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

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จากห้องสมุด cDNA จากเม็ดเลือดกุ้งกุลาดำปกติและติดเชื้อวิบริโอได้แยกหายีนที่เกี่ยวข้องกับภูมิคุ้มกันของกุ้งกุลาดำ โดยเทคนิค Expressed Sequence Tags (ESTs) พบโคลนของตัวยับยั้งเซรีนโปรติเนส จำนวน 6 โคลน ในจำนวนนี้เป็นยีนที่สมบูรณ์จำนวน 4 โคลน ซึ่งมีความคล้ายคลึงกับตัวยับยั้งเซรีนโปรติเนส (serine proteinase inhibitor หรือ SPI) ชนิด Kazal ที่มีรายงานใน crayfish โดยทั้ง 4 โคลนอาจมีความแตกต่างกันที่จำนวนหรือความสมบูรณ์ของ Kazal domain การศึกษานี้เลือกศึกษายีนจากโคลนที่มีจำนวนโดเมน 5 โดเมน (โคลน SH415) ซึ่งมีบริเวณ open reading frame ที่มีขนาด 801 คู่เบส ซึ่งแปลรหัสให้โปรตีนที่มีกรดอะมิโนจำนวน 266 ตัว โดยทำการแสดงออกใน *Escherichia coli* และใช้ pTrcHis 2C เป็นเวคเตอร์ในการแสดงออก การทดลองนี้ทำการแสดงออกของตัวยับยั้งเซรีนโปรติเนสโดยตัดส่วนที่คาดว่าเป็น signal sequence ออก แล้วจึงโคลนเข้าสู่เวคเตอร์ ทำการคัดเลือกโคลนที่มีรีคอมบิแนนท์ pTrcHis 2C/SPI และวิเคราะห์โปรตีนที่ได้จากการแสดงออก เมื่อเปรียบเทียบระหว่างโคลนที่มี pTrcHis 2C และโคลนที่มีรีคอมบิแนนท์ pTrcHis 2C/SPI พบแถบโปรตีนขนาดประมาณ 35 กิโลดาลตันที่มีความแตกต่างในความเข้มของแถบโปรตีน โดยพบว่ามีความเข้มสูงกว่าในโคลนที่มีรีคอมบิแนนท์ pTrcHis 2C/SPI เมื่อนำโปรตีนหยาบที่ได้ไปทดสอบแอคติวิตีในการยับยั้งเซรีนโปรติเนสชนิดต่าง ๆ โดยวิธี proteinase inhibitor activity gelatin/SDS-PAGE assay พบแถบของเจลาตินที่ไม่ถูกย่อยขนาดประมาณ 35 กิโลดาลตันในส่วนที่เป็นโปรตีนหยาบจากโคลนที่มีรีคอมบิแนนท์ pTrcHis 2C/SPI เมื่อทดสอบกับ trypsin, chymotrypsin และ subtilisin โดยไม่พบแถบโปรตีนในโคลนที่มี pTrcHis 2C เมื่อทดสอบแอคติวิตีในการยับยั้งเซรีนโปรติเนสของโปรตีนหยาบโดยวิธี inhibitory spectrum assay พบว่าแอคติวิตีของเอนไซม์ลดลง 89 %, 70 % และ 8 % เมื่อทดสอบกับ trypsin, chymotrypsin และ subtilisin ตามลำดับ แต่ไม่พบการลดลงของแอคติวิตีเมื่อทดสอบกับ elastase

