การแสดงออกและลักษณะสมบัติของตัวยับยั้งเซรีนโปรติเนสของกุ้งกุลาดำ *Penaeus monodon*

นางสาวนวรัตน์ สมประสงค์

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EXPRESSION AND CHARACTERIZATION OF SERINE PROTEINASE INHIBITOR OF THE BLACK TIGER SHRIMP *Penaeus monodon*

Miss Nawarat Somprasong

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By	Miss Nawarat Somprasong
Field of study	Biochemistry
Thesis Advisor	Associate Professor Anchalee Tassanakajon, Ph.D.
Thesis Co-advisor	Assistant Professor Vichien Rimphanitchayakit, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……..……...….. Deputy Dean for Administrative Affairs,
Acting Dean, The Faculty of Science
(Associate Professor Tharapong Vitidsant, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Aran Incharoensakdi, Ph.D.)

…….. Thesis Advisor
(Associate Professor Anchalee Tassanakajon, Ph.D.)

…….. Thesis Co-advisor
(Assistant Professor Vichien Rimphanitchayakit, Ph.D.)

…….. Member
(Assistant Professor Suganya Soontaros, Ph.D.)

…….. Member
(Mr. Sirawut Klinbunga, Ph.D.)

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ทำการโคลน และแสดงออกของยีนตัวยับยั้งเซรีนโปรติเนสจากกุ้งกุลาดำที่มี Kazal domain จำนวน 5 โดเมน ใน *Escherichia coli* สายพันธุ์ Rosetta (DE3) pLysS และใช้ pET-22b(+) เป็นเวคเตอร์ การทดลองนี้ทำการแสดงออกของตัวยับยั้งเซรีนโปรติเนสโดยตัดส่วนที่คาดว่าเป็น signal sequence ออก โดยยีนนี้มีบริเวณ open reading frame ขนาด 747 เบส ซึ่งแปลรหัสให้โปรตีน ที่มีกรดอะมิโนจำนวน 248 ตัว แล้วโคลนเข้าสู่เวคเตอร์ระหว่าง *pelB* signal sequnce และ His•Tag จากการวิเคราะห์โปรตีนที่ได้จากการแสดงออก พบโปรตีนที่คาดว่าน่าจะเป็นรีคอมบิแนนท์ของตัวยับยั้งเซรีนโปรติเนสในรูปของ inclusion body ภายในเซลล์ของ *E.coli* ที่มี pET-22b(+)-SPI อยู่ และได้ยืนยันการแสดงออกของยีนด้วยการทำ Western blot analysis ทำการละลาย inclusion body โดยการใช้ denaturing condition และ nondenaturing condition ซึ่งพบว่าแอคติวิตีในการยับยั้งโปรติเนสของการใช้ denaturing conditon มีค่าต่ำกว่าการใช้ nondenaturing condition ประมาณ 2 เท่า ดังนั้นจึงเลือกใช้ nondenaturing condition เพื่อทำให้โปรตีนรีคอมบิแนนท์บริสุทธิ์โดยทำการแยกให้บริสุทธิ์ด้วยวิธีคอลัมน์โครมาโทกราฟีโดยใช้ Sephadex G-100 ซึ่งพบว่า รีคอมบิแนนท์ของตัวยับยั้งเซรีนโปรติเนสมีความบริสุทธิ์เพิ่มขึ้น 1.03 เท่า จากการหามวลโมเลกุลของโปรตีนด้วยวิธี MALDI-TOF Mass Spectrometry พบว่าโปรตีนมีขนาด 29.065 กิโลดาลตัน และเมื่อศึกษาแอคติวิตีในการยับยั้งโปรติเนสพบว่าโปรตีนรีคอมบิแนนท์ที่ทำให้บริสุทธิ์มีแอคติวิตีในการยับยั้ง subtilisin, elastase ได้ดี และ ยับยั้ง trypsin ได้ต่ำกว่า แต่ไม่สามารถยับยั้ง chymotrysin ค่าคงที่ในการยับยั้งของ subtilisin inhibitor complex และ elastase inhibitor complex เท่ากับ 0.62 และ 3.16 นาโนโมลาร์ ตามลำดับ แสดงให้เห็นว่าโปรตีนรีคอมบิแนนท์มีประสิทธิภาพสูงในการยับยั้ง subtilisin และ elastase

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

สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2547.....ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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NAWARAT SOMPROSONG: EXPRESSION AND CHARACTERIZATION OF SERINE PROTIENASE INHIBITOR OF THE BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: ASSOC. PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS COADVISOR : ASIST. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D. 102 pp. ISBN 974-53-1008-5.

The five-domain Kazal-type proteinase inhibitor from the black tiger shrimp *Penaeus monodon*, SH415, was cloned and expressed in the *Escherichia coli* expression system. The 5' terminal truncated serine proteinase inhibitor gene containing 474 base pair open reading frame encoding for 248 amino acid polypeptide was cloned into pET-22b(+) between a *pelB* signal sequence and His•Tag. The pET-22b(+)-SPI clone was expressed in *E. coli* strain Rosetta (DE3) pLysS. The protein was expressed in the *E. coli* containing pET-22b(+)-SPI as the inclusion bodies and the identity of the expressed recombinant protein was confirmed by Western blot analysis. Two conditions were used to solubilize the inclusion bodies, the denaturing and non-denaturing or native conditions for further purification. The inhibitory activity of the recombinant protein, purified using denaturing condition was about 2 folds lower than that purified using native condition. The recombinant protein prepared under the native condition was further purified using Sephadex G-100 chromatrography. It was purified up to 1.03 folds with 17% recovery. The molecular mass of recombinant SPI was determined by using MALDI-TOF Mass Spectrometry to be 29.065 kDa. From the proteinase inhibitory assay, the purified protein showed substantial inhibitory activity agianst subtilisin and elastase and low activity against trypsin. The inhibition constant (Ki) of subtilisin and elastase inhibitor complex were 0.62 and 3.16 nM, respectively.

Department…..BIOCHEMISTRY… Student's signature.....…..
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List of Abbreviations

°C	degree celsius
µl	microlitre
µM	micromolar
bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	ethylene diamine tetraacetic acid (disodium salt)
IPTG	isopropyl-thiogalactoside
kb	kilobase pair
mg	milligram
min	minute
ml	millilitre
mM	millimolar
nmole	nanomole
nM	nanomolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction

CHAPTER I
INTRODUCTION

One of the most favorite seafood in the world history is shrimp or species-alike. It is therefore not surprising that the world consumption increased yearly. From fishery to culturing, the shrimp production has increased steadily. It has expanded from 2.4 million metric tones in 1987 to 4.2 million metric tones in 2000. Most of the shrimp production takes place in the Pacific region, and most of production growth is from this area (Helga, 2004).

The one shrimp species produced is the black tiger shrimp, *Penaeus monodon*. The production grew very strongly in the late 1980s, bloomed by the growing culture industry. In recent years, however, incident of shrimp infectious diseases occurred in the main producing countries leading to the decline in the production. These infectious diseases are considered the most limiting factor for further development in culture industry. Even worse, epidemiological surveys and knowledge of factors that determine the health status of shrimp are scarce such that adequate measures to control diseases other than management practices are not available. Moreover, shrimp farmers still rely mainly on wild animals for the production of seedstock, and this thus makes the genetic selection of resistant domesticated shrimp stock not feasible. Nevertheless, methods to prevent and control diseases are essential and need to be developed for a sustainable shrimp culture sector.

1.1 Taxonomy of *Penaeus monodon*

Penaeid shrimp belongs to the largest phylum in the animal kingdom, the Arthropoda. This group of animal is characterized by the presence of pair appendages, and by a protective cuticle of exoskeleton that covers their whole body. The subphylum Crustacea is made up of 42,000, predominantly aquatic species, that is divided into 10 classes. Within the class Malacostraca, shrimp, crayfish, lobster and crab belong to the order Decapoda. The taxonomic definition of the giant tiger shrimp, *Penaeus monodon* is as follow (Bailey-Brook and Moss, 1992):

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

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

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

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

1.2 Morphology

The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmented abdomen (Figure 1.1). Most organs, such as gills, digestive system and heart, are located in the cephalothorax, while the muscles concentrate in the abdomen. Appendages of the cephalothorax vary in appearance and function. In the head region, antennules and antennae perform sensory functions. The mandibles and the two pairs of maxillae (not visible in Figure 1.1) form the jaw-like structures that are involved in food uptake (Solis, 1988). In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five

pairs of swimming legs (pleopods) are found on the abdomen (Bell and Lightner, 1988; Baily-Brock and Moss, 1992).

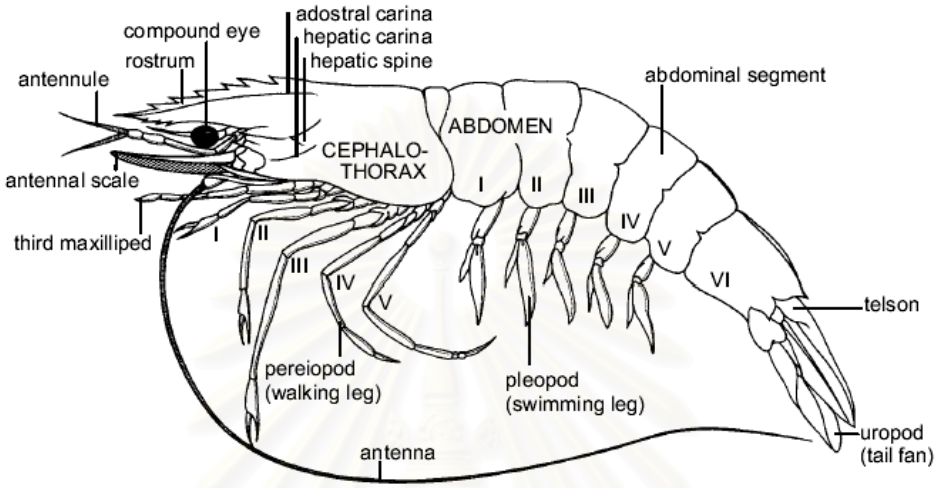

Figure 1.1 Lateral view of the external morphology of *Penaeus monodon* (Primavera, 1990).

The internal morphology of penaeid shrimp is outlined in Figure 1.2. Penaeids and other arthropods have an open circulatory system and, therefore, the blood and the blood cells are called haemolymph and haemocytes, respectively. Crustaceans have a muscular heart that is dorsally located in the cephalothorax. The valved haemolymph vessels leave the heart and branch several times before the haemolymph arrives at the sinuses that are scattered throughout the body where exchange of substances takes place. After passing the gills, the haemolymph returns to the heart by means of three wide non-valved openings (Bauchau, 1981). A large part of the cephalothorax in penaeid shrimp is occupied by the hepatopancreas. This digestive gland consists of diverticula of the intestine. Spaces between these hepatopancreatic tubules are haemolymph sinuses. The main functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leaves the heart ends in the lymphoid organ where the haemolymph is filtered. This organ is located ventro-anteriorly to the hepatopancreas. The haemocytes are produced in haematopoietic

tissue. This organ is dispersed in the cephalothorax, but mainly present around the stomach and in the onset of the maxillipeds. Lymphoid organ and haematopoietic tissue are not shown in Figure 1.2.

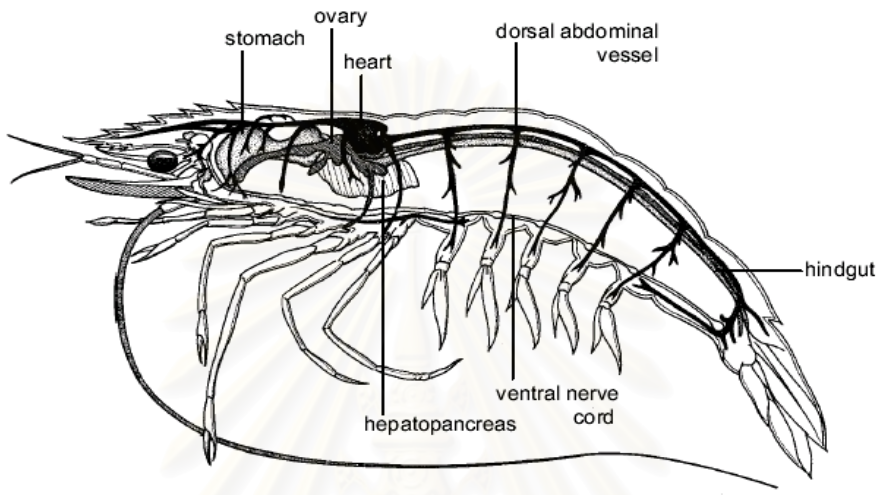

Figure 1.2 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990).

1.3 Distribution and life cycle

The black tiger shrimp is widely distributed throughout the greater part of the Indo-Pacific region, ranging northward to Japan and Taiwan, eastward to Tahiti, southward to Australia and westward to Africa. The penaeid life cycle includes several distinct stages that are found in a variety of habitats (Figure 1.3). Juveniles prefer brackish shore areas and mangrove estuaries as their natural environment. Most of the adults migrate to deeper offshore areas that have higher salinity, where mating and reproduction take place. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). Within 6 hours after fertilization, the eggs hatch into the first larval stage, the nauplius (6 stages in 2 days). The nauplii feed on their reserves for a few days and develop into the protozoeae (3 stages in 5 days). The protozoeae feed on algae and metamorphose into myses (3 stages in 4-5 days). The myses, which have many of the characteristics of adult shrimp, feed on algae and zooplankton. They then develop into megalopas (6-35 days). The megalopa and early

juvenile are call postlarvae. Transition from juvenile to subadult take 135-255 days and subsequently completion of sexual maturity occurs within 10 months (Motoh, 1984).

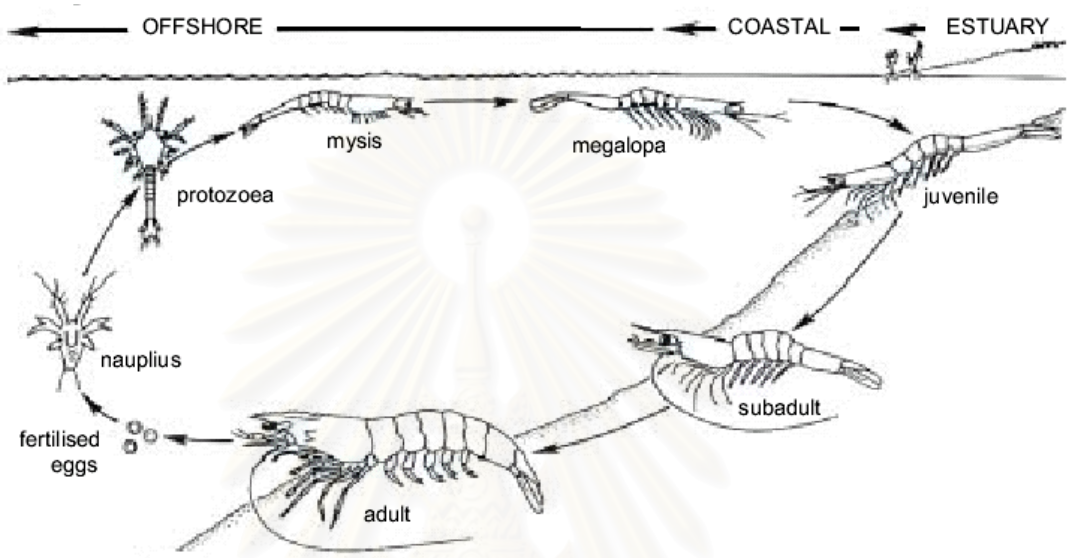

Figure 1.3 The life cycle of *Penaeus monodon* shrimp. Pictures are not in proportion to the actual size. (Baily-Brock and Moss, 1992)

1.4 Exploitation of shrimp culture in Thailand

Thailand has been regarded as the leader of shrimp production for nearly a decade. The success of shirmp industry in Thailand has resulted in the steadily increased income for the nation annually. The main shrimp species was cultured including the black tiger shrimp (*Penaeus monodon*) and the white shrimp (*Penaeus vannamei*). Nevertheless, there are several problem in shrimp industry including environmental degradation, outbreak of diseases, depletion of the wild broodstock that are used to stock commercial hatcheries (Browdy, 1998) and slow growth (Chayaburakul et al., 2004).

The outbreaks of infectious diseases have become more and more serious problem, causing a great deal of loss to the productions (Roch, 1999). The main causes of infectious diseases are white-spot syndrome virus (WSSV), yellow head

virus (YHV) (Flegel, 1997) and luminescent bacteria, *Vibrio* species (Chou et al., 1995; Flegel et al., 1995; Jiravanichpaisal et al., 1994).

Although diagnostic methods of pathogenic agents in *Penaeus monodon* are well developed, the control of diseases is not efficient enough owing to the lacking of knowledge on the mechanism and expression of genes responded to pathogenic infection and the shrimp immune systems. Therefore, more studies on shrimp immune system and genetics are urgently required.

1.5 Shrimp diseases

The outbreaks of infectious diseases become more and more serious in the shrimp industry because of increasing shrimp farming and lack of proper knowledge involving shrimp biology, farm management and diseases. The infectious diseases in *Penaeus monodon* are caused mainly by virus and bacteria belonging to the family Vibrionacea (Lightner et al., 1983; Kroll et al., 1991; Mohney et al., 1994; Hasson et al., 1995; Flegel, 1997).

Outbreaks of yellow head disease were the most serious problem in central and southern Thailand during 1993-1994, while the white spot disease was the most serious problem from 1994 to 1996. Also, from mid 1996 until now, luminescent bacterial disease is increasingly considered to be the cause for the unsuccessful shrimp farming. In 2001-2002 througout in Thailand, black tiger shrimp *Penaeus monodon* farmers reported very unusual retarded growth. This problem was called monodon slow growth syndrome (MSGS). Based on decreased national production, estimated losses due to this phenomenon were in the range of 13000 million baht (approximately US$ 300 million) in 2002. It is possible that MSGS is caused by an unknown pathogen or by some other presently unknown, non-pathogenic factor (Chayaburakul., 2004). These pathogens particularly hamper larval production and lead to stock mortality and consequently profitability problems. They also lead to the over-fishing of wild shrimp larvae and an over-exploitation of broodstock.

1.5.1 Viral disease

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

1.5.2 White spot syndrome (WSS)

White spot syndrome (WSS) is a viral disease, which was first discovered in south-east Asia around 1992. White spot syndrome virus (WSSV) is currently the most serious viral pathogen of shrimp worldwide. It causes up to 100% mortality within 7 to 10 days in commercial shrimp farms, resulting in large economic losses to the shrimp farming industry (Lightner, 1996). This virus belongs to a new virus family, the *Nimaviridae*, and contains a large circular double stranded DNA genome of 292,967 bp (Van Hulten et al, 2001), but isolated with larger genomes have also been identified (AF440570) (Yang et al., 2001). WSSV virions are ellipsoid to bacilliform, enveloped particles with a distinctive tail-like appendage at one end and can be found throughout the body of infected shrimp. The virions contain one nucleocapsid with a typical striated appearance and five major and at least 13 minor proteins (Huang et al., 2002; Van Hulten et al., 2000a, 2000b, 2002).

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

Diseases caused by viruses, especially by white spot syndrome virus (WSSV), are of the greatest challenge to worldwide shrimp aquaculture. The innate immunity of shrimp has attracted extensive attention, but no factor involved in the virus resistance has been reported until 2003. A differential pmAV cDNA cloned from WSSV-resistant shrimp *Penaeus monodon* was found to have an open reading frame (ORF) encoding a 170 amino acid peptide with a C-type lectin-like domain (CTLD) displaying a strong antiviral activity; it inhibited virus-induced cytopathic effect in fish cells *in vitro* (Lua et al., 2003). In addition, two peptides with molecular masses at 73 and 75 kDa were isolated from shrimp *P. monodon* using affinity chromatrography coupled with the purified WSSV or a fish iridovirus (Singapore grouper iridovirus, SGIV), and identified as hemocyanin by mass spectrometry. The results, using fish viruses capable of cell culture, showed for the first time that the hemocyanin had non-specific antiviral properties and no cytotoxicity against host cells. (Zhang et al., 2004)

The development of vaccines against WSSV would be desirable. The idea of vaccination shrimp, or invertebrates in general, seems to be unfeasible since they are assumed to lack an adaptive immune response and rely solely on innate immune response(Kimbrell and Beutler, 2001). However the recent study in the copepod *Macrocyclops albidus* showed that the defense system of this invertebrate species reacted more efficiently after a previous encounter with an antigenically similar parasite, implying that a specific memory may exist (Kurtz et al., 2003). Witteveldt et al. (2004) investigated the potential of oral vaccination of shrimp with subunit vaccines consisting of WSSV virion envelope proteins. *P. monodon* shrimp were fed food pellets coated with inactivated bacteria overexpressing two WSSV envelope proteins, VP19 and VP28. The result suggests that contrary to current assumptions that invertebrates do not have a true adaptive immune system, a specific immune response and protection can be induced in *P. monodon*. These experiments open up new ways to benefit the WSSV-hampered shrimp farming industry.

1.5.3 Yellow-head (YH) disease

Yellow head virus infection has significant losses of cultured shrimp *Penaeus monodon* throughout Asia (Chantanachookin et al., 1993; Flegel, 1997).

