDPVCGSDGVTYSNLCQLEIANCLNGGGISLAYPGECEATDGS
CDIVCTANYDPVCGSDGNTYGNACDLEVADCMSDEDITLAHPGPC
```

SPIPm4
```
MANKVAFLTLLAMAVAASGYG◆KGGDSRL
CAQHCTTI-SPVCGSDGKTYDSRCHLQNAA-CGGVRVTFHHAGPCYPPKR
CPQACPFIYDPVCGTNGKTYSNSCELENDRTCNGAYVSKKHDGPCVDRKTGY
```

SPIPm5
```
MFYKTTLLILFAVAVSGYG◆KGGKFRL
CARFCPLREELPVCGSNGKTYQSRCHLDNEN-CRDESITFVHHGSCEPHQF
CPGVCPTVYD-PVCGTDGKTYSNVCDLVEAARCEGRAVSVDHDGACGRKTSPIKKGY
```

SPIPm6
```
MARFSLAFGLLAVALMASVEA◆RSPIPPGFRPGSN
CGAIACPAIYAPVCGSDGNTYPSRCNLQAARCSNPQLRVVSQGECPSQNN
CPTV-CPENYDPVCGSNGKTYPNECALESDKCSVQGLTLSHRGEC
```

SPIPm7
```
          ...DNKAYGNECKLLQEACRRPGLG-KKNDGPCVSSNN
CQRQ-CSSSYEPVCATDGVSYDNKCHLSIKSCENPSVQ-LRYEGPCAVPANPPS
PTSQPTSTGGSLQPASGSVNKVPQVGQSVLSLVTGAARPQNSAPAPAPITSPTARPASVGNAGGSTPR
CRDE-CQLGFRPVCGTDGKVYANECKLRVASCHDPTIQ-KKNDGVCEKD
CSIA-CPDDVEPVCGSNGVTYRNSCFYSIARCLDPDVT-KVANAPCANRRDTK
CDLA-CDKSINPVCGSDGRTYRNQCELEANACTKTSLI-KLYNGQCQEQQGAR
CDTV-CPNVVEEVCGDDGKTYRSHCDLIVLSCRANVRVNKAYDGRC
```

SPIPm8
```
MSRYHFVLVIFISVSAG◆HYLHTNNRK
CVATCPDLSDPVCGSNQVTYDNDCLLERAQCED-LSLHKVAEGPCE
CNSICNDEQASVCGSDGKTYESACNLQRAKCKDNPTLQQVAQGPCASSDA
CTRECGFALQPVCGSDGVTYRSECELEVAAC...
```

SPIPm9
```
    ...ILNIAICKAQARGVEIQKAYNGECDTATSTRTRPEQQQGEDYGAFAAQDDASD
CPEVCDDAFAPVCGTDSVTYDNLCSLKQSSCLTRAAGGGAPLVTRAFEGPCVAALPRGPI
CKEECVRTFLPVCGSDGETYNNICLLNLADCLNPFVTISLVKEGACTDGDEDSSADSD
EFVGLLSDTFPDPGPPPPPPGGSSSSDLTSSELTPHGGYLPPV
```

Fig. 2. Amino acid sequences of the nine Kazal-type SPIs from the *Penaeus monodon* EST database. The conserved cysteine residues are shaded. Italicized and shaded residues are the reactive P1 residues. Diamonds indicate the potential signal peptide cleavage sites. Dotted lines indicate the incomplete ends of the amino acid sequences.
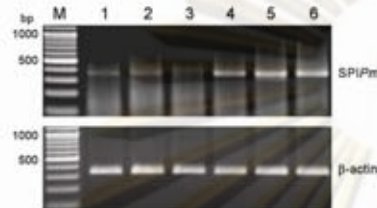
Fig. 3. RT-PCR analysis of SPIPm5 expression in heat-treated shrimp. Six subgroups of 3 individuals were divided into 2 groups, the control (lanes 1–3) and heat-treated (lanes 4–6) groups. The total RNA samples from individuals in each subgroup were pooled for cDNA synthesis for the RT-PCR. Upper and lower panels are the RT-PCR of SPIPm5 and β-actin, respectively. Lane M is the 100-bp ladder.