เมื่อศึกษาการเปลี่ยนแปลงในการแสดงออกของเอ็มอาร์เอ็นเอของตัวยับยั้งเซรีนโปรติเนสในเม็ดเลือดกุ้งกุลาดำที่ติดเชื้อวิบริโอ โดยใช้เทคนิค *in situ* hybridization พบว่ายีนนี้มีการแสดงออกในเซลล์เม็ดเลือดของกุ้งกุลาดำ ค่าเฉลี่ยของจำนวนเม็ดเลือดทั้งหมดในกุ้งที่ติดเชื้อเป็นเวลา 6 ชั่วโมงมีค่าน้อยกว่ากุ้งในกลุ่มอื่น ๆ และพบว่าการแสดงออกตัวยับยั้งเซรีนโปรติเนสในเม็ดเลือดของกุ้งที่ติดเชื้อเป็นเวลา 6 ชั่วโมงลดลงเมื่อเทียบกับการแสดงออกในเม็ดเลือดกุ้งที่เวลาอื่น ๆ นอกจากนี้จำนวนเม็ดเลือดที่มีการแสดงออกของตัวยับยั้งเซรีนโปรติเนสเพิ่มขึ้นอย่างมีนัยสำคัญในกุ้งที่ติดเชื้อเป็นเวลา 24 ชั่วโมง ในขณะที่จำนวนเม็ดเลือดที่มีการแสดงออกของตัวยับยั้งเซรีนโปรติเนสในกุ้งกลุ่มอื่น ๆ มีค่าใกล้เคียงกัน ข้อมูลที่ได้นี้จัดเป็นข้อมูลเบื้องต้นสำหรับการศึกษาตัวยับยั้งเซรีนโปรติเนสชนิดนี้ต่อไป

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KEY WORD : *P. monodon*/ black tiger shrimp/ serine proteinase inhibitor/ haemocytes/ *E. coli* expression system/ *in situ* hybridization

BOONYARIN JARASRASSAMEE : EXPRESSION AND CHARACTERIZATION OF SERINE PROTEINASE INHIBITOR OF THE BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : ASSOC. PROF. Anchalee Tassanakajon, Ph.D., THESIS CO-ADVISOR : Sirawut Klinbunga, Ph.D., 116 pp. ISBN 974-17-3899-4

Expressed Sequence Tags (ESTs) analysis of the normal and *Vibrio harveyi* infected haemocyte cDNA libraries of *Penaeus monodon* identified 6 putative serine proteinase inhibitors that are homologous to a Kazal-type serine proteinase inhibitor (SPI) from crayfish. Four full-length cDNA clones of SPI were obtained. They have the difference in either number of Kazal domain or domain completeness. In this study, we selected a five Kazal-domain clone, sh415, which have 801 bp of open reading frame coding for 266 amino acids. This clone was expressed by using *E. coli* expression system. NH_2-terminal truncated SPI gene, mature protein, was cloned into pTrcHis 2C. A 35 kDa protein band observed in recombinant pTrcHis 2C/SPI clone has higher intensity than that of parental pTrcHis 2C clone. The crude proteins of the recombinant clone were tested for serine proteinase inhibitory activity. In SPI activity gelatin/SDS-PAGE assay, the nondegraded-gelatin band with size of 35 kDa was observed in recombinant clone but not parental clone in trypsin, chymotrypsin, and subtilisin incubation. In inhibitory spectrum assay, trypsin, chymotrypsin, and subtilisin were inhibited their activities to 89 %, 70 %, and 8 %, respectively. The decrease in activity was not observed in elastase.

Moreover, we also determined the change in SPI transcripts in haemocytes of *V. harveyi* challenged *P. monodon* by using *in situ* hybridization. We found that Kazal inhibitor expressed in the haemocytes. We discovered that average total haemocyte number was decreased at 6 h after *V. harveyi* injection. Weak hybridization signal was observed in haemocytes at 6 h after injection indicates the decrease in SPI expression at this time point comparing to others. The number of haemocytes expressing SPI significantly increased at 24 h after injection. Whereas the SPI expressed haemocyte numbers of the others were not significantly different. These results provide preliminary data for further studying SPI.

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List of Abbreviations

ALF	anti-lipopolysaccharide factor
bp	base pair
°C	degree Celcius
DEPC	Diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EtBr	ethidium bromide
LPS	Lipopolysaccharide
M	Molar
ml	Millilitre
MT	metric ton
$MgCl_2$	magnesium chloride
mg	Milligram
mM	Millimolar
ng	Nanogram
O.D.	optical density
PCR	polymerase chain reaction
pfu	Plaque forming unit
proPO	Prophenoloxidase
proppA	prophenoloxidase activating enzyme
RNA	Ribonucleotide
RT	Reverse transcription
SPI	serine proteinae inhibitor
µg	Microgram
µl	Microlitre
µM	Micromolar

CHAPTER I

INTRODUCTIONS

1.1 General introduction

The black tiger shrimp, *Penaeus monodon*, is the most important cultured penaeid species in the Southern Indo Pacific region; such as Australia, Bangladesh, China, India, Indonesia, Philippines, Thailand and Vietnam (Bailey-Brock and Moss, 1992). The world shrimp production in 2000 comprises 56 % of *P. monodon*, 17 % of *P. merguiensis*, 16 % of *P. vannamei* and 11 % of the others (Rosenberry, 2001). *P. monodon* farming has considerably enhanced an economic growth and employment distribution.