Extensive characterization of YHV genome (Sittidilokratna et al., 2002; Cowly and Walker, 2002; Jitrapakdee et al., 2003) has clearly shown that this virus is classified in a new genus *Okavirus* and family *Roniviridae* in the order *Nidovirales* (Mayo, 2002).

Yellow-head principally infects pond-reared black tiger prawn *Penaeus 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). At the onset of Yellow head disease shrimp have been observed consuming feed at an abnormally high rate for several days. Feeding abruptly ceases and within 1 day, a few moribund shrimp appear swimming slowly near the surface at the pond edges. Affected shrimp exhibit light yellow coloration of the cephalothorax area and a generally pale or bleached appearance; they die within a few hours. 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 (Lightner, 1996, Chantanachookin et al., 1993). Moribund shrimp with YHD generally appear pallid in color, with a yellowish, often swollen cephalothorax. Infected shrimp frequently exhibit whitish or pale yellowish to brown gills, and often a pale yellow hepatopancreas (Lightner, 1996).

To diagnose the infection, antibody (Lu et al., 1996; Nadala et al., 1997; Nadala and Loh, 2000; Sithigorngul et al., 2000, 2002; Soowannayan et al., 2003), *in situ* hybridization (ISH) (Tang and Lightner, 1999; Tang et al., 2002; Spann et al., 2003), conventional RT-PCR (Wongteerasupaya et al., 1997; Tang and Lightner, 1999; Cowley et al., 1999, 2000a) and real-time RT-PCR (Dhar et al., 2002) methods have been used to detect YHV. Of the antibody-based diagnostic techniques, immuno-histochemistry using monoclonal antibodies to a surface glycoprotein and the nucleocapsid protein of YHV (Sithigorngul et al., 2000, 2002) has recently allowed the detection and distinction of YHV infections (Soowannayan et al., 2003).

To neutralize virus, the mouse polyclonal antiserum raised against the YHV gp116 or gp64 structural glycoproteins could neutralize YHV infectivity as determined using an in vitro quantal assay in primary cultures of lymphoid organ cells

was investigated. Anti-gp116 antiserum showed virus-neutralizing activity whereas anti-gp64 antiserum failed to inhibit infection. The results suggest that gpl16 antiserum blocks binding of virions to cellular receptors to facilitate YHV entry into lymphoid organ cells. (Assavalapsakul et al., 2005)

1.5.4 Bacterial disease

Vibrio species are normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). Different *Vibrio* species have been reported as opportunist pathogen that cause serious production losses in shrimp farms with mortality to 100%, particularly in postlarvae and juvenile populations (Karunasagar, et al., 1994; Prayitno and Latchford 1995; Vandenberghe, 1998; Sudheesh and Xu 2001).

The luminescent bacterium, *Vibrio harveyi*, frequently related to the outbreaks of luminous vibriosis in cultured *Penaeus monodon* in hatcheries in many countries such as Australia, China, India, Indonesia, Thailand, Philippines and Taiwan (Vandenbreghe et al., 1998). In Thailand, vibriosis is the main cause of production loss in penaeid shrimp farms. The bacterium, *V. harveyi*, is therefore claimed to be the most causative agent for shrimp farming. *V. harveyi* is a rod shape, Gram-negative bacterium with 0.5-0.8 μm width and 1.4-2.6 μm in length. This bacterium is able to emit a blue-green color light. The reaction leading to light emission is catalyzed by the luciferase enzyme. The substrates are reduced flavin mononucleotide ($FMNH_2$), a long chain aldehyde (RCHO), and oxygen, which react according to the following reaction:

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

The disease caused by *Vibrio harveyi* is widely known as luminous disease. The gross signs of localized infection in the cuticle or sub-cuticle also give the names shell disease or black/brown spot disease. These superficial infections can develop into systemic infections under some circumstances and can cause mortality. 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.

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

To control this disease, the antibiotic was used to control the pathogen but it has lead to the problems of drug resistance (Karunasaga et al., 1994). The development and use of probiotics is being recognised as a useful strategy to combat shrimp pathogens. A marine bacterial strain, *Pseudomonas* I-2 produced inhibitory compound against shrimp pathogenic vibrios and has potential applications for control shrimp pathogenic vibrios in aquaculture system (Chythanya et al., 2001). The *in vitro* and *in vivo* antagonistic effect of *Bacillus subtilis* BT23 against the pathogen Vibrios was studied (Vaseeharan et al., 2003). Cell free extracts of *Bacillus subtilis* BT23 showed greater inhibitory effect against the growth of *Vibrio harveyi* isolated by agar antagonism assay from *Penaeus monodon* with black gill disease. The Marine bacterium *Pseudoalteromonas* species strian X153 isolated from a pebble collected at St. Anne du Portzic (France) was purified and partial identified. This bacterium protected bivalve larvae against mortality, following experimental challenges with ichthyopathogenic *Vibrio*. *Pseudoalteromonas* sp. X153 may be useful in aquaculture as a probiotic bacterium (Longeon et al., 2005).

1.6 The crustacean immune system

1.6.1 Evolution of the immune system

Two systems providing internal defense against infectious agents have been selected during evolution, namely the innate (natural) and the acquired (adaptive) immune systems. The innate immune system can be found in all multi-cellular animals and consists of cellular and humoral elements. The most prominent cellular defense reactions against invading microorganisms are phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting. The humoral defense factors, such as clotting proteins, agglutinins (e.g. lectins), hydrolytic enzymes and

antimicrobial peptides are often produced by and act in conjunction with the defensive cells. The acquired immune system is phylogenetically younger, found only in vertebrates and operates through lymphocytes. Even though the immune system of invertebrates has often been described as far less complicated than that of vertebrates, it is still very efficient and complex. Invertebrates have managed to occupy nearly all habitats on earth and, consequently, they have to cope with an extremely large variety of pathogens. The efficacy of their defense system is witnessed by their persistent survival through many million years of evolution (Millar and Ratcliffe, 1994).

1.6.2 Study of the immune system

The extensive study of vertebrate defense, including the origin and development of different blood cell types, results in a fairly uniform scheme of morphological and immuno-functional classification of blood cells. Moreover, the purification and characterization of defense proteins provides many explanations of the immune functions. In contrast, the huge diversity of invertebrates and the limited knowledge of their haemocyte lineage make it, so far, impossible to categorize haemocytes in morphologically well-defined ontogenic classes. In addition, haemocytes are very reactive cells and undergo considerable transformation when they are removed from the haemocoel (Bauchau, 1981), thus functional characteristics of those cells are more difficult to study than those of the vertebrate blood cell. Haemocyte activation results in rapid clotting, cellular degranulation, activation of the proPO system (see below) and subsequently production of sticky molecules (Johansson and Söderhäll, 1992). The labile nature and the low quantity of several defense proteins in the haemolymph also complicate the purification of individual proteins of the invertebrate defense system (Soderhall et al., 1990).

During the last few years, considerable progress has been made in utilizing different anticoagulants and media to keep the haemocytes closer to their natural state (Bachere, 2000), which has provided opportunities for reliable in vitro functional studies. In addition, the cloning and characterization of genes encoding for immunity and subsequent studies of the expression of those genes during infection or defense stimulation also lead to a better understanding of the functioning of the defense system in invertebrate. Non-specific molecules including phenoloxidase, bactericidins

and lectins, specifically antibacterial antiviral and antifungal peptides, have been documented and some genes have been characterized (Cuthbertson et al., 2002; Destoumieux et al., 2002; Destoumieux-Garzon et al., 2001; Gross et al., 2001; Rojtinnakorn et al., 2002; Roux et al., 2002 and Zhang et al., 2004). The combination of different approaches highly contributes to the advance in this field.

1.6.3 Functioning of the crustacean defense system

The hard cuticle, a physical barrier that may also contain antimicrobial factors, can be considered as the external defense in crustaceans. The haemocytes, on the other hand, play an important and central role in the internal defense. Although until now three different cell types have been commonly described, a universally accepted haemocyte classification scheme is not yet available for penaeid shrimp. In general, the hyaline cell is the smallest cell type with a high nucleus/cytoplasm ratio and no or few cytoplasmic granules. The granular cell is the largest cell type with a relatively smaller nucleus and fully packed with granules. The semigranular cell is an intermediate between the hyaline and the granular cell (Bauchau, 1981; Söderhäll and Cerenius, 1992).

A schematic overview of the most important factors in the crustacean defense system, which are known until now, is given in Figure 1.4. The first and essential internal defense process is the recognition of invading microorganisms, which is mediated by the haemocytes and plasma proteins (Vargas-Albores and Yepiz-Plascencia, 2000). The invertebrate immune system presumably recognizes large groups of pathogens, represented by fixed common molecular patterns, rather than fine structures, specific for particular microbes (Söderhäll et al., 1996). Several types of recognition proteins have been described and are called pattern recognition proteins (PRPs). The PRPs recognize carbohydrate moieties of cell wall components of microorganisms, like lipopolysaccharides (LPS) or peptidoglycans (PG) from bacteria, or β-1,3-glucans from fungi (Söderhäll et al., 1996; Vargas-Albores et al., 1996; 1997). Some of the PRPs are lectins and can work directly as agglutinins or opsonins (Kopácek et al., 1993; Söderhäll et al., 1996). After binding of the PRP ligand with the microbial component, a second site becomes active for cellular

binding. Haemocyte activation is generated after this second binding step (Vargas-Albores and Yepiz-Plascencia, 2000) (Figure 1.4a). Recently, the cDNA encoding β-1,3-glucan binding protein of *Penaeus monodon* was cloned and sequenced (Sritunyalucksana et al., 2002).

After detection of foreign material, haemocytes migrate to the site of invasion by a process of chemotaxis and result in inflammation. This event also appears in vertebrates. The open circulatory system in invertebrate demands a rapid and efficient defense, in which the proteolytic cascades play an important role (Sritunyalucksana and Söderhäll, 2000). The haemocytes are involved in the synthesis, storage and, upon activation, discharge of proenzymes and substrates of the clotting and proPO cascades (Johansson and Söderhäll, 1992; Söderhäll et al., 1996; Sritunyalucksana and Söderhäll, 2000).

The clotting mechanism entraps foreign material and prevents loss of haemolymph. The transglutaminase (TGase)-dependent clotting reaction of crustaceans is best described in the freshwater crayfish *Pacifastacus leniusculus* (Kopácek et al., 1993; Hall et al., 1998). The clotting reaction is induced when TGase is released from the haemocytes or tissues. The Ca^{2+}-dependent TGase catalyses polymerization of the clotting protein in the plasma to form a gel-like substance (Kopácek et al., 1993; Yeh et al., 1998) (Figure 1.4b).

The proPO-activating system in a crustacean, the freshwater crayfish *Pacifastacus leniusculus*, has been studied most extensively (Söderhäll et al., 1996; Söderhäll and Cerenius, 1998). Proteins of the proPO system occupy a very prominent position in non-self recognition, haemocyte communication and the production of melanin. Upon activation and degranulation of the haemocytes, the inactive proPO is converted to the active phenoloxidase (PO) by prophenoloxidase activating enzyme (ppA). The PO enzyme catalyses the stepwise oxidation of phenols to quinones, followed by several intermediate steps that lead to the formation of melanin (Figure 1.4c). During this formation, antimicrobial factors are also formed (Söderhäll et al., 1996; Söderhäll and Cerenius, 1998). Melanin is a dark brown pigment that sequesters the pathogens, thus preventing their contact with the host. Melanized matter can often be seen as dark spots in or under the cuticle of arthropods.

An important factor that is associated with the proPO system is peroxinectin, which was recently cloned from *Penaeus monodon* (Sritunyalucksana et al., 2001). Peroxinectin has two different functions, cell-adhesion and peroxidase activity. Crayfish peroxinectin is synthesized in the haemocytes, stored in the secretory granules in an inactive form, released in response to stimuli and activated outside the cells. Transmembrane receptors of the integrin family on the haemocytes play an important role in the cell adhesion function of peroxinectin (Johansson, 1999). The cell-adhesion is involved in attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination (aggregation), while the antimicrobial properties of the peroxidase activity of the protein might help to kill invading microorganisms (Johansson and Söderhäll, 1988; 1989; Kobayashi et al., 1990; Thörnqvist et al., 1994) (Figure 1.4d). Phagocytosis is the internalization of small foreign particles by individual cells. After phagocytosis, shrimp haemocytes, like vertebrate blood cells, use cytotoxic oxygen radicals to kill the foreign material (Song and Hsieh, 1994; Munoz et al., 2000). If large amounts of foreign particles enter the body or if they are too large to be internalized, several haemocytes cooperate to seal off the pathogens. These phenomena are called nodule formation and encapsulation, respectively (Söderhäll et al., 1996).

Enzyme inhibitors, also produced by the haemocytes, are necessary to regulate the proteinase cascades and prevent over-activation and damage to the host tissue. Serine proteinase inhibitors from the Kazal and Serpin families have been identified in crustaceans (Kanost, 1999). The α-macroglobulin, which serves as a broad-spectrum protease-binding protein, is stored in the haemocyte granules (Armstrong and Quigley, 1999). In addition, haemocytes play an important role in the production and discharge of agglutinins such as lectins (Kopácek et al., 1993), of antibacterial peptides (Destoumieux et al., 1997; 2000) and of cytotoxic molecules such as lysosomal enzymes (lysozyme, esterases, phosphatases, phospholipases, peroxidases and proteases) (Millar and Ratcliffe, 1994). For an efficient immune defense, all different components of the immune system must work together.

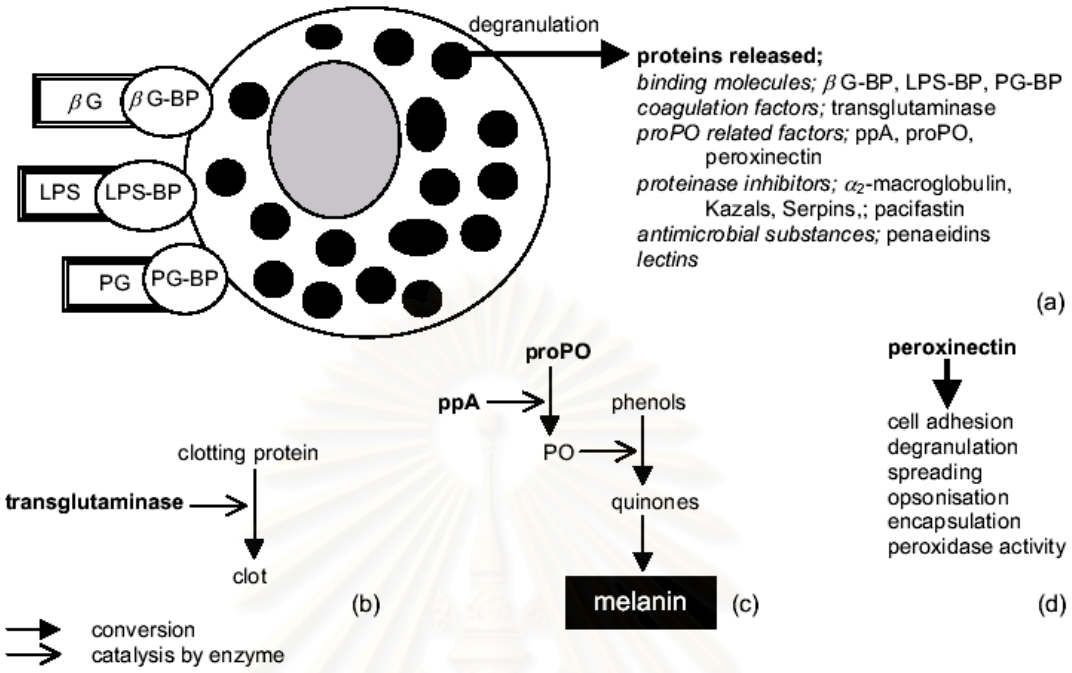

Figure 1.4 Simplified overview of present-day knowledge of the most important defense factors of decapod crustaceans that are mediated by the haemocytes. Different pattern recognition proteins in the haemolymph recognize and bind cell wall components of microorganisms. Subsequently their cellular binding is induced, which operates as elicitor of defense responses. The haemocytes degranulate and release different proteins (a). The proteins that are released are involved in the clotting (b), the prophenoloxidase activating (c) system or in other cellular activation processes (d). The proteins involved in those processes that are released from the haemocytes are indicated in bold letters. Activation of cascade processes is regulated by different proteinase inhibitors. βG, β-1,3-glucan; βG-BP, β-1,3-glucan binding protein; LPS, lipopolysaccharide, LPS-BP, lipopolysaccharide binding protein; PG, peptidoglycan; PG-BP, peptidoglycan binding protein; PO, phenoloxidase; ppA, prophenoloxidase activating enzyme; proPO, prophenoloxidase.

1.7 Serine proteinase inhibitors in arthropod immunity

Arthropod haemolymph contains proteins with serine proteinase inhibitory activity. These inhibitors may exist in plasma or in haemocyte granules. They are members of several different gene families (Kanost and Jiang, 1996; Palanoski and Wilusz, 1996). According to their characteristics, serine proteinase inhibitors can be divided into three families, inhibitors that contain a small disulfide-rich domain, α-macroglobulins and serpins.

1.7.1 Inhibitors containing a small, disulfide-rich domain

Low molecular weight serine proteinase inhibitors from several gene families have been identified. They may occur as single, small proteins or in some cases as chains of inhibitor domains that are part of the same polypeptides. Some are plasma proteins and others are located within the haemocytes. The inhibitors in this family comprise Kazal, Kunitz and light chain of pacifastin (Johansson et al., 1994; Nakamura et al., 1987 and Liang et al., 1997). They mostly consist of several repeated domains, each made up of 50-60 amino acid residues with six conserved cysteine residues forming three intradomain disulfide bridges, which stabilize the domain (Bode, and Huber, 1992)

A proteinase inhibitor from the Kazal family was isolated from the haemocytes of the crayfish. This protein contained four Kazal family domains and was shown to inhibit chymotrypsin and subtilisin (Johansson et al., 1994). Many Kazal serine proteinase inhibitors from several multicellular organisms have been identified and characterized. Mammalian Kazal inhibitors contain one inhibitory domain except the submandibular inhibitor of dog, which contains two Kazal domains. Several inhibitory domains are common in ovomucoid and ovoinhibitors from birds (Laskowski, 1987). In several insect species, many Kazal inhibitors have been identified and characterized (Kanost and Jiang, 1996, Polanowski and Wilusz, 1996). Detection of Kazal inhibitors in invertebrates including sea anemaone (Kolkenbrock and Tschesche, 1987), leech (*Bdellins*) (Fink et al., 1986), and the insect *Rhodnius prolixus* (Friedrich et al., 1993) suggests that the gene is phylogenetically ancient. However, the serine proteinase inhibitors in crustacean are

poorly characterized except those of crayfish (Johansson et al., 1994) and horseshoe crab (Nakamura et al., 1987). From the haemocyte library of the crayfish, a cDNA with an open reading frame of 684 base pairs was isolated. It codes for a signal sequence and a mature protein of 209 amino acid residues. The amino acid sequence consists of four repeated stretches, indicating that the protein has four domains. The domains have significant sequence similarity to serine proteinase inhinitor of the Kazal family. The three first domains have a leucine residue in the putative reactive site, suggesting that the protein is a chymotrypsin inhibitor. Recently a cDNA clone coding for a four Kazal protein was isolated from a cDNA library of *Litopenaeus vannamei* hemocytes (Jimenez-Vega and Vargas-Albores, 2005). The third and fourth domains have the sequences CPEIYAPVC and CPKNYRPVC, indicating that they may inhibit subtilisin- and trypsin-like proteins, respectively.

Proteinase inhibitors from the Kunitz family have been characterized as haemolymph proteins from a horseshoe crab (Nakamura et al., 1987). They contain a single domain and are inhibitors of trypsin or chymotrypsin. Another new family of serine proteinase inhibitors has been discovered in the crayfish, named pacifastin. It is an inhibitor of trypsin and chymotrypsin. Pacifastin has unique combination of the proteinase inhibitors in its light chain together with a covalently linked heavy chain that is a transferrin (Liang et al., 1997).

1.7.2 α-macroglobulin

In comparison to the group of inhibitors described above, α-macroglobulin are much larger proteins**.** Each α-macroglobulin subunit contains an exposed bait region that is susceptible to proteolytic cleavage. Cleavage of the bait region by a proteinase leads to a conformational change, thus trapping the proteinase in the cavity formed by the α-macroglobulin dimer (Sottrup-Jensen, 1989). The change in conformation also leads to the formation of covalently cross-links between the thiol ester region of α-macroglobulin and lysine side chains of the proteinase, thereby resulting in irreversible inhibition of the proteinase, even though its active site is not affected.

α-macroglubulins have been identified and characterized in horseshoe crabs and other crustaceans. A cDNA for *Limulus polyphemus* α-macroglobulins was

cloned (Iwaki et al., 1996). The amino acid sequence of horseshoe crab protein was 28-29% identical to mammalian α-macroglobulins and consists of a conserved bait region, a thiol ester site and a receptor-binding domain. Like the mammalian proteins, *L. polyphemus* is related in sequence to complement proteins C3 and C4. It is expressed in haemocytes and stored in the large granules.