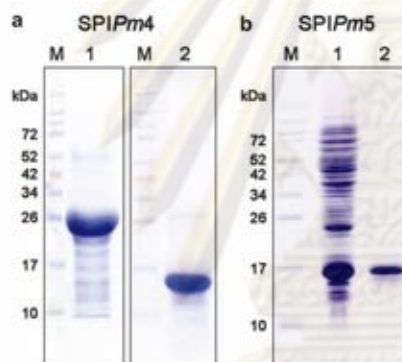

Fig. 4. Recombinant expression of rSPIPm4 and 5. The cell cultures were grown for 3 h after IPTG induction and harvested. The rSPIs were purified. For SPIPm4 (a), the fusion protein was purified through nickel-NTA column (lane 1). After digestion with enterokinase, the SPIPm4 was purified again with nickel-NTA column (lane 2). For SPIPm5 (b), the cell lysate (lane 1) was purified through a nickel-NTA column giving a considerable pure SPIPm5 preparation (lane 2). The rSPIs were kept track of using 18% SDS-PAGE. Lanes M are protein size market.
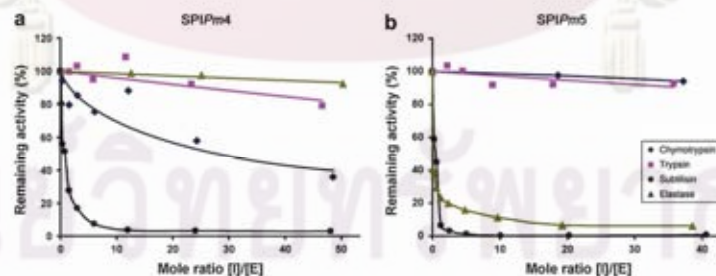
For the proteinase inhibition study, the expression clones for the two SPI genes were engineered. The recombinant proteins were produced and tested for their inhibitory activities against four proteinases: subtilisin, chymotrypsin, trypsin and elastase. It was found that the SPIPm4 strongly inhibited subtilisin and weakly inhibited chymotrypsin. The SPIPm5 strongly inhibited subtilisin and elastase.

It is well known that SPIs with P1 Lys and Arg are apt to inhibit trypsin. Those with P1 amino acid having bulky hydrophobic side chains, for example Tyr, Phe and Leu, inhibit chymotrypsin [32]. It is possible too that the Kazal domain in certain sequence contexts may be inactive or moderately active like that observed in particular domains of SPI from the crayfish *Pacifastacus leniusculus*, SPIPm2 from *P. monodon* and the MRPINK from *M. rosenbergii* [8,24,33]. In this study, the P1 Phe in SPIPm4 might contribute to the weak inhibition of chymotrypsin. The strong inhibition of subtilisin was somewhat puzzling. The two domains might contribute to the strong inhibition of subtilisin. This was possible because the P1 specificity against subtilisin was somewhat broader. For example, the P1 Ala and Glu in SPIPm2 [24], Thr in GmSPI 2 from *Galleria mellonella* L. [34], Leu in greglin from the desert locust *Schistocerca gregaria* [35], Asp in EPI1 from the oomycete *P. infestans* [5] and even Arg in CmPI-II from the marine snail *Cenchritis muricatus* [36] were P1 amino acids of subtilisin inhibitors.

For the SPIPm5 whose P1 amino acids were Leu and Thr, strong inhibition of subtilisin and elastase was observed. It was tempting to conclude that the Thr was responsible for subtilisin inhibition and Leu for elastase inhibition. The clue was from the study of a four-domain Kazal SPI TgPI-1 from an obligate intracellular parasite of human *T. gondii* [7]. The inhibitor with its P1 Arg, Arg, Leu and Leu was able to inhibit trypsin, chymotrypsin, pancreatic elastase, and neutrophil elastase. Nevertheless, the actual inhibitory activities of the Kazal domains remained to be elucidated.