The black tiger shrimp farming is one of the most common aquacultural industries in some tropical countries. In Thailand, *P. monodon* have long been cultured intensively. Approximately 60 % of the total shrimp harvests obtain from cultivation. Shrimp farms and hatcheries are dispersed along the coastal areas. Southern provinces (Nakon Sri Thammarat and Surat Thani) yield the majority of harvests whereas East and Central provinces (Samut Sakhon and Samut Songkhram) yield the minority in terms of number.

Thailand is now one of the top of shrimp cultivating countries. In the year 2001, the shrimp production from Thailand is 280,000 metric tons following by the other major exporting countries; such as China, India and Indonesia (Table 1.1). In many countries, diseases are a major constraint to aquaculture production. Especially in the shrimp production sector, infectious diseases are considered the most limiting factor for further development. In Thailand, a severe outbreak occurred during

Table 1.1 The world total shrimp production. Estimates on shrimp aquaculture production (in 1,000 metric tons).

Country	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**	**2000**	**2001**
Thailand	120.0	150.0	225.0	220.0	160.0	150.0	210.0	220.0	250.0	280.0
China	220.0	55.0	35.0	70.0	80.0	80.0	80.0	85.0	85.0	100.0
Indonesia	150.0	80.0	100.0	130.0	90.0	80.0	80.0	85.0	850.0	90.0
India	42.0	60.0	70.0	70.0	70.0	75.0	70.0	75.0	80.0	100.0
Bangladesh	27.0	29.0	30.0	30.0	35.0	34.0	38.0	45.0	45.0	55.0
Ecuador	110.0	90.0	100.0	100.0	120.0	130.0	155.0	80.0	40.0	20.0
Vietnam	39.0	41.0	50.0	50.0	30.0	30.0	25.0	35.0	35.0	42.0
Mexico	6.0	6.5	12.0	12.0	12.0	16.0	17.0	20.0	25.0	32.0
Philippines	25.0	20.0	18.0	25.0	25.0	10.0	15.0	20.0	20.0	25.0
Coloumbia	10.0	12.0	18.0	20.0	20.0	18.0	18.0	18.0	20.0	25.0
Taiwan	25.0	20.0	15.0	7.0	6.0	14.0	10.0	9.0	10.0	10.0
Honduras	5.2	5.7	6.5	10.0	10.0	12.0	12.0	10.0	10.0	12.0
Panama	4.2	4.4	4.6	10.0	10.0	10.0	10.0	9.0	8.0	5.5
Guatemala	2.5	2.7	3.0	7.0	7.0	7.0	7.0	6.0	6.0	4.5
Peru	5.6	5.8	6.0	8.0	8.0	6.0	6.0	5.0	5.0	2.5
Japan	3.5	3.5	3.6	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Others	45.0	51.2	50.0	14.0	30.0	35.0	55.0	54.0	50.0	47.0
Total	840.0	636.8	746.7	788.0	718.0	712.0	813.0	781.0	779.0	855.5

Source : Globefish

1995-1997 caused the decrease in the shrimp production. However, Thailand is still the largest *P. monodon* cultivator.

The United States of America and Japan are 2 major shrimp markets (Table 1.2). Approximately two-third of *P. monodon* exported from Thailand are imported to these countries, thereby worthing 38,859 million baht. The rest of the shrimp markets are Europe, Asian countries, Australia and others.

The *P. monodon* cultivation in Thailand has been skyrocketly generating large annual production. There are several advantages including the appropriate farming areas without the serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils and terrain for pond construction, thus enabling prosperous shrimp cultivation. Besides the culturing systems have been developed, established, and expanded in many shrimp farming areas. Culture of *P. monodon* increases national revenue, therefore, this penaeid shrimp species is economically important species in Thailand.