1.7.3 Serpins

The serpins make up a superfamily of proteins, most of which function as serine proteinase inhibitors. Serpins contain an exposed reactive site loop, which interacts with the active site of a proteinase, leading to the formation of a very stable serpin-proteinase complex (Stone et al., 1997). It appears likely to be an acyl enzyme complex that represents a normal intermediate on the substrate pathway of a serine proteinase (Olson et al., 1995). For this reason, serpins have been called suicide substrate inhibitors, since they develop their inhibitory propensity only after the initial interactions with the proteinase as a normal substrate. Recently, the cDNAs for two new serpins (serpin-4 and serpin-5) from the tobacco hornworm, *Manduca sexta* were cloned (Tong and Kanost, in press). Serpin-4 and serpin-5 mRNAs are constitutively expressed at a low level in larval hemocytes and fat body, and increase dramatically upon bacterial challenge. Recombinant serpin-4 and serpin-5 decreased proPO activation when added to plasma, but they did not directly inhibit the proPO-activating proteases (PAPs). Instead, they apparently regulate the pathway by inhibiting one or more target proteases upstream of PAPs.

1.8 Roles of serine proteinase inhibitors in arthropod immunity

1.8.1 Protection against microbial proteinases

Proteinases function in many pathogenic fungi to aid in penetrating the cuticle of their arthropod hosts. Proteinases also contribute to the virulence of bacterial pathogens. Some of the proteinase inhibitors in haemolymph may defend the host against such microbial proteinases. For example, the silk worm (*Bombyx mori*) serine proteinase inhibitor is active against proteinases from fungal pathogens (Eguchi et al., 1993). Several of *Manduca sexta* serpin gene-1 variants inhibit bacterial and

fungal serine proteinases (Jiang et al., 1997). Proteinase inhibitors in the cuticle or at the surface of the integument might also function in protection against fungal infection. An external secretion from grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity (Polanowski et al., 1997).

1.8.2 Regulation of endogenous proteinases

A clue to the function of proteinase inhibitors in arthropod haemolymph may lie in the knowledge of the roles of serpins in vertebrate plasma. Injury and microbial infection in vertebrates lead to activation of the blood coagulation and complement systems. Both of these systems employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides) resulting in rapid and efficient responses to the threats to health (Whaley et al., 1993; O'Brien et al., 1993). Blood clotting and complement 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 serpins also in the plasma (Potempa et al., 1994). Such proteinase systems, regulated by inhibitors, may be a common evolutionary strategy in innate immune responses and maintaining homeostasis in animals.

1.8.3 Haemolymph coagulation

The haemolymph coagulation system in horseshoe crab is the most thoroughly characterized pathways in invertebrates. The horseshoe crab coagulation system is composed of a complex pathway of serine proteinases of high specificity, leading to proteolytic activation of a coagulogen and production of a clot. When horseshoe crab haemocytes are exposed to bacterial lipopolysaccharide or β(1-3) glucan (from fungal cell walls), they release five clotting factors stored in the membrane-bound granules. Four of these factors are serine proteinase zymogens, and the other is coagulogen, a clotting protein precursor analogous to fibrinogen (Kawabata et al., 1996). Exposure to lipopolysaccharide (LPS) causes conversion of factor C to its active form, and exposure to β(1-3) glucan converts factor G to active factor G. These proteinase zymogens act as sensors for the presence of

microorganisms in the haemolymph. The pathway consists of two branches, which converge upon the activation of a proteinase called clotting enzyme. In the glucan-stimulated branch, factor G activates proclotting enzyme to produce clotting enzyme. Clotting enzyme then cleaves coagulogen to produce coagulin, which forms an insoluble gel-like clot. In the LPS-activated branch of the clotting pathway, factor C activates factor B, and the active factor B cleaves the proclotting enzyme. All of these proteins have been purified, their cDNAs have been cloned, and the activation reactions have been biochemically characterized (Kawabata et al., 1996).

When degranulation occurs and the clotting factors are released, three serpins, LICI-1, LICI-2 and LICI-3, are also released from the granules. LICI-2 has a high inhibition rate for clotting enzyme, LICI-1 for factor C and LICI-3 for factor G (Miura et al., 1994; Miura et al., 1995 and Argarwala et al., 1996). Thus, each proteinase has it own counter serpin that may regulate its activity in vivo, in a manner analogous to the vertebrate plasma serpins in regulating coagulation.

1.8.4 Phenoloxidase activation

Melanization is a response to wounding and infection in insects and crustaceans, which involves serine proteinases. Wounding or infection leads to rapid activation of a proteinase that in turn activates a phenoloxidase zymogen (prophenoloxidase; PPO). The active phenol oxidase then oxidizes phenols leading to the production of quinines that are subsequently polymerized to form melanin. The proteinases in this system have not been well characterized, but there is evidence that activation of PPO, like blood clotting, involves a serine proteinase cascade (Söderhäll et al., 1996 and Ashida et al., 1997) Like the horseshoe crab clotting system, PPO activation can be stimulated by LPS or β(1-3) glucan. Recognition of these microbial polysaccharides results in activation of a serine proteinase (pro-phenol oxidase activating enzyme: PPAE) that cleaves PPO at a specific peptide bond near its amino terminus, which then activates this enzyme.

Knowledge of the PPO activation pathway is much less advanced than that of the horseshoe crab clotting system. It is not known with certainty how many steps may occur between recognition of polysaccharide by a haemolymph protein and

activation of PPAE, although two different serine proteinase activities have been detected after treating silkworm plasma with bacterial cell walls (Ashida et al., 1997). Enzymes with PPAE activity have been purified from haemolymph of a crayfish (Aspán et al., 1990), from *Bombyx mori* cuticle (Dohke, 1973), and from a homogenate of *Drosophila melanogaster* pupae (Chosa et al., 1997). These are all ~30 kDa serine proteinases that cleave very specifically at an Arg-Phe bond approximately 50 residues from the amino terminus of prophenoloxidase, activating the zymogen. Recently a cDNA for a PPO activating proteinase was isolated from *Manduca*. sexta (Jiang et al., 1998). This proteinase is similar in domain structure to the horseshoe crab proclotting enzyme and factor B.

Like blood clotting, phenoloxidase activation is normally regulated in vivo as a local reaction of brief duration. Also comparable to blood clotting, the regulation may be due in part to serine proteinase inhibitors in plasma (Kanost et. al., 1996). Pacifastin (Hergenhahn et al., 1987) and, to the lesser degree, α-macroglobulin inhibit crayfish PPO activation (Aspán et al., 1990). Among the low molecular weight inhibitors from insect haemolymph, Kunitz family inhibitors from *Manduca sexta, Sarcophaga bullata* and *Bombyx mori* (Sugumaran et al., 1985; Saul et al., 1986 and Aso et al., 1994) and the 4 kDa locust inhibitors (Boigegrain et al., 1992) can interfere with PPO activation. Serpin-1J from haemolymph of *M. sexta* inhibits the activity of a serine proteinase linked to prophenoloxidase activation (Jiang et al., 1997). Recently, the *Manduca sexta* serpin-6 was isolated from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3)(Wang and Jiang, 2004). In addition, its structure and function was further characterized by cloning and expression in *E.coli* expression system.(Zou and Jiang, in press). The results suggest that serpin-6 plays important roles in the regulation of immune proteinases in the hemolymph. It appears likely that each proteinase in the PPO cascade is regulated by one or more specific inhibitors present in plasma or in haemocyte granules.

1.8.5 Proteolytic activation of cytokines

In *Drosophila* embryos, dorsal/ventral development is regulated by a signal transduction system that depends on an extracellular serine proteinase cascade.

An inactive protein called spatzle is eventually cleaved, thus making it competent to bind to a receptor named Toll (Belvin and Anderson, 1996). Toll is homologous in sequence to the mammalian interleukin-1 receptor. Binding of spatzle to Toll initiates a signal transduction pathway that leads to the activation of a transcription factor named Dorsal. In mammalian, binding of interleukin-1 to its receptor can stimulates expression of many mammalian acute phase proteins. This evolutionarily conserved pathway also functions in the regulation of immune protein genes in *Drosophila* (Hoffmann and Reichhart, 1997). Injection of bacteria into lepidopteran insects leads to a rapid decrease in the number of plasmatocytes in circulation (Chain and Anderson, 1983; Geng and Dunn, 1989). A peptide that has the properties of a plasmatocyte depletion factor, named plasmatocyte-spreading peptide, has been isolated from plasma of *Pseudoplusia includens* (Clark et al., 1997). This peptide is first synthesized as precursor. It is cleaved by a proteinase to liberate the C-terminal peptide, and can then act on a target like plasmatocytes. One can expect that such a proteinase and other proteinases yet to be discovered involved in proteolytic activation of cytokines would be regulated by proteinase inhibitors.

1.9 Previous studies

In previous studies, the expressed sequence tags (ESTs) from the haemocyte of black tiger shrimp *Penaeus monodon* were generated in order to identify genes associated with shrimp immunity (Supungul et al., 2002). Two haemocyte cDNA libraries were constructed from the haemocytes of normal and *Vibrio harveyi* challenged shrimps. Randomly selected cDNA clones with inserts over 500 base pairs in length were sequenced. The partial nucleotide sequences of cDNA clones were compared with sequences in the GenBank database using the BLAST program. From these ESTs, approximately 9% of the matched genes are involved in defense reactions. These are the components of the proPO system including PO and proPO activation enzymes; the components of the clotting system including glutamine γ-glutamyl transferase and haemocyte protease; antioxidative enzymes such as peroxidase and catalase; antimicrobial peptides such as anti-lipopolysasccharide

factor, penaeidins, 11.5 kDa antibacterial peptides and lysozyme; and finally serine proteinase inhibitors.

The deduced amino acid sequences from the four full-length cDNA clones of serine proteinase inhibitors isolated from the haemocyte cDNA library were analyzed. They show high homology to the four-domain Kazal proteinase inhibitor from crayfish, *Pacifastacus leniusculus*. The amino acid sequences of these cDNA clones indicates a variation in the number of Kazal domains. One clone named SH415 contains 5 Kazal domains and has an open reading frame of 801 base pairs encoding a protein of 266 amino acid residues with a predicted molecular mass of 28.9 kDa. The 18 amino acid signal sequence truncated SH415 clone was subcloned and expressed in *Escherichia coli* expression system using the pTrcHis2C as vector (Jarasrassamee et al., in press). For unknown reasons, the recombinant protein was not over-expressed as expected and provided limited studies of its activity.

1.10 The aim of this thesis

In this study, the SH415 was subcloned into another expression vector pET-22b(+) in order to over-express the multiple Kazal-type inhibitor for further characterization. The gene product was purified and characterized for the inhibitory activity against various types of serine proteinases and for the kinetics of inhibition.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Amicon® Ultra Centrifugal Filter Devices (Millipore, USA)

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

Automatic micropipettes P10, P100, P200, and P1000 (Gilson®, France)

Fraction collector FRAC-100 (Amersham Biosciences, Sweden)

-20°C Freezer (Whirlpool)

-80°C Freezer (ThermoForma)

Hot plate (CERAMAG Midi, IKA® WORKS, USA)

Incubator 37°C (Memmert)

Larminar 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)

Minicentrifuge (Costar, USA)

Mini-Protein®3 electrophoresis cell (Bio-RAD Laboratories, USA)

Nitrocellulose transfer membrane PROTRAN® (Schleicher&Schuell, Germany)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

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

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

Peristaltic pump P-1 (Amersham Biosciences, Sweden)

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

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

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Sonicator Bransonic 32 (Bandelin, Germany)

Spectrophotometer DU650 (Beckman, USA)

Touch mixer Model # 232 (Fisher Scientific, USA)

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

White/UV transilluminator: UVP ImageStore 7500 (Misubishi Electric Corporation, Japan)

2.1.2 Chemicals and Reagents

Absolute ethanol, C_2H_5OH (BDH)

Absolute methanol, CH_3OH (Merck)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide (Pharmacia)

Agarose (Sekem)

Ammonium persulfate (USB)

Amplicillin (BioBasic)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Bromophenol blue (Merck)

Chloramphenicol (Sigma)

Chloroform, $CHCl_3$

Coomassie brilliant blue U-250 (BDH)

dATP, dCTP, dGTP and dTTP (Promega)

Diaminobenzidine (Sigma)

DTT (Dithiothreitol) (Bio Basic INC.)

Ethidium bromide (Sigma)

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

Formaldehyde (BDH)

Glycerol (Scharlau)

Glycine (Scharlau)

Hydrogenperoxide (Merck)

Isopropanol (Merck)

Isopropyl-β-D-thiogalactoside (IPTG), $C_8H_{18}O_5S$ (USBiological)

2-mercaptoethanol (Scharlau)

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

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

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

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

N, N', methylenebisacrylamide (Fluka)

Phenol crystals, C_6H_5OH (Carlo Erba)

Sodium acetate (Merck)

Sodium carbonate (Carlo Erba)

Sodium chloride (Carlo Erba)

Sodium dodecyl sulfate (Sigma)

Sodium hydrogen carbonate (AnalaR)

Sodium hydroxide, NaOH (Eka Nobel)

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

TritonX-100 (Merck)

Tween-20 (LABCHEM)

2.1.3 Enzymes

AmpliTaq DNA polymerase (Perekin-Elmer Cetus, USA)

*Bam*HI (Biolabs)

Pfu DNA polymerase (Promega)

Sal I (Biolabs)

T_4 DNA ligase

Trypsin (Sigma)

α-Chymotrypsin (Sigma)

Elastase (Sigma)

Subtilisin (Sigma)

2.1.4 Bacterial Strains

Escherichia coli Rosetta (DE3) pLysS (Novagen) Genotype; F^{-}*ompT* $hsdS_B(r_B^{-}m_B^{-})$ *gal dcm lacY1* (DE3) pRARE (Cm^R)

Escherichia coli XL1 blue Genotype; *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F *proAB* $lacI^q$ *ZΔM15 Tn10* (Tet^R)]

2.1.5 Kits

Nucleospin® Extraction Kit (MACHEREY-NAGEL, Germany)

QIAprep® Spin Miniprep Kit (QIAGEN GmbH, Germany)

pGEM®-T Easy Vector System I (Promega corporation, USA)

2.2 Methods

2.2.1 Construction of recombinant pET-22b(+)SPI

2.2.1.1 Preparation of DNA fragment containing serine proteinase inhibitor (SPI) gene

5'-terminal truncated SPI gene was constructed by polymerase chain reaction (PCR). A pBlueScript SK plasmid containing SPI gene was used as template. The reaction was performed in a final volume of 30 µl containing 25 ng of DNA template, 0.45 µM of each primer, 0.2 mM of each dNTP and 0.45 unit of *Pfu* polymerase (Promega). The PCR amplification was run for 35 cycles of 45s at 95°C, 60s at 56°C and 60s at 72°C. The amplified product was analyzed using agarose gel electrophoresis. The size of the product was compared with standard a Gene Ruler™ 100 bp DNA ladder Plus (Fermentas).

The following primers (PROLIGO) were used for PCR amplifications. Restriction sites, *Bam* HI and *Sal* I were incorporated at the 5'-ends of primers 1 and 2, respectively.

1. Forward primer 1 with *BamH*I site. This primer was designed to delete the putative signal sequence of the SPI.

```
          BamHI
5′-CCATGGATCCGGGCTACGGAAAAGGGGGGAAAATCC-3′
```

2. Reverse primer 2 with *Sal*I site

```
          SalI
5′-ATGGTCGACTAGGTACAGTCTGCGACCACAGATTCC-3′
```

The amplified SPI gene was purified by phenol-chloroform precipitation (see Appendices). After ethanol precipitation, the DNA pellet was dissolved in 40 µl TE buffer.

The purified SPI gene was tailed with an adenine nucleotide for ligation into the pGEM®-T Easy vector (see appendices). The recombinant plasmids were transformed into *Escherichia coli* strain XL1-Blue using electroporation as described in 2.2.1.5. The transformants were selected using blue white screening on the ampicillin agar plates. A single white colony of *E. coli* strain XL1-Blue harboring a recombinant plasmid was grown in 1.5 ml LB broth containing 100 µg/ml ampicillin at 37ºC overnight with shaking. The recombinant plasmids were isolated and cut with *Bam* HI and *Sal* I at 37°C for overnight The restriction enzyme digestion was analyzed by 1.2% agarose gel electrophoresis. The band of approximately 750 bp was purified using Necleospin extraction kit (MACHEREY-NAGEL) as describe below.

After electrophoresis, a piece of agarose gel containing the 750 bp DNA fragment was excised. The weight of the gel slice was determined and transferred to a clean 1.5 ml microcentrifuge tube. Three hundred microlitres of NT1 buffer was added into the tube for each 100 mg of agarose gel. The sample was incubated at 50°C with occasional vortexing until the gel pieces was dissolved for 5-10 minutes. A NucleoSpin extract column was placed into a 2 ml collecting tube, the sample was loaded into the column and centrifuged at 10,000×g for 1 minute. The flow-through was discarded. Five hundred microlitres of NT2 buffer was added into the column and centrifuged as describe above. Six hundred microlitres of NT3 buffer was added into the column and centrifuged. The flow-through was discarded. Two hundred microlitres of NT3 buffer was added into the column and centrifuged at 11,000×g for 2 minutes to remove NT3 buffer. The column was placed into a clean 1.5 ml microcentrifuge tube. Forty microlitres of elution buffer NE was added into the

column and leaved at room temperature for 1 minute. The column was centrifuged at 11,000×g for 1 minute. The flow-through containing the DNA fragment was then stored at 4°C until used. DNA concentration was estimated under the UV transilluminator by comparing with the 100 bp DNA ladder. The DNA fragment containing the SPI gene was subsequently ligated to the pET-22b(+) expression vector.

2.1.1.2 Preparation of pET-22b(+) expression vector

The expression vector used in this experiment was pET-22b(+). The expression of the gene of interest was under the T7 promoter. As shown in Figure 2.1, the vector comprised of *lac* operator (*lacO*), ribosome binding site (*rbs*), *pelB*, initiation ATG, multiple cloning site (MCS), polyhistidine region (6×His), T7 terminator, f1 origin, ampicillin resistance gene (β-lactamase), pBR322 origin and *lac* repressor gene (*lacI*). The restriction sites used to clone the SPI gene into vector was *Bam* HI and *Sal* I

A single colony containing the plasmid pET-22b(+) was inoculated into 1.5 ml of LB broth containing 100 μg/ml of ampicillin. The culture was grown overnight at 37°C with shaking at 250 rpm. The plasmid was extracted using QIAprep® Spin Miniprep Kit as described below.

The culture was transferred to 1.5 ml microcentrifuge tube and centrifuged at 800×g for 5 minutes. The supernatant was discarded. The pellet was resuspended in 250 μl of Buffer P1. Two hundred and fifty microlitres of Buffer P2 was added and mixed gently by inverting the tube 4-6 times. Three hundred and fifty microlitres of Buffer N3 was added and the tube was gently inverted immediately for 4-6 times. The sample was centrifuged for 10 min at 17900×g in a table-top microcentrifuge. The supernatant liquid was loaded into the QIAprep spin column and centrifuged as described above. The flow-through was discarded. Five hundred microlitres of Buffer PB was added into the column and centrifuged for 1 minute. The flow-through was discarded. Seven hundred and fifty microlitres of Buffer PE was added and centrifuged for 1 minute. The flow-through was discarded and centrifuged for an additional 1 minute to remove residual wash buffer. The OIAprep column was

then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, forty microlitres of Buffer EB was added to the center of QIAprep spin column, let stand for 1 minute and centrifuged for 1 minute.

The pET-22b(+) was cut with *Bam* HI and *Sal* I and purified by phenol-chloroform extraction. After ethanol precipitation, the DNA pellet was dissolved in 40 µl TE buffer. The DNA concentration was estimated using 1.2% agarose gel electrophoresis under UV transilluminator by comparing with 1 kb DNA ladder (Fementas). The digested pET-22b(+) was used for the cloning of SPI gene.