Most Kazal inhibitors are potent inhibitors with the inhibition constants, K_is, in the range of nanomolar. The potent inhibitory activities of SPIPm4 against subtilisin and SPIPm5 against subtilisin and elastase led us to study their kinetics. As anticipated, the inhibition constants of SPIPm4 and 5 against subtilisin were 14.95 and 4.19 nM. That of SPIPm5 against elastase was 59.64 nM. The inhibition constants were higher compared with 0.52 nM against subtilisin and 3.27 nM against elastase of SPIPm2 [20]. In SPIPm2, there are 3 Kazal domains responsible for the inhibition of subtilisin [24]. It is, therefore, not surprising that the inhibition constant is lower. In fact, the inhibition constants against subtilisin of SPIPm4 and 5 were comparable to those individual Kazal domains of SPIPm2. Nevertheless, the inhibition against elastase of SPIPm5 was

Fig. 5. Proteinase inhibition assay of the recombinant SPIPm4 (a) and 5 (b). The inhibitor was incubated at various ratios with chymotrypsin (♦), trypsin (■), subtilisin (●) and elastase (▲) in the reactions containing chromogenic substrates. The reduction in proteinase activity was determined.
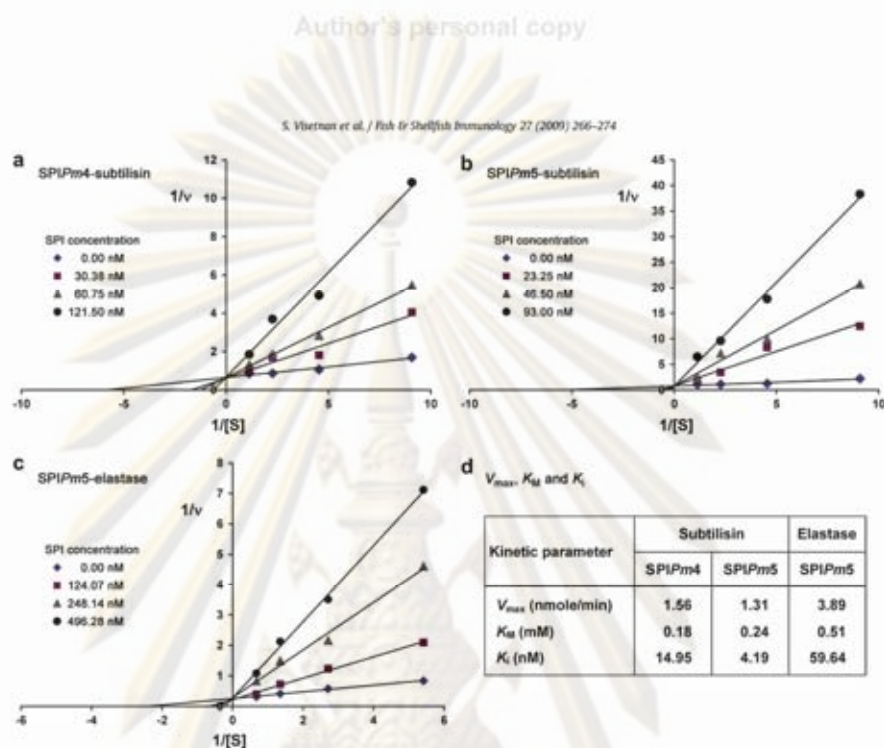
Fig. 6. Lineweaver–Burg plots of the inhibitory reactions between SPIs and proteinases. The inhibitory activity of SPIPm4 and 5 were kinetically studied against subtilisin (a and b, respectively). The SPIPm5 was also studied against elastase (c). The proteinases were assayed at various substrate concentrations in the presence of various concentrations of inhibitors as indicated in the plots. The calculated V_{max}s, K_Ms and K_is are shown (d).

20-fold less active than that of a single responsible domain in SPIPm2.