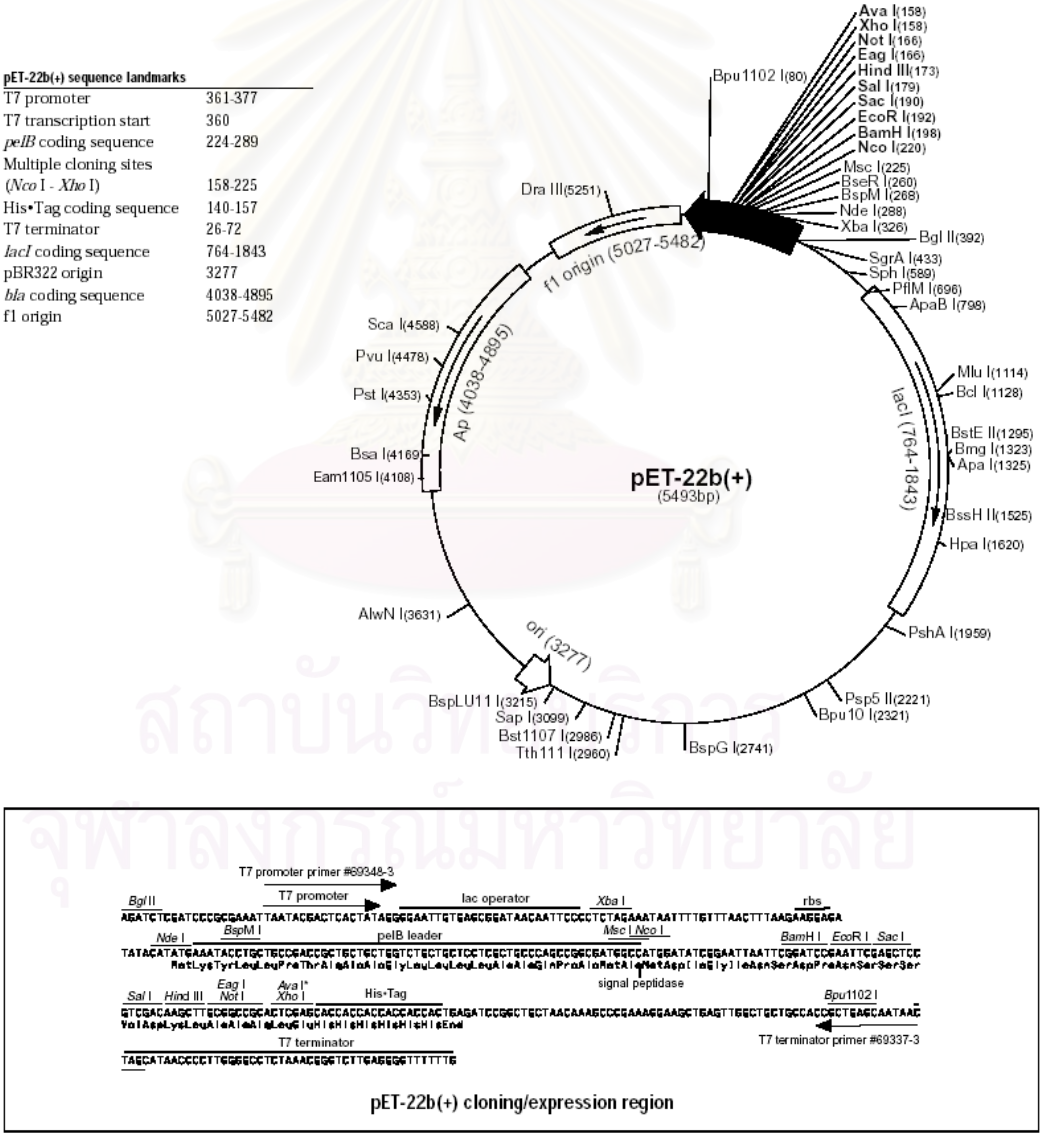

Figure 2.1 pET-22b(+) vector map (Novagen®, Germany)

2.1.1.3 Restriction enzyme digestion

Since the expression vector pET-22b(+) contains *Bam* HI and *Sal* I restriction sites, The two restriction sites were incorporated into the primers used for the amplification of SPI gene. The vector and cloned SPI gene were digested with *Bam* HI and *Sal* I. Approximately 20 µg DNA was digested in a 50 µl reaction volume containing 1× NE buffer 3 (50mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM dithiothreitol), 20 units of *Bam* HI (Biolabs), 20 units of *Sal* I (Biolabs) and 500 µg of BSA. The reaction was incubated at 37^{o}C overnight.

2.1.1.4 Ligation

A suitable molecular ratio between vector and inserted DNA in the mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in the ligation reaction, the following equation was used:

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

The 10 µl ligation reaction was composed of 1 µl of 10× T4 DNA ligase buffer, 1 unit of T4 DNA ligase (USB), 10 ng of *Bam* HI and *Sal* I digested pET-22b(+) and 4 ng of *Bam* HI and *Sal* I digested amplified SPI gene. Sterile distilled water was added to make the final volume of 10 µl. The reaction was mixed, briefly spun for 30 seconds and incubated at 16°C for an overnight. One microlitre of the ligation mixture was transformed into competent *Escherichia coli* strain XL1 blue.

2.1.1.5 Electrotransformation

The competent cells were gently thawed on ice. Forty microlitres of cell suspension were mixed with 1 µl of the ligation mixture, mixed well and placed on ice for 1 minute. The cells were transformed by electroporation in an ice-cold 0.2 cm cuvette with the setting of the electroporater as follows; 25 µF, 200 Ω of the pulse controller unit, and 2.50 kV of the Gene pulser apparatus (Bio-RAD). After one pulse was applied at the above setting, the cells were immediately resuspended with 1 ml of

SOC medium (2% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated at 37°C with shaking at 250 rpm for 1 hour. The cell suspension was spread on the LB agar plates containing 100 μg/ml ampicillin and incubated at 37°C for an overnight. After incubation, colonies were randomly selected for plasmid DNA isolation.

2.1.1.6 Isolation of the recombinant plasmid

The recombinant plasmid containing SPI gene was selected by restriction enzyme digestion using *Bam* HI and *Sal* I. The digested plasmid was analyzed by agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (1 kb DNA ladder, Fermentas). The clone containing the correct DNA fragment of approximately 750 bp was selected. DNA sequencing was performed to confirm the correct junction of the vector and inserted DNA as well as the sequence of the SPI gene.

2.1.2 Expression of recombinant serine proteinase inhibitor

After the correct expression clone was identified, a recombinant pET-22b(+) SPI isolated was used for transformation into an expression host. The expression host used in this experiment was *Escherichia coli* strain Rosetta (DE3) plysS which provides rare codon tRNAs and chloramphenicol-resistant plasmid.

2.1.2.1 Competent cell preparation

The expression host cells were made competent by treatment with calcium chloride in the early log phase of growth. A single colony of *Escherichia coli* strain Rosetta(DE3)plysS was inoculated into 2 ml of LB broth containing 34 μg/ml of chloramphenicol. Then the culture was grown overnight at 37°C with shaking at 250 rpm. Four hundred microlitres of culture was inoculated into 10 ml of LB broth containing 34 μg/ml of chloramphenicol and incubated at 37°C until the optical density at 600 nm (OD_{600}) of the cells reached 0.2-0.6 (about 2-3 hours). Cells were then chilled on ice for 10 minutes and harvested by centrifugation at 5,000×g for 5

minutes at 4°C. The pellet was washed with 1/2 volume of the ice-cold 10 mM $CaCl_2$ and centrifuged as described above. The pellet was resuspended with 1/20 volume of the ice-cold 0.1 M $CaCl_2$ and left on ice at least 30 minutes. The cell was ready for transformation.

2.1.2.2 Calcium chloride transformation

A hundred microlitres of competent cell were mixed with 2 µl of recombinant pET-22b(+) SPI, mixed well and placed on ice for 30 minutes. The mixture was incubated for 1 minutes at 42°C immediately and 0.9 ml LB broth was added. The cell suspension was incubated with shaking at 250 rpm for 1 hour. After incubation, the 20 microlitres of the cell suspension was spread on LB agar plates containing 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol and incubated at 37°C overnight. The colonies were selected for protein expression.

2.1.2.3 Recombinant protein expression

After the recombinant pET-22b(+)SPI was established in the expression host, *Escherichia coli* Rosetta(DE3)pLysS, expression of the serine proteinase inhibitor gene was induced by the addition of IPTG to a growing culture.

A single colony from a freshly streaked plate was picked, inoculated into 2 ml LB broth containing 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol and incubated with shaking at 37°C until the OD_{600} reaches 0.6-1.0. The cell suspension was inoculated into 50 ml LB medium. The culture was incubated with shaking at 37°C until the OD_{600} reached 0.6. IPTG was then added from a 100 mM stock to a final concentration of 1 mM for induction. The culture was incubated further with shaking for 0, 1, 2, 3 and 4 hours, respectively. An aliquot of 1 ml of the culture was sampled at each time point and pelleted at 6,000×g for 10 min. After centrifugation, the supernatant was discarded. The cell pellet was stored at -80°C until required for further analysis.

2.1.3 Protein analysis

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

SDS-PAGE was conducted in a discontinuous system. The gel solutions were prepared as shown in Appendices.

After the glass plates and spacers were assembled, the components of the separation gel solution were mixed thoroughly and pipetted into the gel plate setting. Then a small amount of distilled water was careful layered over the top of the 12% separation gel solution to ensure that a flat surface of gel would be obtained. When the polymerization was complete, the water was poured off. The 5% stacking gel solution was prepared, mixed thoroughly and poured on the 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.

2.1.3.2 Sample preparation

Protein samples were prepared by resuspending in 1×sample buffer (12 mM Tris-HCl, pH6.8, 5% glycerol, 0.4%SDS, 2.88 mM 0.02% bromophenol blue, 2-mercaptoethanol). The samples were then boiled for 5 min in capped microtubes. After centrifugation at 10,000×g for 5 min, the samples were either held at room temperature or kept at 0^{o} C until loaded onto a gel.

2.1.3.3 Analysis of the recombinant protein by SDS-PAGE

After boiling, the protein samples and the prestained protein marker were loaded into the wells. Electrophoresis was conducted in 1× protein running buffer [25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS] at constant current of 36 mA until the tracking dye (bromophenol blue) reached the bottom of the separation gel. The gels were either stained with 0.1% (w/v) Coomassie brilliant blue R250 or Western blotted.

2.1.3.4 Coomassie brilliant blue staining

The gel was placed in Coomassie blue staining solution [0.1% (w/v) Coomassie brilliant blue R250, 10% (v/v) acetic acid, 45% (v/v) methanol] at room temperature with gentle shaking 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 hours. 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.

2.1.3.5 Detection of the recombinant protein by Western blot

The recombinant proteins were separated on 12% SDS-PAGE gels as described in section 2.2.3.1-2.2.3.3. The gel slab was removed from the glass plates and incubated for 10 min in a tank-blotting buffer (25 mM Tris base, 150 mM glycine and 20% methanol). The filter paper and membrane were soaked in tank-blotting buffer. Four sheets of filter paper (Whatman® No.1) were placed on the fiber pad, followed by the gel, the membrane, 4 sheets of filter paper, and finally the other fiber pad (Figure 2.2).

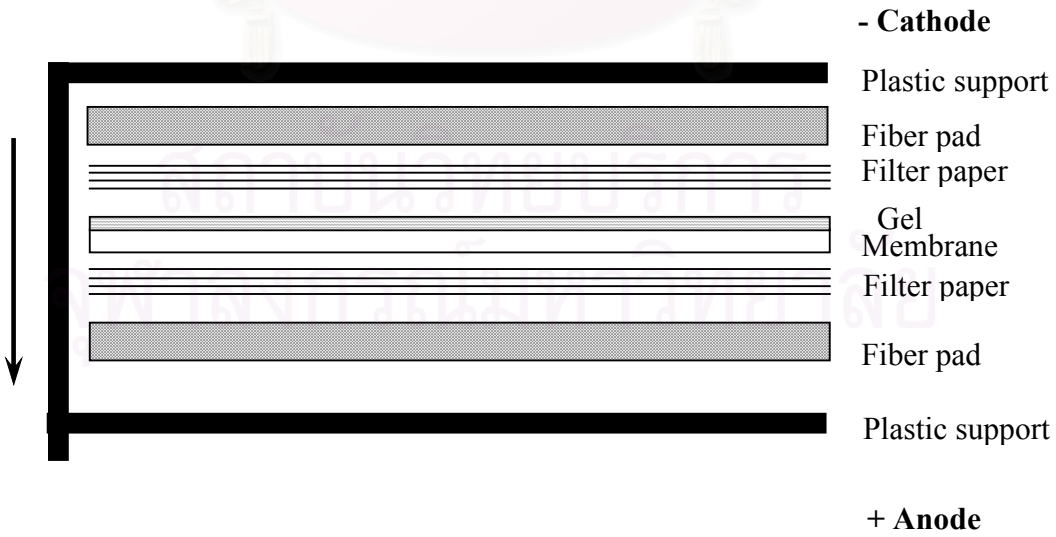

Figure 2.2 Western transfer cassette

The cassette was placed in the chamber. Protein transfer was performed at constant voltage of 60 V at 4°C from cathode towards anode. After transfer, the orientation of the gel was marked on the membrane. The membrane was washed twice for 10 minutes each time with TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) at room temperature and then incubated for an overnight in blocking buffer (3% (w/v) BSA in TBS buffer) at room temperature. The membrane was washed twice for 10 min each time in TBS-Tween buffer (20 mM Tris-HCl, 500 mM NaCl, 0.025% (v/v) Tween 20, pH 7.5) and incubated in anti-His antibody, 1/1200 dilution in blocking buffer at room temperature for 3 hours. The membrane was then washed twice for 10 min each time in TBS-Tween buffer at room temperature and washed again for 10 min with TBS buffer at room temperature. After that it was incubated with secondary antibody solution, 1/10000 dilution in blocking buffer, for 1 hour at room temperature. The membrane was washed four times for 10 minutes each time in TBS-Tween buffer at room temperature. The membrane was stained with HRP staining solution [18 mg diaminobenzidine (DAB) dissolved in 30 ml Tris-saline [9% (w/v) NaCl in 1 M Tris-HCl, pH 8.0] followed by 60 µl of 30% hydrogen peroxide (H_2O_2) until the signal was clearly visible (approximately 5-15 min). The chromogenic reaction was stopped by rinsing the membrane twice with water.

2.2.3.6 Determination of protein concentration

Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard. The 100 µl of sample was mixed with Bradford working buffer and left for 2 minutes before the absorbance at 595 nm was measured. The 100 ml of Bradford working buffer 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.

2.2.3.7 Solubilization of the recombinant protein

After 3 hours of IPTG induction, the culture was centrifuged at 6,000×g for 10 minutes. The cell pellet was collected, frozen completely at -80°C,

thawed at room temperature and resuspended by pipetting up and down in lysis buffer (50 mM Tris-HCl, pH 8, 5% glycerol, 50 mM NaCl). The suspension was incubated on a shaking platform for 20 minutes at room temperature and centrifuged at 10,000× g for 20 minutes to remove the insoluble proteins. The cell lysate of *Eschericia coli* and insoluble protein were analyzed by electrophoresis on a 12% SDS-PAGE.

2.1.4 Purification of recombinant protein

2.1.4.1 Denaturing condition

1. Crude extract preparation

A few microlitres of cell suspension from a glycerol stock was inoculated into 40 ml LB medium containing 100 µg/ml of ampicilin and 34 µg/ml of chloramphenicol and incubated with shaking at 37°C until the OD_{600} reached 0.6-1.0. The cell suspension was inoculated into 1 liter LB medium. The culture was incubated with shaking at 37°C until the OD_{600} reaches 0.6. IPTG was then added from a 100 mM stock to a final concentration of 1 mM for induction. The culture was incubated further with shaking for 3 hours. The culture was placed on ice for 5 min and the cells were harvested by centrifugation at 5000×g for 5 min at 4°C. The supernatant was removed and the cells were stored as a frozen pellet for 1 h at -80°C

The frozen cell pellet was completely thawed and resuspended in lysis buffer (50 mM Tris-HCl, pH 8, 5%glycerol, 50 mM NaCl). The cell suspension was incubated for 1 hour at room temperature with gentle shaking and then sonicated with a Bransonic 32 (Bandelin, Germany) for 2-4 min. The cell lysate was centrifuged at 15,000 rpm for 15 min and the pellet containing the inclusion bodies was washed twice with 0.5 M NaCl, 2% Triton X-100, twice with 0.5 M NaCl and twice with distilled water. The inclusion bodies were solubilized in 3 ml of 50 mM Tris-HCl buffer, pH 8 containing 6M urea at room temperature overnight. The insoluble material was removed by centrifugation and the supernatant was stored at 4°C until further used.

2. Ni-NTA agarose column chromatography

Ni-NTA agarose (Pharmingen) was gently resuspended in 6×His wash buffer (50 mM Tris-HCl, 300 mM NaCl, 10%glycerol, 6 M urea and 20 mM immidazole, pH 8) and packed into a column (1×5 cm). The column was washed with 5 bed volumes of 6×His wash buffer. The recombinant protein, which was solubilized in 50 mM Tris-HCl containing 6 M urea, pH 8, was applied to the column at a flow rate of 5 ml/h. The column was washed with 6×His wash buffer until the absorbance at 280 nm of eluate was less than 0.01. After washing, the column was eluted with 6×His elution buffer (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 6 M urea and 100 mM immidazole, pH 6) at 60 ml/h until the eluate absorbance at 280 nm was zero. The eluted protein was pooled and slowly dialysed against 50 mM sodium carbonate buffer, pH 10 containing 1 mM DTT. The partially purified protein was tested for serine proteinase inhibitory activity.

2.1.4.2 Non denaturing condition

The crude extract was prepared as described in 2.2.4.1. In stead of solubilizing the inclusion bodies with 50 mM Tris-HCl containing 6 M urea, pH 8, the inclusion bodies was solubilized with the carbonate buffer, pH 10, at room temperature overnight. The insoluble material was removed by centrifugation at 10000xg for 20 min and the supernatant was stored at 4°C until further used.

1. Sephadex G-100 column chromatography

Sephadex G-100 was swelled in distilled water for 24 hours at 50°C. The swelled gel was degassed and packed into a glass column at a flow rate of 25 ml/h using a peristaltic pump. The Sephadex G-100 column (2 × 60 cm) was equilibrated with elution buffer (50 mM sodium carbonate buffer, pH 10) for 3 column volumes at a flow rate of 20 ml/h to stabilize the gel bed volume in the column. The column was calibrated with molecular weight marker proteins (bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 27 kDa; myoglobin, 17.5 kDa; cytochrome C, 12.5 kDa) at the flow rate of 20 ml/h at room temperature. Blue dextran 2000 and potassium dichromate were respectively used to determine the void volume and the total volume of the column. Fractions of 3 ml were

collected using a fraction collector. The elution profile was monitored for protein at 280 nm and SPI activity. The fractions showing serine proteinase inhibitor band in SDS-PAGE and SPI inhibitory activity were pooled and concentrated for further analysis.

2.1.5 Molecular mass determination of recombinant SPI by using MALDI-TOF Mass Spectrometry

MALDI-TOF Mass spectrometry is a technique for accurate determination of protein molecular mass. It was performed in the commercial facility of the Proteomic Service Center, Bioservice Unit (BSU) (BIOTEC, Pathumthani, Thailand).

2.1.6 Serine proteinase inhibitory assay

The inhibitor activity of recombinant protein towards serine proteinases was tested using a modified procedure from Manickam et al. (1985). The reaction mixture consisted of 0.017 μM of inhibitor, 0.02 μM of the proteinase and 146.8 μM of chromogenic substrate (N-benzoyl-Phe-Val-Arg-p-nitroanilide for subtilisin and trypsin, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin) in a total volume of 100 μl using 50 mM Tris-HCl, pH 8. The reaction was incubated at 30°C for 15 minutes and then terminated by adding 50 μl of 50% acetic acid. The absorbance of p-nitroaniline formed was measured at 410 nm using a spectrophotometer. One unit of inhibitory activity is the amount of SPI that reduces the inhibition of proteinase activity per minute by 10% under the above assay condition.

The purified protein obtained from nondenaturing purification was further assayed against various types of proteinase, trypsin, chymotrypsin, subtilisin and elastase. In this assay, 293.6 and 146.8 μM of N-benzoyl-Phe-Val-Arg-p-nitroanilide was used as substrate for subtilisin and trypsin, respectively, 147.3 μM of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin and 443.1 μM of N-succinyl-Ala-Ala-Ala-p-nitroanilide for elastase. The concentrations of subtilisin, trypsin, chymotrypsin, and elastase were 0.010, 0.005, 0.003, and 0.039 μM, respectively. The said condition of the assay gave comparable absorption at 410 nm. The proteinase

was incubated with different concentrations of inhibitor, 0, 0.026, 0.052, 0.130, 0.207, and 0.413 μM in the presence of chromogenic substrate at 30^{o} C for 15 min. The reaction was terminated by adding 50 μl of 50% acetic acid. The absorbance of p-nitroaniline formed was measured at 410 nm using spectrophotometer. Remaining activity was calculated and plotted against inhibitor concentrations.

2.1.7 Kinetic studies

The purified recombinant SPI was shown to inhibit subtilisin and elastase. The kinetic studies were therefore carried out in order to determine the inhibition constant (K_i) of the inhibitor against both proteinases.

2.1.7.1 Determination of subtilisin inhibitor constant

The studies were composed of four sets of reactions in which each set consisted of seven concentrations of N-benzoyl-Phe-Val-Arg-p-nitroanilide, 0, 0.147, 0.294, 0.440, 0.587, 0.734 and 1.028 mM in the presence of 9.2 nM of subtilisin and a fixed amount of inhibitor. Four different concentrations of inhibitor, 0, 0.67, 1.34 and 2.68 nM were used. The reaction was made a total volume of 100 μl using 50 mM Tris-HCl, pH 8. The reactions were initiated by the addition of subtilisin. After incubating at 30°C for 15 minutes, they were arrested with 50 μl of 50% acetic acid. The absorbance of p-nitroaniline formed was spectrophotometrically measured at 410 nm using spectrophotometer. The amount of p-nitroaniline was calculated using an milimolar extinction coefficient of 8.8. 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 latter was constructed for the calculation of inhibition constant (K_i).