Since the SPIPm4 and 5 potently inhibited subtilisin, it was thought that they might possess bacteriostatic activity against some bacteria, particularly *B. subtilis*, like the SPIPm2 [24]. It was found, however, that they did not inhibit the growth of bacteria both Gram-positive and Gram-negative. Thus, the notion that subtilisin inhibitors inhibit the growth of *B. subtilis* did not always hold true [28].

Acknowledgements

References

[1] Jiravanichpaisal P, Lee BL, Söderhäll K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 2006;211:213–36.
[2] Iwanaga S, Lee BL. Recent advances in the innate immunity of invertebrate animals. J Biochem Mol Biol 2005;38:128–50.
[3] Christeller JT. Evolutionary mechanisms acting on proteinase inhibitor variability. FEBS J 2005;272:5710–22.
[4] Zheng QL, Chen J, Nie ZM, Lv ZB, Wang D, Zhang YZ. Expression, purification and characterization of a three-domain Kazal-type inhibitor from silkworm pupae (*Bombyx mori*). Comp Biochem Physiol B Biochem Mol Biol 2007;146:234–40.
[5] Tian M, Huitema E, Da Cunha L, Torto-Alalibo T, Kamoun S. A Kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. J Biol Chem 2004;279:26370–7.
[6] Tian M, Benedetti B, Kamoun S. A second Kazal-like protease inhibitor from *Phytophthora infestans* inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato. Plant Physiol 2005;138:1785–93.
[7] Morris MT, Coppin A, Tomavo S, Carruthers VB. Functional analysis of *Toxoplasma gondii* protease inhibitor 1. J Biol Chem 2002;277:45259–66.
[8] Li Y, Qian YQ, Ma WM, Yang WJ. Inhibition mechanism and the effects of structure on activity of male reproduction-related peptidase inhibitor Kazal-type (MRPINK) of *Macrobrachium rosenbergii*. Mar Biotechnol (NY) 2009;11:252–9.
[9] Słowińska M, Olczak M, Wojtczak M, Glogowski J, Jankowski J, Watorek W, et al. Isolation, characterization and cDNA sequencing of a Kazal family proteinase inhibitor from seminal plasma of turkey (*Meleagris gallopavo*). Comp Biochem Physiol B Biochem Mol Biol 2008;150:207–15.
[10] Mende K, Lange U, Nowak G. Three recombinant serine proteinase inhibitors expressed from the coding region of the thrombin inhibitor dipetalogastin. Insect Biochem Mol Biol 2004;34:971–9.
[11] Campos IT, Amino R, Sampaio CA, Auerswald EA, Friedrich T, Lemaire HG, et al. Infestin, a thrombin inhibitor presents in *Triatoma infestans* midgut, a Chagas' disease vector: gene cloning, expression and characterization of the inhibitor. Insect Biochem Mol Biol 2002;32:991–7.
[12] Rawlings ND, Tolle DP, Barrett AJ. Evolutionary families of peptidase inhibitors. Biochem J 2004;378(Pt 3):705–16.
[13] Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. MEROPS: the peptidase database. Nucleic Acids Res 2008;36(Database issue):D320–5.
[14] van de Locht A, Lamba D, Bauer M, Huber R, Friedrich T, Kröger B, et al. Two heads are better than one: crystal structure of the insect derived double domain Kazal inhibitor rhodniin in complex with thrombin. EMBO J 1995;14:5149–57.
[15] Lu W, Apostol I, Qasim MA, Warne N, Wynn R, Zhang WL, et al. Binding of amino acid side-chains to S1 cavities of serine proteinases. J Mol Biol 1997;266:441–61.

[16] Bode W, Huber R. Structural basis of the endoproteinase–protein inhibitor interaction. Biochim Biophys Acta 2000;1477:241–52.

[17] Wang B, Zhao J, Song L, Zhang H, Wang L, Li C, et al. Molecular cloning and expression of a novel Kazal-type serine proteinase inhibitor gene from Zhikong scallop Chlamys farreri, and the inhibitory activity of its recombinant domain. Fish Shellfish Immunol 2008;24:629–37.