2.1.7.2 Determination of elastase inhibitor constant

The studies were composed of four sets of reactions in which each set consisted of seven concentrations of N-succinyl-Ala-Ala-Ala-p-nitroanilide, 0, 0.443, 0.886, 1.772, 2.658, 3.544 and 4.431 mM in the presence of 19.3 nM of elastase and a fixed amount of inhibitor. Four different concentrations of inhibitor, 0, 1.34, 2.68 and 5.36 nM were used. The reaction was made a total volume of 100 μl using 50 mM Tris-HCl, pH 8. The reactions were initiated by the addition of elastase.

After incubating at 30°C for 15 minutes, they were arrested with 50 µl of 50% acetic acid. The absorbance of p-nitroaniline formed was spectrophotometrically measured at 410 nm using spectrophotometer. The amount of p-nitroaniline was calculated using an milimolar extinction coefficient of 8.8. 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 latter was constructed for the calculation of inhibition constant (K_i).

CHAPTER III
RESULTS

The expressed sequence tag (EST) analysis of the haemocyte cDNA libraries of *Peneous monodon* (Supangul et al., 2002) using the BLASTX program has identified 6 putative serine proteinase inhibitors. They show 50-58% homology to a Kazal serine proteinase inhibitor from crayfish. The open reading frame (ORF) of each clone was identified by using the Genetyx program (ABI). Four full-length cDNA clones of serine proteinase inhibitor including SH415, SH610, SH1064 and SH1069 were obtained. In this study, the complete five Kazal domain clone SH415 was selected for cloning and expression (Figure 3.1). The SH415 cDNA clone consists of an open reading frame of 801 base pairs coding for a protein of 266 amino acids, 5'-untranslated region and very long 3'-untranslated region (Figure 3.2). Since the Kazal inhibitor reported in the crayfish contains signal sequence, the shrimp inhibitor was very likely to contain the signal peptide. The deduced amino acid of clone SH415 was used to predict the signal peptide using a SignalP prediction server (Nielsen et al., 1997). The putative signal cleavage site is between amino acid Ser18 and Gly19. Two oligonucleotides were designed. The 5' oligonucleotide was designed such that the signal peptide was excluded and a restriction site *Bam*HI was included (5'-CCATGGATCCGGGCTACGGAAAAGGGGGGAAAAT CC-3') for the cloning into the pGEM®-T Easy vector and subsequently into the pET-22b(+) expression vector, which resulted in a fusion protein among *pel*B signal sequence, SPI gene and His•Tag® sequence. The 3' oligonucleotide was designed to include the *Sal*I site (5'-ATGGTCGACTAGGTACAGTCTGCGACCACAGATTCC-3') for cloning into the *Sal*I site of pGEM®-T Easy vector and the pET-22b(+) expression vector. The latter cloning resulted in the fusion of the C-terminal of SPI to the His•Tag® sequence.

Penaeus monodon SPI(SH415)

```
MANKVALLILLAVAVAVSGYGKGGKIRL
CAKH--CTTIS-PVCGSDGKTYDSRCHLENAA-CGGVSVTFHHAGPCPPPKR        Domain 1
CPGI--CPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSKKHDGRCG            Domain 2
CNPIVACPEIYAPVCGSDGKTYDNDCYFQAAV-CKNPDLKKVRDGNCD            Domain 3
CTPLIGCPKNYRPVCGSDGVTYNNDCFFKVAQ-CKNPALVKVSDTRCE            Domain 4
CNHV--CTEEYYPVCGSNGVTYSNICLLNNAA-CLDSSIYKVSDGICGRRLYLZ      Domain 5
```

Pacifastacus leniusculus SPI

```
MMLSLLTWITTLLVVVASTAAR
CPSI--CPLNYKPVCGSDLKTYGNSCQLNAAI-CRNPSLKKLYDGP—-CIDKPQPQ   Domain 1
CPSI--CPLDYNPVCGTDGKTYSNLCALRIEA-CNNPHLNLRVDYQGECRPKNQ     Domain 2
CRNG--CTLQYDPKCGTDGKTYSNLCDLEVAA-CNNPQLNLKVAYKGECKQQNQ     Domain 3
CPTI--CTQQYDPVCGTDGKTYGNSCELGVAA-CNNPQLNLKIAYKGACNFPQQQT   Domain 4
```

Figure 3.1 The putative five kazal domains of the shrimp *Penaeus monodon* serine proteinase inhibitor clone SH415 compared to the four kazal domain proteinase inhibitor of the crayfish *Pacifastacus leniusculus* The putative reactive site (P1 residue) are boxed.

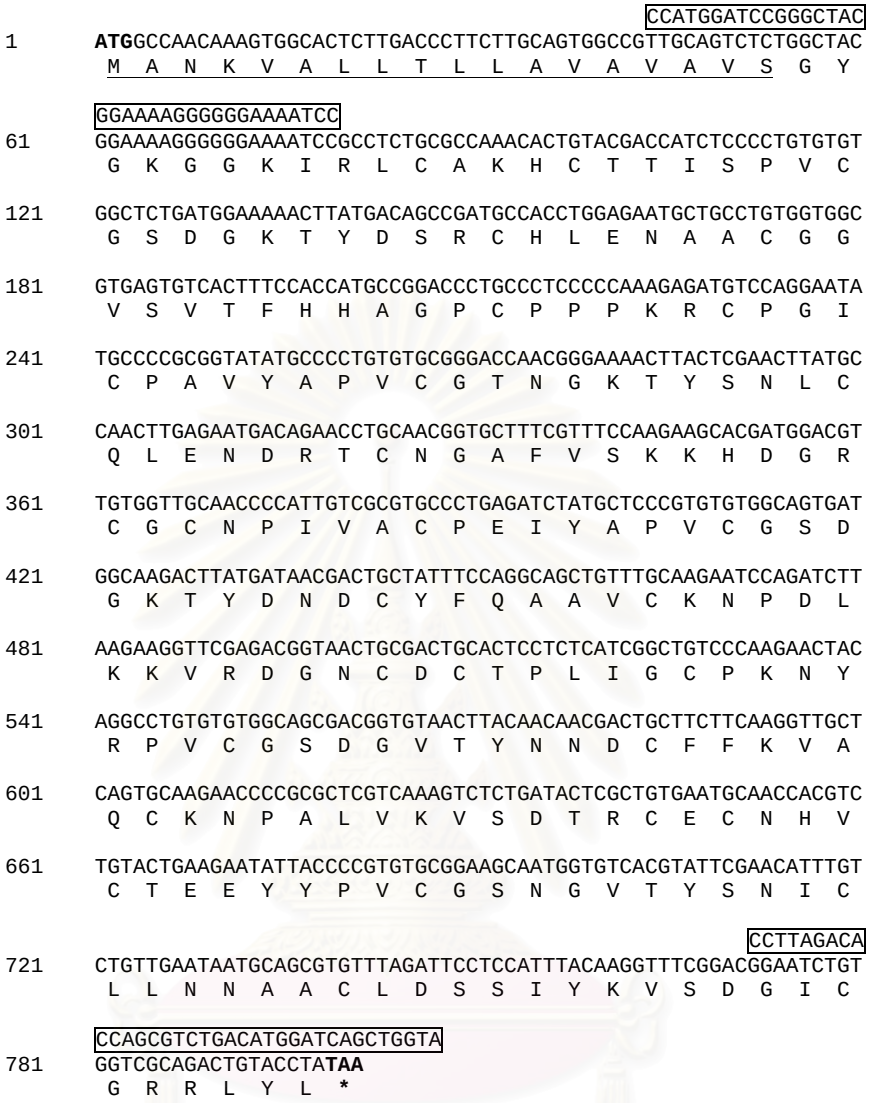

Figure 3.2 Nucleotide and amino acid sequences of the ORF of cDNA clone, SH415, encoding serine proteinase inhibitor from the black tiger shrimp *Penaeus monodon*. Amino acids are shown as single letter abbreviation. Start and stop codons are in bold letter. The putative signal peptide is underlined. The primers used for PCR amplification are boxed.

3.1 Recombinant expression of serine proteinase inhibitor (SPI) in *Escherichia coli* expression system

3.1.1 Amplification of the truncated SPI gene

The 5'-terminal truncated SPI gene was constructed by PCR amplification using pSH415 as a template and the two oligonucleotide primers described above. The resulting PCR products were separated on a 1.2% agarose gel and a specific 750 bp fragment of expected size was observed (Figure3.3).

3.1.2 Construction of recombinant plasmid pGEM-T-SPI

The 750 bp PCR fragment was purified and ligated into pGEM®-T easy vector. After ligation, the reaction mixture was transformed to *Escherichia coli* strain XL1 Blue. The recombinant clone was first selected with LB agar plate containing amplicilin, X-Gal and IPTG. White colonies were randomly selected and cultured in LB medium containing 100 μg/ml ampicillin. The plasmids were extracted from the selected colonies and cut with *Bam* HI and *Sal* I. The digested plasmids were analyzed on 1.2% agarose gel electrophoresis (Figure 3.4). The clone containing the 750 bp inserted DNA was selected for augmentation and named pGEM-T-SPI. After restriction digestion and electrophoresis of the clone, the 750 bp inserted DNA was purified from the agarose gel for further ligation to pET-22b(+).

3.1.3 Construction of recombinant plasmid pET-22b(+)-SPI

The truncated SPI insert excised from the pGEM-T-SPI using *Bam* HI and *Sal* I. was subsequently cloned into pET-22b(+) expression vector at the *Bam* HI and *Sal* I sites. This plasmid vecter carries an N-terminal *pel*B signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence (Figure 3.5) After ligation, the reaction mixture was transformed to *Escherichia coli* strain XL1 Blue. The recombinant clone was selected with LB agar plate containing 100 μg/ml amplicillin. The plasmids were extracted from the selected colonies and cut with *Bam* HI and *Sal* I. The digested plasmids were analyzed on 1.2% agarose gel electrophoresis (Figure 3.6). The correct construct was named pET-22b(+)-SPI.

3.2 Recombinant protein expression in *Escherichia coli* Rosetta (DE3) pLysS

Recombinant plasmid pET-22b(+)-SPI were transformed into an *Escherichia coli* Rosetta (DE3) pLysS to test for the induction of SPI synthesis. After induction with 1 mM isopropyl thiogalactoside (IPTG) for 0, 1, 2, 3 and 4 hours, the cells were harvested by centrifugation. The cells were then solubilized with the SDS-PAGE loading buffer and analyzed by electrophoresis on a 12% SDS-PAGE. Cultures containing the parental plasmid pET-22b(+) with IPTG induction were used as a negative control.

Following staining of the gels with Coomassie brilliant blue, an approximately 31 kDa protein was induced in the host cell lysate containing pET-22b(+)-SPI but absent in that containing the parental plasmid pET-22b(+) (Figure 3.7). The protien was detected after 2 hours of induction. At 3 hours after induction, the intensity of 31 kDa protein band was enough for further studies, then this condition was used to prepare the recombinant protein for further characterization.

3.2.1 Detection of recombinant protein using Western blot analysis

Since the SPI gene was fused to the His tag at its C-terminus, the induced protein can be identified using Western blot analysis. The expressed His tagged protein was specifically detected using Anti-His antibody and a second antibody tagged with horseradish peroxidase (HRP). The protein bands were localized in the gels with colorimetric method using diaminobenzidine (DAB) as substrate.

Cells containing either the parental plasmid pET-22b(+) or the recombinant plasmid pET-22b(+)-SPI were grown, and the synthesis of protein was induced with 1 mM IPTG for 3 hours. The cell lysates were prepared and analyzed using 12% SDS-PAGE. The approximately 31 kDa protein band was detected in the cell lysate of cells containing the pET-22b(+)-SPI but not the parental plasmid pET-22b(+)(Figure 3.8). This indicated that the serine proteinase inhibitor was successfully expressed in the host cell.

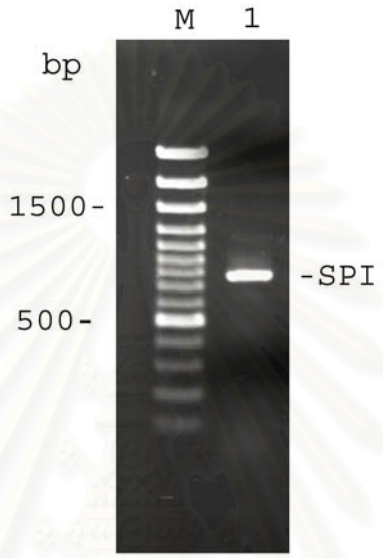

Figure 3.3 Agarose gel electrophoresis of PCR amplification product of truncated segment of the serine proteinase inhibitor-coding region

Lane M = 100 bp Ladder (Fermentas)
Lane 1 = Truncated segment of SPI PCR product

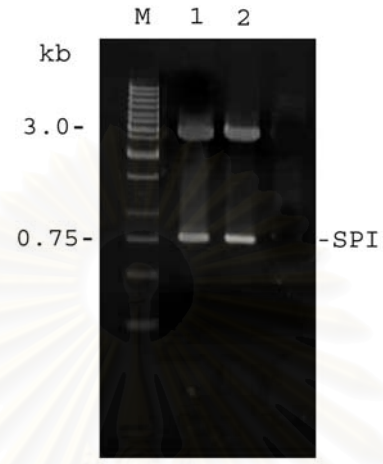

Figure 3.4 Agarose gel electrophoresis illustrating the products of *Bam*HI–*Sal*I digestion of pGEM®-T-SPI

Lane M = 1 kb Ladder (Fermentas)
Lanes 1, 2 = pGEM-T-SPI following digestion with *Bam*HI and *Sal*I

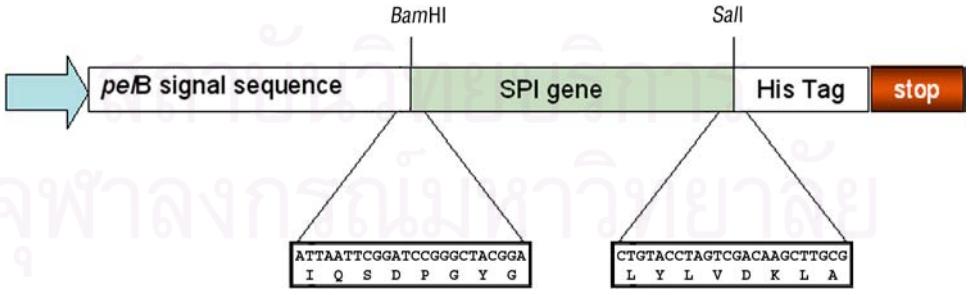

Figure 3.5 Schematic representation of pET-22b(+)-SPI showing the *pel*B signal sequence, cloning sites and His •Tag sequence

Figure 3.6 Agarose gel electrophoresis illustrating the products of *Bam* HI-*Sal* I digestion of pET-22b(+)-SPI

Lane M = 1 kb Ladder (Fermentas)
Lanes 1-4 = pET-22b(+)-SPI following digestion with *Bam*HI and *Sal*I

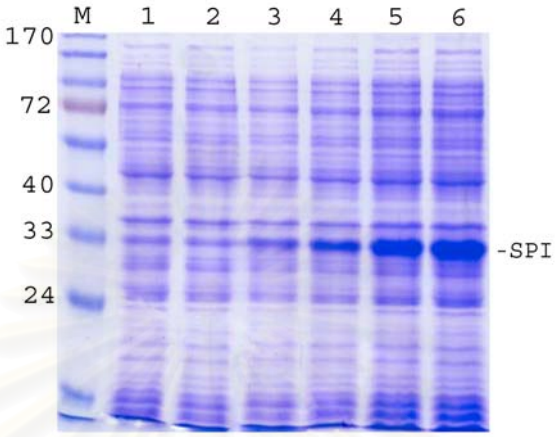

Figure 3.7 SDS-PAGE analysis of serine protinase inhibitor expressed in *Escherichia coli* Rosetta (DE3) pLysS at various time of induction

Lane M: Prestained protein marker (Fermentas)
Lane 1: *E.coli* lysate of the cells containing parental plasmid pET-22b(+)
Lanes 2-6: *E.coli* lysate of the cells containing recombinant plasmid pET-22b (+)-SPI at 0, 1, 2, 3, and 4 hours after induction, respectively

3.2.2 Solubility of the recombinant protein

The induced protein was suspected to be insoluble in the form of inclusion bodies. To verify the notion, the culture was grown, induced with IPTG for 3 hours and harvested by centrifugation. The cell pellet was completely frozen and thawed. The lysis buffer was added, and the cell suspension was centrifuged to separate the insoluble proteins. The cell lysate of *Escherichia coli* and insoluble proteins were analyzed by electrophoresis on a 12 % SDS-PAGE. The approximately 31 kDa protein band was observed mainly in the insoluble protein fraction (Figure 3.9) and probably accounted for more than 80 % of the insoluble protein. The protein was thus expressed and aggregate in the cells as inclusion bodies.

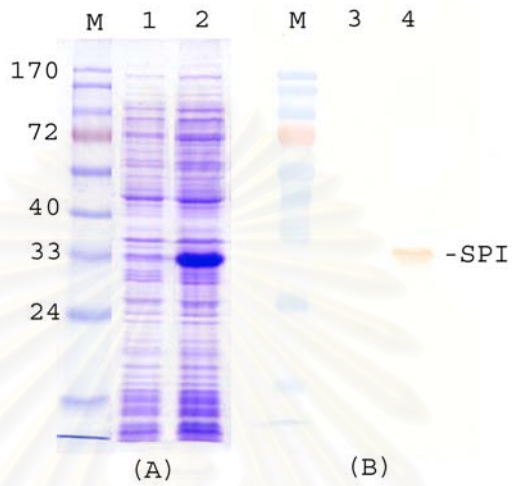

Figure 3.8 SDS-PAGE (A) and Western blot analyses (B) of serine proteinase inhibitor expressed in *Escherichia coli* Rosetta (DE3)pLysS

The *E.coli* lysates were subjected to 12% SDS-PAGE, blotted onto a nitrocellulose membrane and detected with anti-6×his antibody, then goat anti-mouse antibody linked with horse-radish peroxidase(HRP) and colorimetric reaction using diaminobenzidine (DAB) as substrate.

Lane M: Prestained protein marker (Fermentas)
Lanes 1, 3: *E.coli* lysate of the cells containing parental plasmid pET-22b(+)
Lanes 2, 4 : *E.coli* lysate of the cells containing recombinant plasmid pET-22b(+) SPI

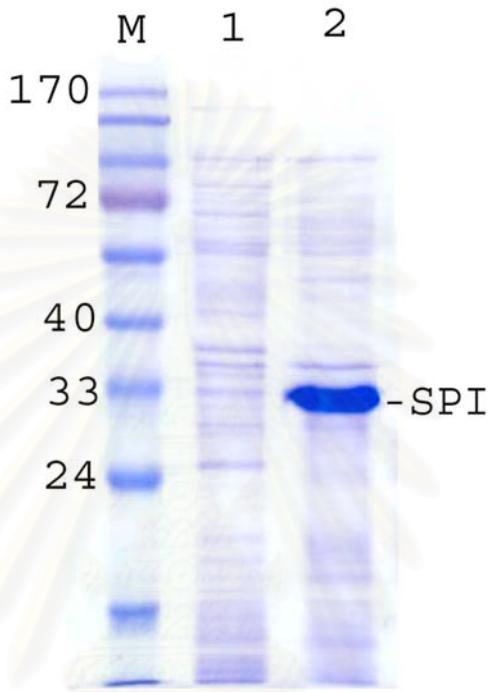

Figure 3.9 SDS-PAGE analysis of the soluble and insoluble protein fractions from the cell lysate of *Escherichia coli* containing the recombinant SPI

Lane M: Prestained protein marker (Fermentas)
Lane 1: Soluble protein of *E.coli* lysate
Lane 2: Insoluble protein of *E.coli* lysate

3.3 Purification of the recombinant protein

3.3.1 Denaturing condition

As recommended by researchers working with inclusion bodies (Light, 1985), the inclusion bodies of serine proteinase inhibitor were solubilized in 50 mM Tris-HCl, pH 8, containing 6 M urea and was applied to a Ni-NTA agarose column as described in section 2.2.4.1. The chromatographic profile was shown in Figure 3.10.

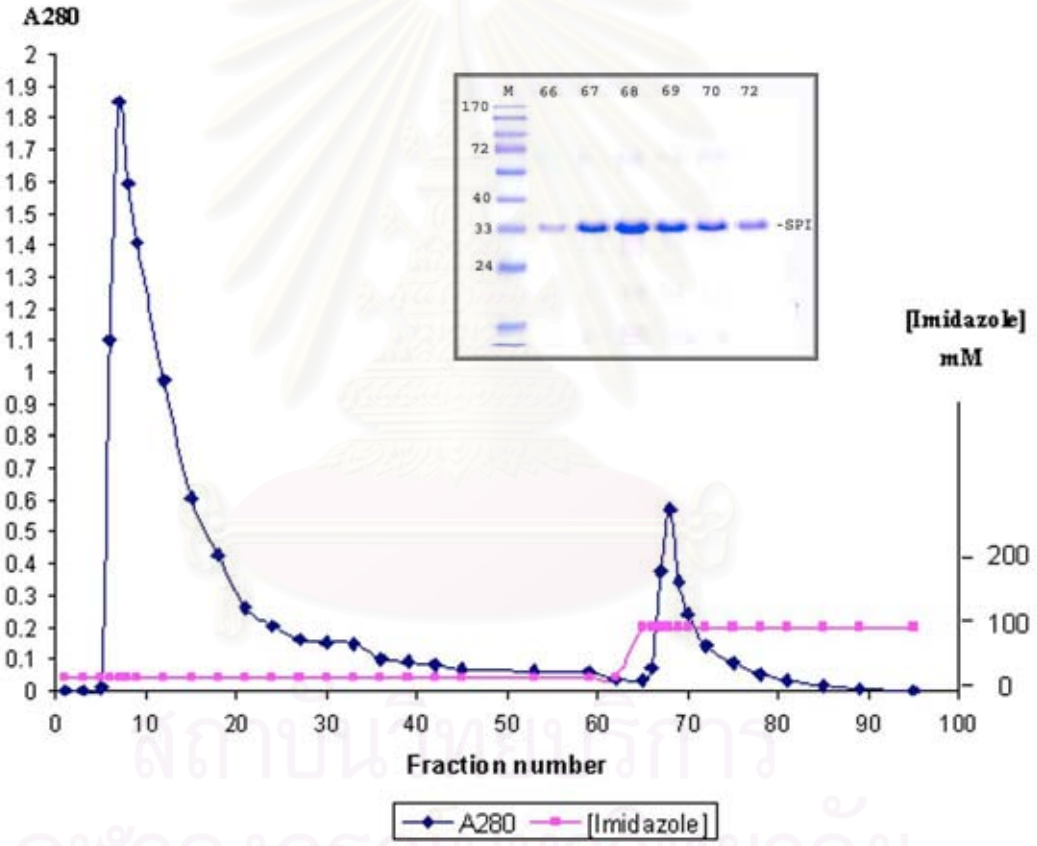

Figure 3.10 Chromatographic profile of Ni-NTA agarose column of crude extract solubilized in 50 mM Tris-HCl, pH 8, containing 6 M urea
The inset shows the SDS-PAGE analysis of the eluted fractions (66-70 and 72) showing the 31 kDa serine proteinase inhibitor band.

The unbound proteins were eluted from the Ni-NTA agarose column with 6×His wash buffer. The bound proteins were then eluted with 6×His elution buffer containing 100 mM imidazole. Since the inhibitory activity could not be assayed under the denaturing condition, the bound fractions were analyzed for the 31 kDa protein on a 12% SDS-PAGE (Figure 3.10 inset). The bound fractions containing the 31 kDa protein were pooled and dialysed slowly against 50 mM sodium carbonate buffer, pH 10, 1 mM DTT. The protein was renatured upon dialysis. The dialysate was tested further for the serine proteinase inhibitory activity.

3.3.2 Nondenaturing condition

Alternatively, the inclusion bodies obtained after cell lysis were solubilized in 50 mM sodium carbonate, pH 10. The crude preparation of serine protienase inhibitor was tested for activity as described in section 2.2.6. One unit of the inhibitor was defined as the amount that reduces the proteinases activity per min by 10%. The total 143,839.8 inhibitory activity units of serine proteinase inhibitor were obtained with the total protein of 0.81 mg. The specific activity of the inhibitor was then 177,580 units/mg protein.

The crude protein solubilized in 50 mM sodium carbonate, pH 10, was further purified by Sephadex G-100 column as described in section 2.2.4.2. The chromatographic profile is shown in Figure 3.11. The column was calibrated with various standard molecular weight markers: bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome C and molecular weight calibration curve was constructed (Figure 3.12). The molecular weight of serine proteinase inhibitor was determined to be 31.4 kDa. The eluted fractions were also analyzed by SDS-PAGE. The fractions showing serine protienase inhibitory activity and protein band in SDS-PAGE (Figure 3.11 inset) were pooled and concentrated. About 24,745.5 activity units of the serine proteinase inhibitory activity was recovered with the protein of 0.136 mg. The specific activity of the inhibitor in this step was 182,220 units/mg protein. The inhibitor was enriched as the purification fold increased about 1.03 whereas the recovery was about 17% (Table 3.1).

Partial purified serine proteinase inhibitor was subjected to 12% SDS polyacrylamide gel electrophoresis comparing with the crude protein (Figure 3.13).

Since the serine proteinase inhibitor purified under the denaturing condition gave low inhibitory activity as compared to that purified under the native condition, it was suspected that the refolding of the SPI was inadequate to give the native conformation. The SPI purified under the native condition was then more suitable for the further studies.

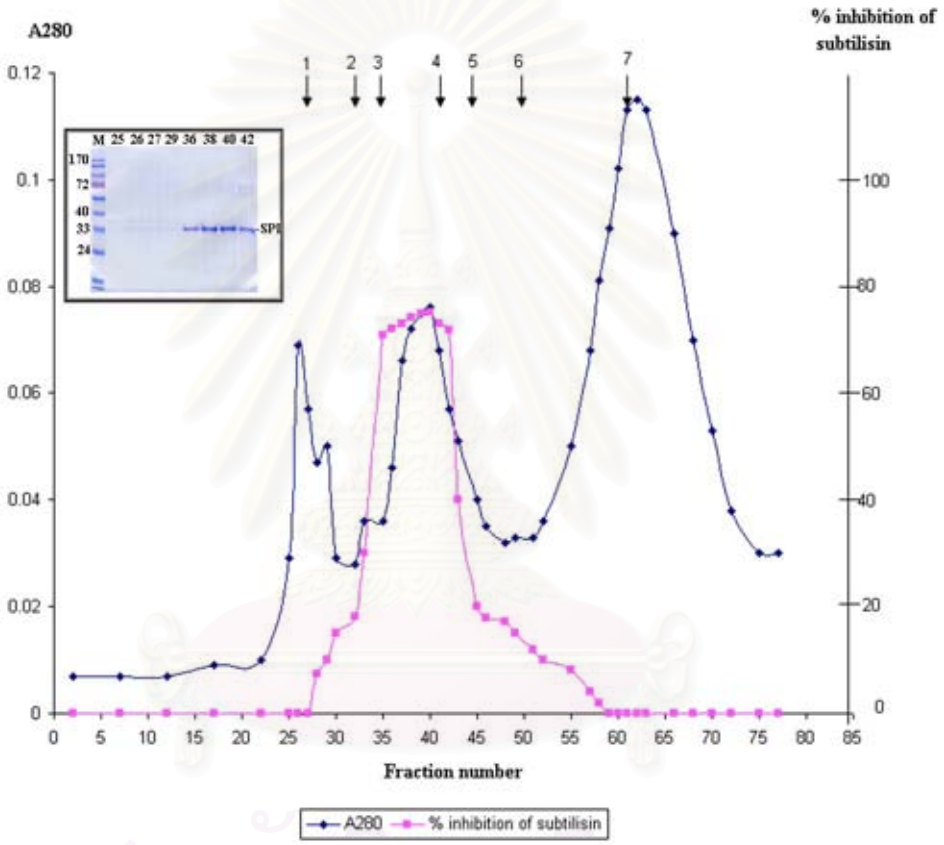

Figure 3.11 The chromatographic profile on Sephadex G-100 of crude recombinant protein solubilized in 50 mM sodium carbonate buffer, pH 10
The inset shows the SDS-PAGE analysis of the eluted fractions (25-27, 29, 36, 38, 40 and 42) showing the serine proteinase inhibitor band. The fraction size is 3 ml. The numbers 1-7 represent the elution peaks of blue dextran, bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsinogen (27 kDa), myoglobin (17.5 kDa), cytochrome c (12.5 kDa) and potassium dichromate, respectively.

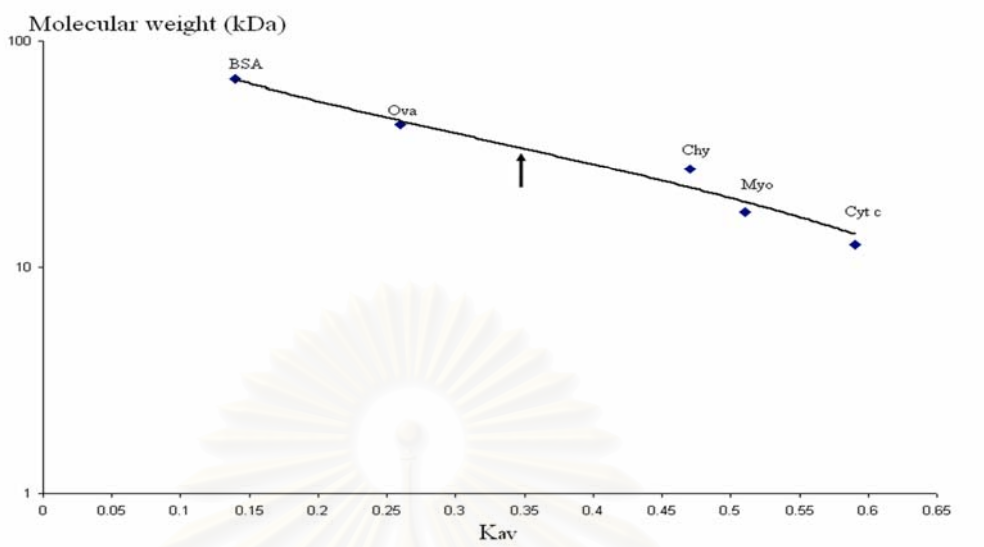

Figure 3.12 Calibration curve for molecular weight determination for the chromatography on Sephadex G-100 column of the recombinant SPI

Arrow indicates the Kav of the recombinant SPI
BSA: Bovine serum albumin MW = 68 kDa
Ova: Ovalbumin MW = 43 kDa
Chy: Chymotrpsinogen MW = 27 kDa
Myo: Myoglobin MW = 17.5 kDa
Cyt c: Cytochrome c MW = 12.5 kDa

Table 3.1 Purification table of the recombinant serine proteinase inhibitor

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification fold	Recovery (%)
Crude extract	2.7	0.81	143,839.8	177,580	1	100
SephadexG-100	0.97	0.136	24,745.5	182,220	1.03	17

Inhibitor activity unit: The amount of SPI that reduces the subtilisin activity per min by 10%
Specific activity: The activity unit per mg protein
Chromogenic substrate: N-benzoyl-Phe-Val-Arg-p-nitroanilide for subtilisin

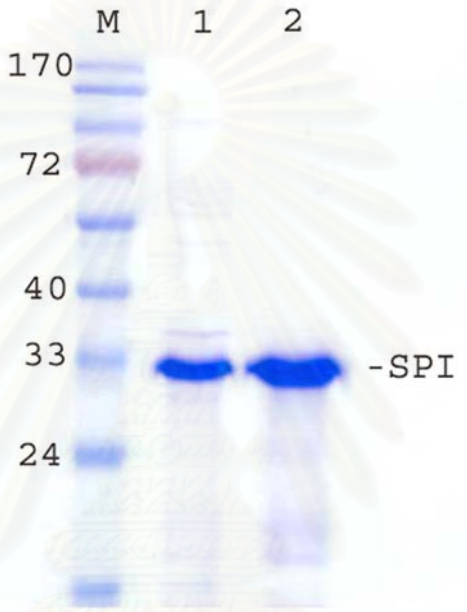

Figure 3.13 SDS-PAGE analysis of the purified recombinant protein. The proteins from crude extract (lane 1) and Sephadex G-100 pool (lane 2) were subjected to 12% SDS-PAGE. Lane M, Prestained protein marker (Fermentas).

3.3.3 Molecular mass determination of recombinant SPI by using MALDI-TOF Mass Spectrometry

The molecular mass of recombinant SPI was determined by using MALDI-TOF Mass Spectrometry. The MALDI-TOF spectra for recombinant SPI was shown in Figure 3.14. This result indicated that the molecular mass of recombinant SPI was 29.065 kDa. The molucular mass of recombinant SPI was used to calculate the inhibitor concentration.

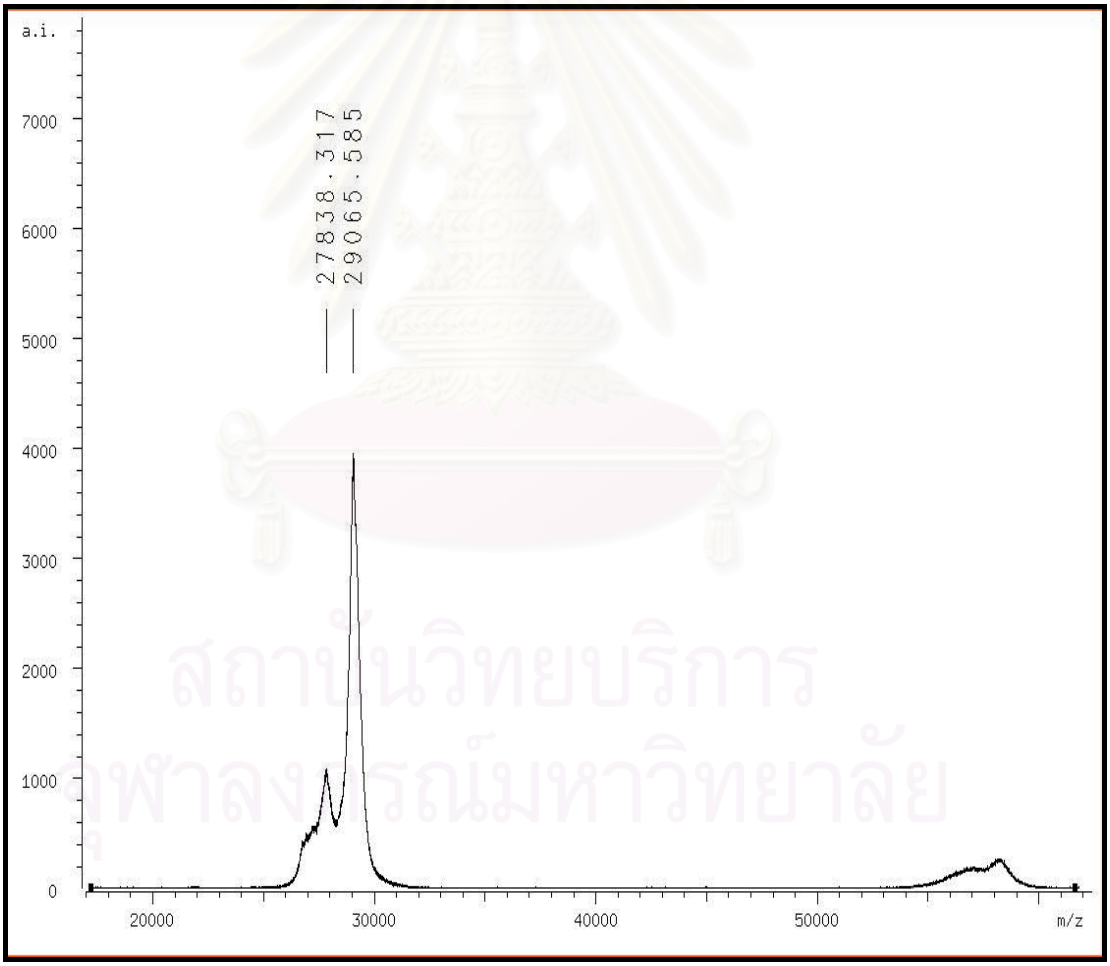

Figure 3.14 The MALDI-TOF spectra for recombinant SPI

3.3.4 Serine proteinase inhibitory assay

The inhibitor activity of recombinant serine proteinase inhibitor towards serine proteianase was tested by incubation of recombinant serine proteinase inhibitor and serine proteianase. The residual serine proteinase activity was determined by addition of chromogenic substrate which can be cleaved by the serine proteinase and released p-nitroanilide. Absorbance of chromophore p-nitroanilide produced was measured at 410 nm. Therefore, inhibition of serine proteinase hydrolysis of chromogenic substrate can be measured by following the change in absorbance 410 nm. The purified recombinant protein from Ni-NTA agarose column (denaturing condition) and Sephadex G-100 column (nondenaturing condition) were tested for serine proteinase inhibitory activity. The 0.017 μM inhibitor was used in this experiment. A decrease in absorbance was observed when the protein incubated with subtilisin but not trypsin and chymotrypsin (Table 3.2). The percentage inhibition of subtilisin was two folds lower than that of native condition. This assumed that it may be caused by the incorrect folding of the protein.

Table 3.2 Serine protienase inhibitory activity of partially purified recombinant protein by using Ni-NTA column chromatography (denaturing condition) and Sephadex G-100 column chromatography, solubilized in 50 mM sodium carbonate buffer, pH 10 (nondenaturing condition)

Proteinase (0.02 μM)	**Percentage of inhibition**	
	Denaturing condition	**Nondenaturing condition**
Subtilisin	41.1	91.1
Trypsin	0	0
Chymotrypsin	0	0

Chromogenic substrate: N-benzoyl-Phe-Val-Arg-p-nitroanilide for subtilisin and trypsin, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin

To further investigate the inhibitory activity of the inhibitor, increasing amounts of the purified protein obtained from nondenaturing purification was assayed against various types of proteinase, trypsin, chymotrypsin, subtilisin and elastase. In this assay, 293.6 and 146.8 μM of N-benzoyl-Phe-Val-Arg-p-nitroanilide was used as substrate for subtilisin and trypsin, respectively, 147.3 μM of N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin and 443.1 μM of N-succinyl-Ala-Ala-Ala-p-nitroanilide for elastase. The concentrations of subtilisin, trypsin, chymotrypsin, and elastase were 0.010, 0.005, 0.003, and 0.039 μM, respectively. The said condition of the assay gave comparable absorption at 410 nm. The proteinase was incubated with different concentrations of inhibitor, 0, 0.026, 0.052, 0.130, 0.207, and 0.413 μM in the presence of chromogenic substrate at 30^{o} C for 15 min. The absorption at 410 nm was determined and the remaining activity (%) was ploted against the concentrations of inhibitor (Figure 3.15). This result indicated that the recombinant SPI had inhibitory activity against subtilisin and elastase but low against trypsin. The recombinant SPI had no inhibitory activity against chymotrypsin.

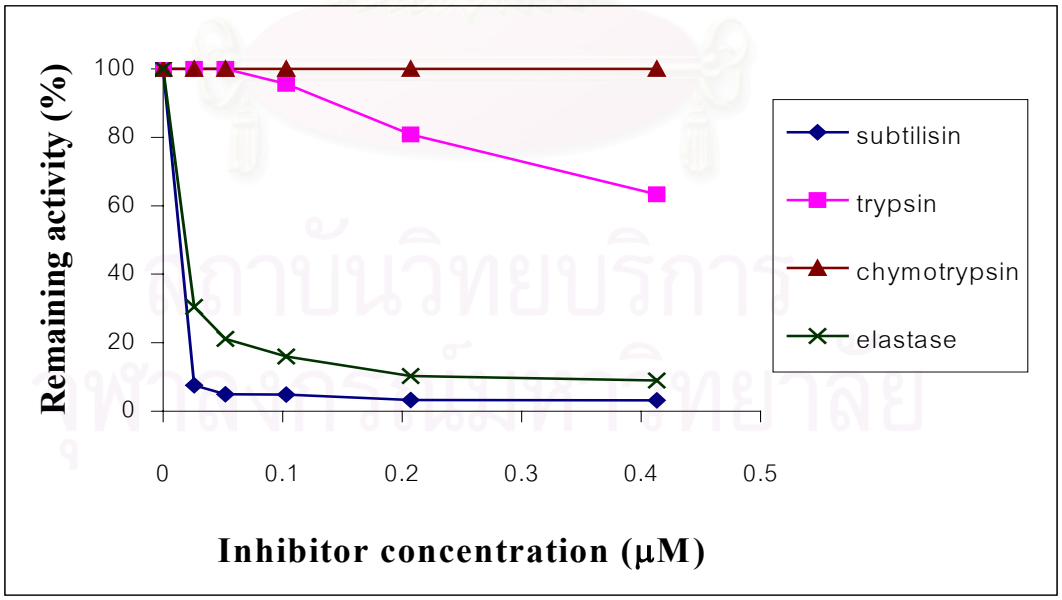

Figure 3.15 The inhibitory affect of serine proteinase inhibitor on various proteinases. Chromogenic substrate: N-benzoyl-Phe-Val-Arg-p-nitroanilide for subtilisin and trypsin, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin, and N-succinyl-Ala-Ala-Ala-p-nitroanilide for elastase. The concentrations of enzymes and substrates were adjusted to give comparable absorption at 410 nm.

3.4 Kinetic studies

3.4.1 Determination of subtilisin inhibition constant

To gain more insight on the inhibition of subtilisin by the serine proteinase inhibitor, 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 2.2.7.1. The substrate saturation curve of subtilisin inhibition was plotted as shown in Figure 3.16 as well as the Lineweaver-Burk plot (Figure 3.17). The K_M and V_{max} for the substrate was determined as shown in Table3.3. The inhibition profile was of competitive type of inhibiton. Therefore the following Michaelis-Menten equation was applied to determine the dissociation constant of the subtilisin-inhibitor complex, K_i.