[18] Kong HJ, Cho HK, Park EM, Hong GE, Kim YO, Nam BH, et al. Molecular cloning of Kazal-type proteinase inhibitor of the shrimp Fenneropenaeus chinensis. Fish Shellfish Immunol 2009;26:109–14.

[19] Supungul P, Klinbunga S, Pichyangkura R, Jitrapakdee S, Hirono I, Aoki T, et al. Identification of immune-related genes in hemocytes of black tiger shrimp (Penaeus monodon). Mar Biotechnol (NY) 2002;4:487–94.

[20] Somprasong N, Rimphanitchayakit V, Tassanakajon A. A five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp Penaeus monodon and its inhibitory activities. Dev Comp Immunol 2006;30:998–1008.

[21] Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, Jitrapakdee S, et al. Penaeus monodon gene discovery project: the generation of an EST collection and establishment of a database. Gene 2006;384:104–12.

[22] Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng 1997;10:1–6.

[23] Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, et al. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003;31:3497–500.

[24] Donpudsa S, Tassanakajon A, Rimphanitchayakit V. Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp Penaeus monodon. Dev Comp Immunol 2009;33:481–8.

[25] Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, et al. Protein identification and analysis tools on the ExPASy server. In: Walker JM, editor. The proteomics protocols handbook. Humana Press; 2005. p. 571–607.

[26] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[27] Hergenhahn HG, Aspan A, Söderhäll K. Purification and characterization of a high-Mr proteinase inhibitor of pro-phenol oxidase activation from crayfish plasma. Biochem J 1987;248:223–8.

[28] Han Y, Yu H, Yang X, Rees HH, Liu J, Lai R. A serine proteinase inhibitor from frog eggs with bacteriostatic activity. Comp Biochem Physiol B Biochem Mol Biol 2008;149:58–62.

[29] Lowther WT, Matthews BW. Structure and function of the methionine aminopeptidases. Biochim Biophys Acta 2000;1477:157–67.

[30] Jarasrassamee B, Supungul P, Panyim S, Klinbunga S, Rimphanitchayakit V, Tassanakajon A. Recombinant expression and characterization of five-domain Kazal-type serine proteinase inhibitor of black tiger shrimp (Penaeus monodon). Mar Biotechnol (NY) 2005;7:46–52.

[31] Jiménez-Vega F, Vargas-Albores F. A four-Kazal domain protein in Litopenaeus vannamei hemocytes. Dev Comp Immunol 2005;29:385–91.

[32] Laskowski Jr M, Kato I. Protein inhibitors of proteinases. Annu Rev Biochem 1980;49:593–626.

[33] Johansson MW, Keyser P, Söderhäll K. Purification and cDNA cloning of a four-domain Kazal proteinase inhibitor from crayfish blood cells. Eur J Biochem 1994;223:389–94.

[34] Nirmala X, Kodrik D, Zurovec M, Sehnal F. Insect silk contains both a Kunitz-type and a unique Kazal-type proteinase inhibitor. Eur J Biochem 2001;268:2064–73.

[35] Brillard-Bourdet M, Hamdaoui A, Hajjar E, Boudier C, Reuter N, Ehret-Sabatier L, et al. A novel locust (Schistocerca gregoria) serine protease inhibitor with a high affinity for neutrophil elastase. Biochem J 2006;400:467–76.

[36] González Y, Pons T, Gil J, Besada V, Alonso-del-Rivero M, Tanaka AS, et al. Characterization and comparative 3D modeling of CmPI-II, a novel 'non-classical' Kazal-type inhibitor from the marine snail Cenchritis muricatus (Mollusca). Biol Chem 2007;388:1183–94.

BIOGRAPHY

Miss Suwattana Visetnan was born on April 26, 1982 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of Biology, Field of study Applied Biology (Microbiology), Faculty of Science and Technology, Suan Sunandha Rajabhat University in 2004. She has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 2006.

She had published her works in the research journals on the topics of "Kazal-type serine proteinase inhibitors from the black tiger shrimp *Penaeus monodon* and the inhibitory activities of SPI*Pm*4 and 5".