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

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

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

The inhibition constant was determined from the replot of the apparent $K_{M,app}$ values against the inhibitor concentrations [I] at which they were obtained (Figure 3.18). By extrapolating the plot to where the $K_{M,app}$ equal zero, the value of K_i is obtained. The inhibition constant for the inhibitor in complex with subtilisin (K_i) was was shown in Table3.3

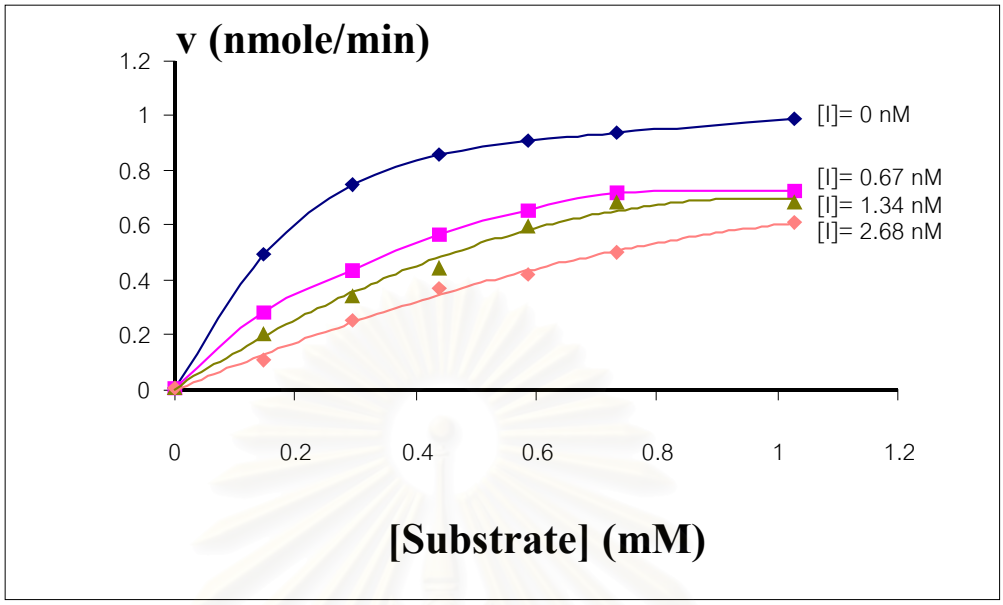

Figure 3.16 Substrate saturation curve of subtilisin with and without SPI

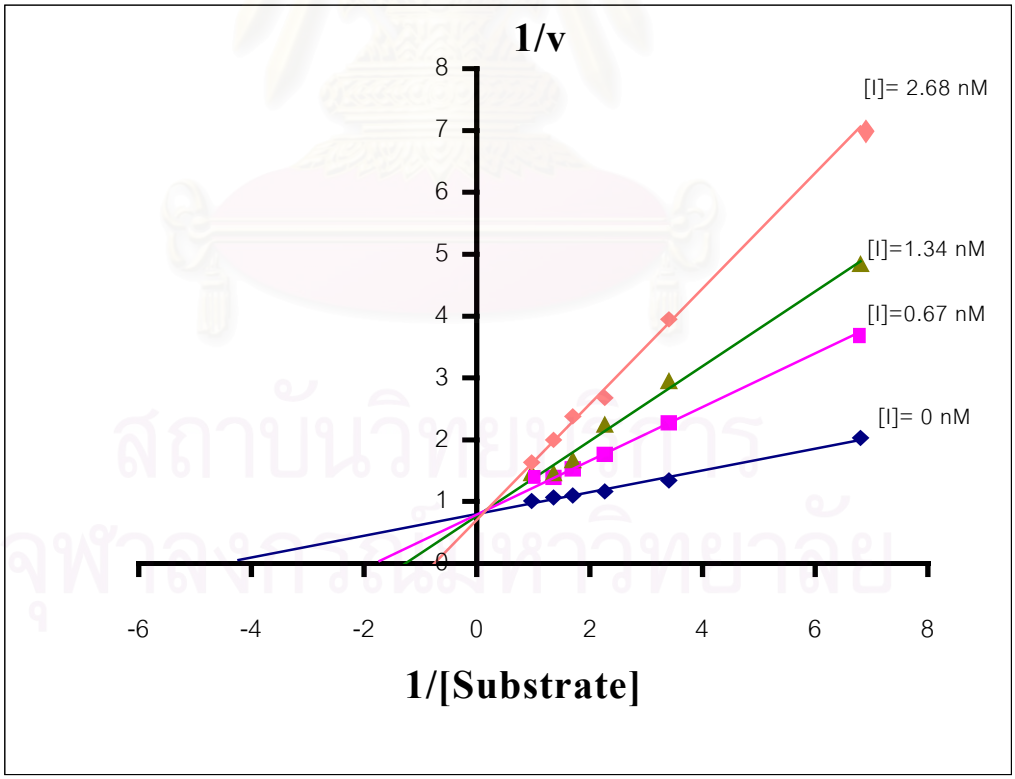

Figure 3.17 Lineweaver-Burk plots of subtilisin activity at different concentrations of the serine proteinase inhibitor (0, 0.67, 1.34 and 2.68 nM)

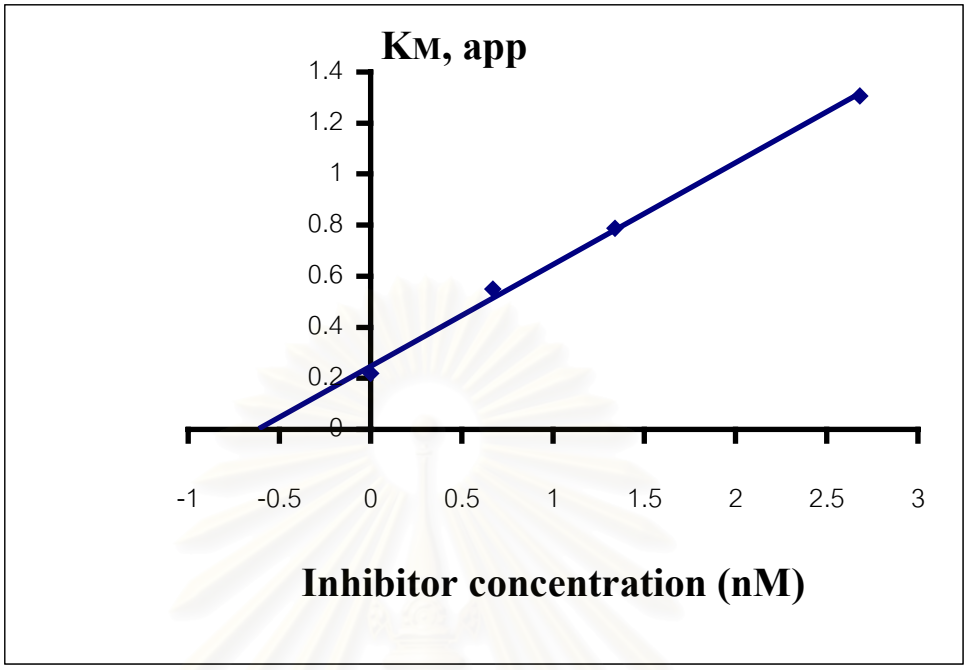

Figure 3.18 The plot of $K_{M,\ app}$ of subtilisin inhibition against the concentration of inhibitors for the determination of K_i

3.4.2 Determination of elastase inhibition constant

To gain more insight on the inhibition of elastase by the serine proteinase inhibitor, the inhibition constant was determined by measuring its inhibitory effect on enzymatic hydrolysis of N-succinyl-Ala-Ala-Ala-p-nitroanilide substrate at 30°C for 15 min as described in section 2.2.7.2. The substrate saturation curve of elastase inhibition was plotted as shown in Figure 3.19 as well as the Lineweaver-Burk plot (Figure 3.20). The K_M and V_{max} for the substrate was determined as shown in Table3.3 The inhibition profile was of competitive type of inhibiton. Therefore the Michaelis-Menten equation was applied to determine the dissociation constant of the elastase-inhibitor complex, K_i.

The inhibition constant was determined from the replot of the apparent $K_{M,app}$ values against the inhibitor concentrations [I] at which they were obtained (Figure 3.21). By extrapolating the plot to where the $K_{M,app}$ equal zero, the value of K_i is obtained. The inhibition constant for the inhibitor in complex with elastase (K_i) was shown in Table3.3

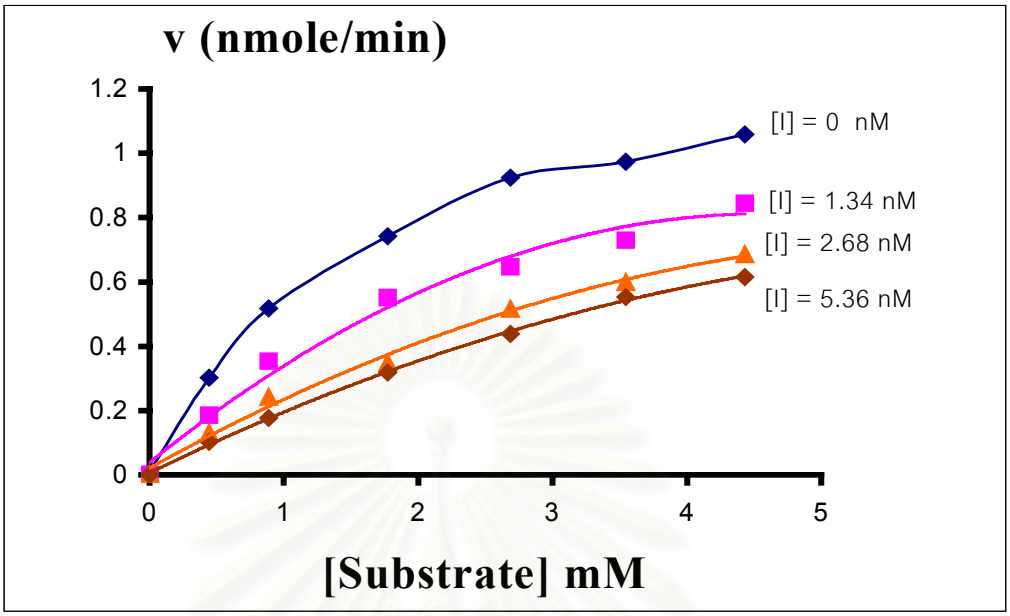

Figure 3.19 Substrate saturation curve of elastase with and without SPI

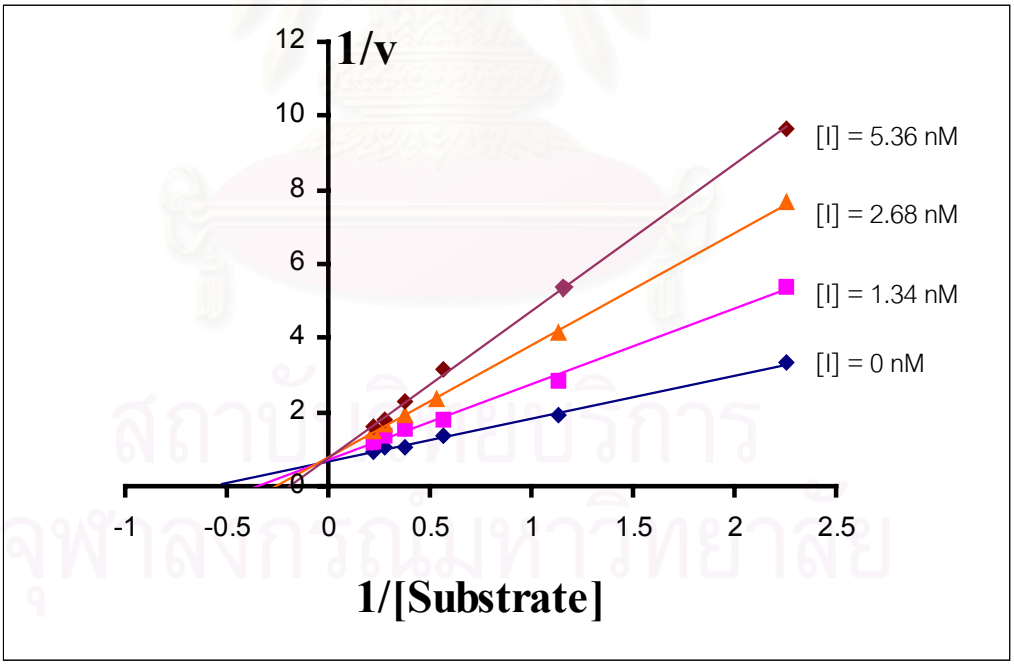

Figure 3.20 Lineweaver-Burk plots of elastase activity at different concentrations of the serine proteinase inhibitor (0, 1.34, 2.68 and 5.36 nM)

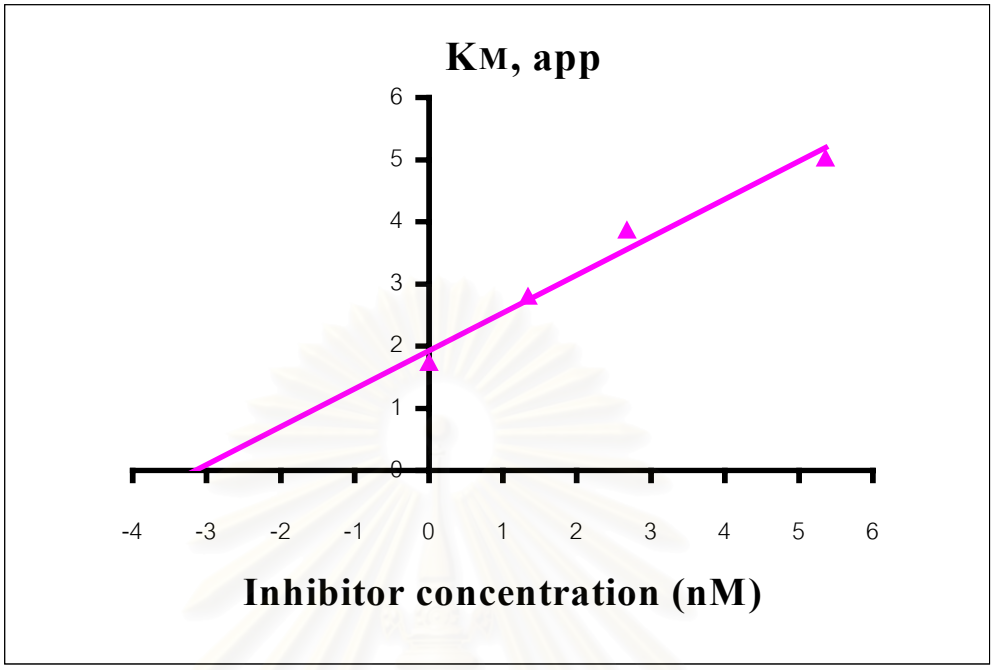

Figure 3.21 The plot of $K_{M, app}$ of elastase inhibition against the concentration of inhibitors for the determination of K_i

Table 3.3 The summary of the kinetic constants

	Subtilisin	**Elastase**
Vmax (nmole/min)	1.25	1.49
K_M (M)	0.22×10^{-3}	1.73×10^{-3}
Ki (M)	0.62×10^{-9}	3.16×10^{-9}

CHAPTER IV
DISCUSSIONS

Proteinases feature a wide array of important physiological function, including digestion, blood coagulation, fibrinolysis, fertilization, embryonic development and immunity in various organisms (Rawlings et al., 1993; Krem et al., 2002). Beside their physiological necessity, proteinases are potentially hazardous to their proteinaceous environment. When not properly controlled, proteinase can be detrimental and responsible for several serious diseases. As such, their activity must be precisely controlled by means of, for example, counteraction proteins known as proteinase inhibitors. Among these, serine proteinase inhibitors (SPIs) are known to widely distribute in living organisms and their major physiological function appears to be to prevent unwanted proteolysis. In arthropod hemolymph, SPIs are plentifully found and believed to have an inhibition role against proteinases from microorganisms or be regulators of host-defense reactions involving in blood coagulation, prophenoloxidase activation, or cytokine activation. A growing number of SPIs have been continuously reported and divided into established inhibitor families, namely, Kazal-type, Kunitz-type, serpins and α-macroglobulins (Kanost, 1999).

From the Expressed Sequence Tags (ESTs) analysis of *P. monodon* haemocytes, six cDNA clones were demonstrated as putative serine proteinase inhibitors by using BLASTX program (Supungul et al., 2002). They showed 50-58% homology to a Kazal serine proteinase inhibitor of crayfish *P. leniusculus*, which was a small, disulfide rich protein found in Arthropod hemolymph. The open reading frames (ORF) of the clones were identified using a Genetyx program. Four full-length cDNA clones were identified. The four clones contained the open reading frames with different length. The corresponding Kazal domains of putative *P. monodon* SPIs were demonstrated by aligning the amino acid sequences to that of proteinase inhibitor from the crayfish (Johansson et al., 1994). The four clones differed from each other in the number of Kazal domains. By comparing to the amino acid sequence and the

structure of third domains of silver pheasant ovomucoid inhibitor, the secondary structures of these Kazal domains are expected to contain an α-helix and three β-sheet structures (Figure 4.1) (Bode et al., 1985). The six conserved cysteine residues formed three intra-disulfide bridges, which stabilized the secondary structure, especially the inhibitory binding loop (Scott et al., 1987).

A full-length cDNA clone SH415 containing 5 complete Kazal domains of *P. monodon* is potentially an interesting clone because this clones was the only one that have five complete Kazal domains with diverse P1 residues or the putative reactive sites. This clone consists of an open reading frame of 801 base pairs encoding a 266 amino acid residue polypeptide as shown in Figure 4.1. The entire amino acid sequence of this protein shows 52% homology to the Kazal proteinase inhibitor sequences from the crayfish (Johansson et al., 1994). Like that of the crayfish, the Kazal protienase inhibitor from the SH415 is predicted using the SignalP 1.1 to contain the N-terminal 18 amino acid residue signal sequence (Neilsen et al., 1997). Recently a new SignalP 3.0 has been put into service (Bendtsen et al., 2004). The new predicted signal sequence is 19 amino acid longer or in the other word the mature polypeptide is 1 amino acid shorter. The 18 amino acid signal sequence truncated clone was subcloned and expressed in *Escherichia coli* expression system using the pTrcHis2C as vector (Jarasrassamee et al., in press). For unknown reasons, the recombinant protein was not over-expressed as expected and provided limited studies of its activity. Nevertheless, the crude recombinant protein has inhibitory activity against trypsin and chymotrypsin but not subtilisin and elastase.

In this study, the SH415 was subcloned into another expression vector pET-22b(+) in order to over-express the multiple Kazal-type inhibitor for further purification and characterization. The 5'-teriminal signal sequence truncated SPI gene was amplified with PCR using designed primers which incorporated restriction enzyme sites at their 5' ends, *Bam* HI and *Sal* I which resided respectively at 5' and 3' ends of the amplified fragment. The signal sequence-free fragment were 747 base pairs in length, which encoding a polypeptide of 249 amino acid residues. With 2 different restriction sites incorporated, the gene could be cloned directionally into the vector. For some reasons, the restriction digested PCR product could not be cloned

directly into the pET-22b(+) expression vector. Thus, the PCR product was first cloned into a pGEM-T easy vector and then subcloned into the pET-22b(+) expression vector.

A

```
                                    1 2
P. monodon SPI MANKVALLTLLAVAVAVS▼G▼
               YGKGGKIRLC-AKH-CTTI-SPVCGSDGKTYDSRCHLENAA-CGGVSVTFHHAGPC
                   PPPKRC-PGI-CPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSKKHDGRC
                       GCNPIVACPEIYAPVCGSDGKTYDNDCYFQAAV-CKNPDLKKVRDGNC
                       DCTPLIGCPKNYRPVCGSDGVTYNNDCFFKVAQ-CKNPALVKVSDTRC
                       EC-NHV-CTEEYYPVCGSNGVTYSNICLLNNAA-CLDSSIYKVSDGICGRRLYL

Ovomucoid      LAAVSVDCSEYPKPACTMEYRPLCGSDNKTYGNKCNFCNAVVESNGTLTLSHFGKC
                                  P1
```

B

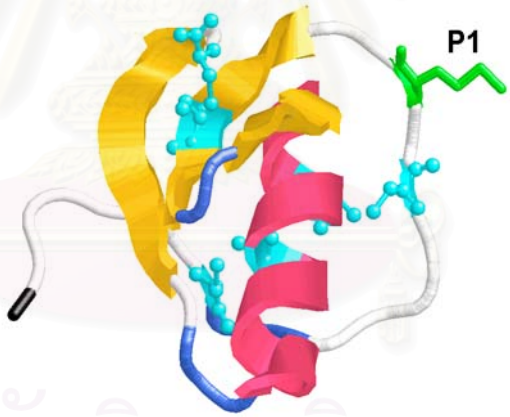

Figure 4.1 The Kazal domain of *P.monodon* SPI comparing with Ovomucoid 3rd domain (A). 1 and 2 indicated the signal sequence predicted using SignalP1.1 and SignalP3.0, respectively. Three dimensional structure of Ovomucoid 3rd domain (B). P1 is the putative reactive amino acid residue.

Since the PCR amplification of SPI segment used the *Pfu* DNA polymerase. There were no 3' terminal adenine added to both ends of the PCR fragment. Therefore, the adenine nucleotides were enzymatically added and the PCR fragment was then ligated to the pGEM-T easy vector, which contained the 3'-thymidine

nucleotide overhangs at the insertion site. The recombinant construct, named pGEM-T-SPI, contained the expected 750 bp gene fragment. The truncated SPI gene was then cloned into the pET-22b(+) by means of *Bam* HI and *Sal* I digestion and T4 ligase. The cloning of the gene fragment between the 2 restriction sites, *Bam* HI and *Sal* I, put the SPI amino acid sequence downstream of the *pel*B signal sequence which was for the periplasmic localization, and upstream of the His•Tag® sequence for the purification of the recombinant protein. The recombinant clone was named pET-22b (+)-SPI

In order to over-express the recombinant SPI, an *Escherichia coli* strain Rosetta (DE3) pLysS was used as a host. The Rosetta strains are designed to enhance the expression of eukaryotic proteins that usually contain codons rarely used in *E. coli* (Brinkmann et al., 1989; Seidel et al., 1992 Kane, 1995; Kurland and Gallant, 1996). The strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC and GGA on a compatible chloramphenicol resistant plasmid, pLysS. Supplying of the rare tRNA results in an increase in the expression of the eukaryotic proteins (Brinkmann et al., 1989; Seidel et al., 1992; Rosenberg et al., 1993; Del Tito et al., 1995). The expression of protein of interest is driven by the T7 polymerase and controlled by the *lac* repressor which can be induced by a synthetic inducer, isopropyl thiogalactoside (IPTG). However, even in the absence of IPTG, there is low level expression of T7 RNA polymerase under a strong *lacUV5* promoter in the λDE3 lysogens. This results in the basal expression of the target protein from the recombinant plasmid. If the foreign recombinant protein expressed in *E. coli* interfere with the normal function of the cell, it may be toxic to the host bacteria. The basal level expression can therefore be enough to prevent vigorous growth and the establishment of plasmid in λDE3 lysogens. To prevent such basal expression, a small amount of T7 lysozyme, a natural inhibitor of T7 RNA polymerase, is provided via a compatible chlorampheinicol-resistant plasmid, pLysS (Moffatt and Studier, 1987; Studier, 1991). T7 lysozyme is a bifunctional protein: it cuts a specific bond in the peptidoglycan layers of the *E.coli* cell wall (Inouye et al., 1973), and it binds to T7 RNA polymerase, inhibiting transcription (Zhang and Studier, 1997; Huang et al., 1999).

To verify whether the recombinant protein was expressed, the pET-22b(+)-SPI transformant was cultured and induced with IPTG. The total cell pellets were solubilized in SDS-PAGE sample buffer and subjected to 12% SDS-PAGE. An approximately 31 kDa protien was detected after 2 hours of induction in the cell lysate of *Escherichia coli* containing pET-22b(+)-SPI but absent in that containing the parental plasmid pET-22b(+). At 3 hours after induction, the intensity of 31 kDa protein band was enough for further studies, then this condition was used to prepare the recombinant protein for further characterization.

The bacterial expression system is particularly convenient for preparing very large quantities of protein under well-defined conditions. Unfortunately, bacterially expressed proteins are often difficult to purify because of their tendency to precipitate within the cell. The precipitated protein forms inclusion bodies, the dense, granular structures distributed throughout the cytoplasm (Marston, 1986; Krueger et al., 1989; Hockney, 1995). The formation of inclusion bodies is especially common for non-bacterial proteins. The recombinant SPI is no exception. To verify whether the recombinant SPI was in the inclusion bodies, the cell lysate was subjected to centrifugation to separate the supernatant liquid and insoluble precipitate. They were analyzed by 12 % SDS-PAGE. The approximately 31 kDa protein band was observed mainly in the insoluble fraction (Figure 3.9) which probably accounted for more than 80 % of the insoluble proteins. The 31 kDa protein was thus expressed and aggregated in the cells as inclusion bodies.

Since the recombinant protein obtained from pET-22b(+)-SPI contained six histidine residues at the C-terminus, the Western blot analysis could be used to confirm the identity of recombinant protein. The cell extracts were electrophoresed on a SDS-PAGE and transferred to a nitrocellulose membrane where they were probed with anti-His antibody and a second antibody tagged with horseradish peroxidase (HRP). The positive protein bands were localized in the membrane with colorimetric method using diaminobenzidine (DAB) as substrate. The approximately 31 kDa protein band was also detected in the lysate of cells containing the pET-22b(+)-SPI but not the parental plasmid pET-22b(+) (Figure 3.8). The results indicated that the 31

kDa protein was actually the recombinant SPI and successfully over-expressed in the *Escherichia coli* expression system.

The recombinant SPI was further purified. Two conditions had been used to solubilize the inclusion bodies, the denaturing and nondenaturing or native conditions. Under the denaturing condition, the inclusion bodies were solubilized using 6 M urea, and the recombinant SPI was purified with the Ni-NTA agarose column for it was tagged at the C-terminus with polyhistidine (Delcarte et al., 2003). The eluted fractions were selectively analyzed by the SDS-PAGE. The fractions containing the 31 kDa protein were pooled and renatured by dialysis slowly against the sodium carbonate buffer, pH 10 in the presence of dithiothreitol (DTT). For the non-denaturing condition, the inclusion bodies were solubilized using the alkaline carbonate buffer at pH 10. The recombinant SPI was further purified using the Sephadex G-100 chromatrography. The gel filtration did remove higher MW proteins as shown in Figure 3.13 but very small amount of the smaller MW proteins remained. As depicted in Table 3.1, the recombinant SPI was only slightly purer with the purification of 1.03 fold but lost substantially with the recovery only 17 %. Since the crude preparation contained the SPI as a major protein, probably more than 90 %, it is not surprising that further purification would give low purification fold. Substantial loss occurred possibly during the concentration of the protein after the gel filtration.

By using several standard proteins to calibrate the gel filtration column and SDS-PAGE analysis, the molecular weight of the recombinant SPI was estimated to be 31.4 and 31 kDa, respectively. The predicted MW of the protein with or without the *pelB* signal sequence from the amino acid sequence by using Genetyx program was 31.4 and 29.2 kDa, respectively. From this result, it was likely that the inhibitor contained the *pel*B signal sequence. However, the actual molecular mass of recombinant protein was not known. In order to determine the actual molecular mass of the inhibitor, MALDI-TOF Mass Spectrometry was performed. The MALDI-TOF spectra revealed a peak of the recombinant SPI with the molecular mass of 29.065 kDa. This result indicated that the *pelB* signal sequence was absent from the mature protein. The molecular mass of recombinant SPI determined by MALDI-TOF Mass Spectrometry was then used to calculate the inhibitor concentration.

The inhibitor activity of the recombinant serine proteinase inhibitor of dialysate from Ni-NTA agarose column (denaturing condition) and Sephadex G-100 column (nondenaturing condition) toward serine proteianase was tested using a spectrophotometric method. The method monitored the release of a color product, p-nitroaniline, which absorbed light at 410 nm, from the chromogenic substrate. Upon serine proteinase inhibition, the decrease in absorbance at 410 nm was measured. Compared to the proteinase reaction without the inhibitor, the percentage of inhibition was calculated. As shown in Table 3.2, the inhibition of proteinase was only observed when the SPI was incubated with subtilisin but not with trypsin and chymotrypsin. However, the subtilisin inhibitory activity of the SPI purified under the denaturing condition was about two folds lower than that of the SPI purified under the native condition. The results suggested that the recombinant SPI folding under this condition was not fully active or only certain amount of the SPI protein was correctly refolded. It may be due to the presence of several cysteine residues in this protein rendering the polypeptide more difficult to refold correctly.

The purified protein obtained from nondenaturing purification was further assayed against various types of proteinase, trypsin, chymotrypsin, subtilisin and elastase. Each proteinase was incubated with different concentration of recombinant SPI. The recombinant SPI had activity against elastase, subtilisin and low activity against trypsin. There was no activity against chymotrypsin. This result indicated that the recombinant SPI was both subtilisin and elastase inhibitor.

The Kazal inhibitors react with cognate proteinases according to the common, substrate-like standard mechanism (Bode and Huber, 1992). The reactive site is a single special peptide bond in each inhibitor molecule or domain, which serves the cognate enzyme as a high-affinity substrate (Saxena et al., 1997). Primary determinant of Kazal inhibitory specificity is believed to be the P1 amino acid of the reactive site which is involved in enzyme-inhibitor contact and highly variable among the Kazal inhibitors (Figure 4.1) (Laslowski, 1980; Scott et al., 1987; and Saxena et al., 1997). The P1 residue is the third amino acid residue after the second cysteine of each Kazal domain. For an inhibitor with multiple Kazal domain, each domain can possibly function independently. From the previous studies, if the Kazal domain

contains arginine or lysine residue at the P1 site, the inhibitor tends to inhibit trypsin. In the same manner, if P1 residue is tyrosine, methionine, leucine or phenylalanine; the inhibitor is most likely to inhibit chymotrypsin, subtilisin and/or elastase. If the Kazal domain contains alanine or serine, the inhibitor is supposed to inhibit elastase (Saxena et al., 1997). If the Kazal domian contains glutamate, the inhibitor is supposed to inhibit subtilisin (Lu et al., 2001). In the four Kazal domain proteinase inhibitor of crayfish, the P1 residues in the three Kazal domains are leucine residues and the other one is glutamine residue. The activity of the crayfish Kazal inhibitor was expected to inhibit chymotrypsin. Ascribing to the independently inhibitory function of the three crayfish Kazal domain containing leucine residues at the P1 sites, one inhibitor is anticipated to bind three chymotrypsin molecules. However, Johansson et al. (1994) reported that the crayfish Kazal inhibitor bound at 1:1 ratio to chymotrypsin and it was suspected that the other two domains might be inactive.

From the amino acid sequence of the five Kazal domain proteinase inhibitor, SH415 (Figure 4.1), the putative activity of the inhibitor could also be predicted from the P1 residues. Since the P1 residues were threonine, alanine, 2 glutamate, and lysine residues, the inhibitory activity against elastase, subtilisin and trypsin was predicted. From this study, the inhibitor had activity against elastase, subtilisin and low activity against trypsin. There was no activity against chymotrypsin (Figure 3.15). The inhibitory activity of recombinant SPI was conformed to the prediction from P1 residues. Whether or not, the two domains containing glutamate at P1 site were both active against subtilisin need further investigation. The corresponding proteinase for a domain containing threonine at P1 residues is not known.

Recently, Jarasrasamee et al. (in press) reported that the crude SH415 serine proteianase expressed in an *Escherichia coli* expression system using pTrcHis2C as vector had strong inhibitory activity against trypsin and chymotrypsin and weak activity against subtilisin. No inhibitory activity was formed against elastase. These results are in contrast with those of the present study, in which the proteinase has inhibitory activity against elastase, subtilisin and trypsin but not chymotrypsin. The discrepancy may be from a number of reasons. First, the previous work obtained considerably low yield of SPI with a lot of other contaminated proteins. These

proteins may in some ways interfere with the activity assays. On the other hand, the recombinant protein from this study was over-expressed. The crude preparation contained the SPI as a major protein, probably more than 90% and the recombinant SPI was further purified. Second, the differences in the activity may also be resulted from the folding of the recombinant proteins during biosynthesis because SPI is a cysteine-riched protein composing of 30 cysteine residues that may form both inter- and intra-disulfide linkage. Thus, the activity of the recombinant protein may differ from the native protein if different conformations were adopted. Since the nondenaturing condition was used for solubilization of the inhibitor, the protein should be in its native form. Moreover, the inhibitory activities were corresponding to the P1 residues of the inhibitor domains though the trypsin inhibitory activity was low.

To gain more insight on the inhibition of subtilisin and elastase by the serine proteinase inhibitor, the kinetic study was carried out. The inhibitory effect on enzymatic hydrolysis of N-benzoyl-Phe-Val-Arg-p-nitroanilide substrate for subtilisin and N-succinyl-Ala-Ala-Ala-p-nitroanilide for elastase were measured in order to determine the inhibition constant. The substrate saturation curve and the Lineweaver-Burk were plotted. As indicated from the Lineweaver-Burk plot, the SPI is a competitive inhibitor. The inhibition constant (K_i) of subtilisin and elastase were determined to be 0.62 and 3.16 nM, respectively. The inhibitor favored the subtilisin over the elastase. In general, the inhibition constants of the competitive inhibitors ranged from 10^{-9} to 10^{-12} M. Therefore, the recombinant SPI could be considered a strong inhibitor for subtilisin and elastase.

The others Kazal-type proteinase inhibitor cDNA were also isolated from the haemocyte cDNA library. The Kazal domains in each clone contain different P1 amino acids. The presence of several clones indicates that the Kazal-type proteinase inhibitors may have broad activity against proteinases for either defend the host against microbial proteinase or regulate the endogenous proteianses involved in coagulation, prophenoloxidase activition and cytokine activation.

The actual biological function of the black tiger shrimp serine proteinase inhibitor in the blood cells is not known. In arthropod, the serine proteinase inhibitor

in hemolymph are likely to function in protecting their hosts from infection by pathogens or parasites. For example a fungal proteinase specific protein inhibitor of silkworm is active against proteinases from fungal pathogen (Eguchi et al., 1993). The 23 kDa Kazal inhibitor of chymotrypsin and subtilisin from crayfish blood cell was purified and characterized (Johansson et al., 1994). As the crayfish Kazal inhibitor blocked the bacterial subtilisin, its possible biological function is to protect the crayfish against bacteria. In addition, the serine proteinase inhibitors have roles in regulation endogenous proteinase involved in coagulation, prophenoloxidase activation and cytokine activation. This study has proved quite decisively that the SH415 Kazal-type proteinase inhibitor is a subtilisin inhibitor. Subtilisin is an alkaline proteinase known to be secreted by members of bacteria in the genus *Bacillus*; it is therefore very likely that the SH415 SPI functions as an inhibitor to the microbial proteinaseses. Moreover, SH415 Kazal-type proteinase inhibitor is also an elastase inhibitor. Elastase has been shown to degrade elastin, cartilage proteoglycans, several collagens and fibronectin (Barret, 1981). Physiologically, it is involved in the degradation of foreign materials ingested during phagocytosis, which it is controlled possibly by the proteinase inhibitor. Our SH415 inhibitor has been shown to be mainly in the hemocyte and hemolymph, it may function as inhibitor for controlling phagocytosis.

CHAPTER V
CONCLUSIONS

1. A five-Kazal domain serine proteinase inhibitor (SPI) from the black tiger shrimp was successfully expressed in the *Escherichia coli* strain Rosetta (DE3) pLysS expression system.
2. The recombinant SPI has a molecular mass of appoximately 31 kDa as estimated by SDS-PAGE and the Sephadex G-100 chromatrography, whereas the molecular mass determined by using MALDI-TOF Mass Spectrometry is 29.065 kDa.
3. The recombinant protein was purified by Sephadex G-100 column chromatrography under the non-denaturing condition. The protein was purified up to 1.03 fold with 17% recovery.
4. The serine proteinase inhibitor was found to substantially inhibit subtilisin, elastase and had low activity against trypsin. There was no activity against chymotrypsin. Thus, the recombinant serine proteinase inhibitor is an inhibitor for subtilisin and elastase.
5. The inhibition constant of subtilisin and elastase inhibitor complex were calculated to be 0.62 and 3.16 nM, respectively.

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Appendices

Appendix I

Phenol-Chloroform extraction of DNA

1. Add an equal volume (equal to sample volume) of Phenol-Chloroform (1:1) to sample.
2. Mix (shake, don't vortex).
3. Take aqueous (upper) layer. (If dirty sample, repeat Phenol-Chloroform step until interface is fairly clean).
4. Add equal volume chloroform, mix (shake, don't vortex).
5. Spin 3 min.
6. Take aqueous (upper) layer. (Optional: repeat Chloroform steps).
7. Add 1/10 volume 3 M Sodium acetate mix (shake).
8. Add 2 volumes ice-cold ethanol (100%), mix (shake).
9. Incubate on ice for 15 to 30 minutes. (Can store on ice or at -20°C at this step).
10. Spin 10 minutes, 4°C.
11. Remove supernatant, being careful not to disturb pellet (DNA).
12. Half-fill tube with 70% EtOH, spin at least 2 minutes at 4°C. (Optional: Re-rinse and spin again).
13. Pipet off the supernatant, careful not to touch pellet.
14. Air-dry (~ 1 hour).
15. Redissolve in 40 μl of TE buffer

Appendix II

A-tailing procedure for blunt-ended PCR fragments

1. Start with 1-7 µl of purified PCR fragment generated by a proofreading polymerase, *Pfu* DNA polymerase.
2. Add 1 µl *Taq* DNA polymerase 10x Reaction Buffer with $MgCl_2$.
3. Add dATP to a final concentration of 0.2 mM.
4. Add 5 units of Taq DNA polymerase.
5. Add deionized water to a final reaction volume of 10 µl.
6. Incubate at 70^{o}C for 15-30 minutes.
7. Use 1-2 µl in a ligation reaction with pGEM®-T Easy vector

Appendix III

LB Broth (per Liter)

10 g of NaCl

10 g of tryptone

5 g of yeast extract

Add deionized H_2O to a final volume of 1 liter and autoclave

LB Agar (per Liter)

10 g of NaCl

10 g of tryptone

5 g of yeast extract

15 g pf agar

Add deionized H_2O to a final volume of 1 liter and autoclave. After, pour into petri dishes (~25 ml/100-mm plate)

LB-Ampicillin Agar (per liter)

Prepare 1 liter of LB agar. Autoclave and cool to 55^{o}C

Add 1 ml of 0.1 g/ml of ampicillin

Pour into petri dishes (~25 ml/100-mm plate)

LB-Ampicillin-Chloramphenicol Agar (per liter)

Prepare 1 liter of LB agar. Autoclave and cool to 55^{o}C

Add 1 ml of 0.1 g/ml of ampicillin

Add 1 ml of 0.034 g/ml of chloramphenicol

Pour into petri dishes (~25 ml/100-mm plate)

Appendix IV

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Solution A (Acrylamide Stock Solution), 100 ml

   30% (w/v) acrylamide, 0.8% (w/v) bis acylamide

   a. 29.2 g acrylamide

   b. 0.8 g bis-acrylamide

   Add distilled water to make 100 ml and stir until completely dissolved

2. Solution B (4x Separating Gel Buffer), 100 ml

   a. 75 ml 2 M Tris-HCl (pH 8.8)

   b. 4 ml 10% SDS

   c. 21 ml H_2O

3. Solution C (4x Stacking Gel Buffer), 100 ml

   a. 50 ml 1 M Tris-HCl (pH 6.8)

   b. 4 ml 10% SDS

   c. 46 ml H_2O

4. 10% ammonium persulfate, 5 ml

   a. 0.5 g ammonium persulfate

   b. 5 ml H_2O

5. Electrophoresis Buffer, 1 litter

   a. 3 g Tris

   b. 14.4 g glycine

   c. 1 g SDS

   d. H_2O to make 1 liter

   pH should be approximately 8.3

6. 5x Sample buffer, 10 ml

   a. 0.6 ml 1 M Tris-HCl (pH 6.8)

   b. 5 ml 50% glycerol

   c. 2 ml 10% SDS

   d. 0.5 ml 2-mercaptoethanol

   e. 1 ml 1% bromophenol blue

f. 0.9 ml H_2O

7. Pouring the 12% separating gel, 10 ml

Solution A	4	ml
Solution B	2.5	ml
H_2O	3.5	ml
10% Ammonium Persulfate	50	µl
TEMED	5	µl

8. Pouring the 5% stacking gel, 4 ml

Solution A	0.67	ml
Solution C	1.0	ml
H_2O	2.3	ml
10% Ammonium Persulfate	30	µl
TEMED	5	µl

Appendix V

Chemicals for preparation of Western blot analysis

1. Tank-bloting transfer buffer

   25 mM Tris-base

   150 mM glycine

   20% methanol

2. TBS buffer

   10 mM Tris-HCl

   150 mM NaCl

3. TBS-Tween

   20 mM Tris-Cl, pH 7.5

   500 mM NaCl

   0.025% (v/v) Tween 20

4. Blocking buffer

   3% (w/v) BSA in TBS buffer

5. 10 x Tris-saline

   9% (w/v) NaCl in 1 M Tris-Cl, pH 8.0

6. Staining solution

   Dissolved 18 mg 3,3'- Diaminobenzidine in 1x Tris-saline. After, add 60 μl of 30% H_2O_2

7. Anti-His antibody

   Diluted 1/1200 of Anti-His antibody in blocking buffer

8. Secondary antibody tagged with horseradish peroxidase (HRP).

   Diluted 1/10000 of Secondary antibody in blocking buffer

BIOGRAPHY

Miss Nawarat Somprasong was born on May 7, 1979 in Kanchanaburi. She graduated with the degree of Bachelor of Science from the Department of Biochemistry, Chulalongkorn University in 2000. She has further studied in Master's degree of Science (Biochemistry), Chulalongkorn University.