The general characteristic of the black tiger shrimp can be briefly summarized as below:

1.2 Taxonomy of *Penaeus monodon*

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

Table 1.2 Thai Frozen Shrimp Export in 2001

Country	Quantity (metric tons)	Amount (million baht)
United States	66,990	27,203
Japan	24,837	11,656
Canada	5,758	2,245
Singapore	6,610	2,129
Taiwan	6,308	1,762
Australia	3,638	1,406
Republic of Korea	4,121	1,270
China	3,412	1,051
Hong Kong	2,610	971
United Kingdom	1,587	598
France	1,553	497
Germany	1,242	474
Italy	876	162
New Zealand	337	115
Others	5,031	1,680
Total	134,910	53,219

Source : Globefish

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 (Phillipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Tim sa (Vietnam).

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

1.3 Morphology

The exterior of penaeid shrimp is distinguished by a cephalothorax with a characteristic hard rostrum, and by a segmented abdomen (Figure 1.1). The thorax is covered by a single, immobile carapace, which protects internal organs and supports muscle origins. Most organs, such as gills, digestive system and heart, are located in the cephalothorax, while

the muscles concentrate on 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 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. 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). A tail fan comprises a telson, which bears the anus, and two uropods attach to the last abdominal segment. The telson has deep medication groove without dorso-lateral spines. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawn (Anderson, 1993).

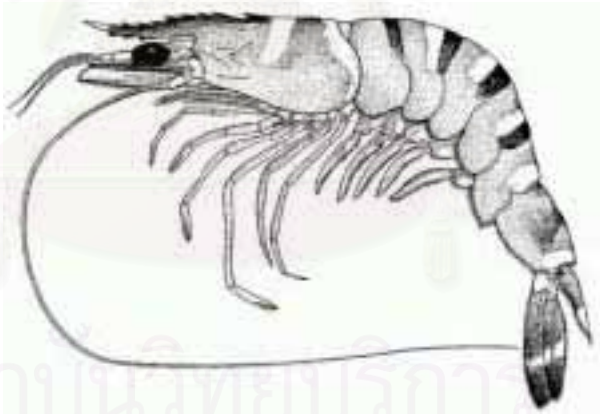


Figure 1.1 The black tiger shrimp (*Penaeus monodon*) derived its name from the huge size and banded tail, providing a tiger-striped appearance to this species (FAO, 2001b).

The cuticle, which is secreted by an epidermal cell layer, consists of chitin and protein in which calcium carbonate and calcium phosphate have been deposited. Epidermis detaches from the inner cuticle layer and

begins to secrete a new cuticle, while the old cuticle is moulted. After moulting the new cuticle is soft and is stretched to accommodate the increase size of the shrimp.

The black tiger shrimp has the following characteristic coloration: generally dark colored with carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow blackish waters or when shrimp are cultured in ponds, the color changes to dark and, often, to blackish brown (Moton, 1981: cited in Solis, 1988).

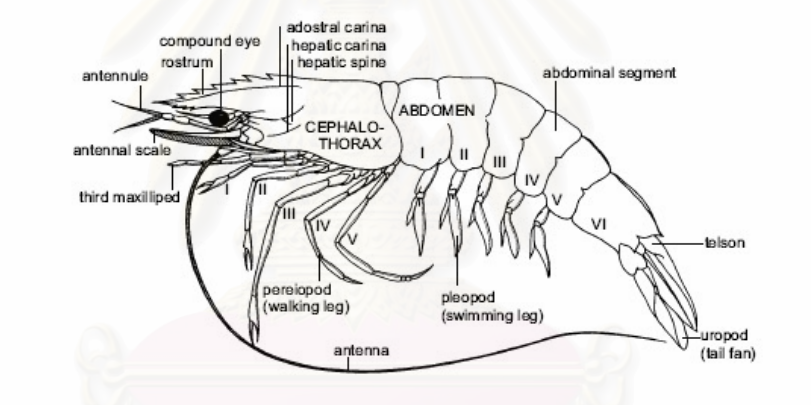


Figure 1.2 Lateral view of the external morphology of *Penaeus monodon* (Primavera, 1990).

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 cephalothorax. The valved haemolymph vessels leave the heart and branch several times before the haemolymph arrives at the sinuses, where exchange of substances takes place, scattering throughout

the body. After passing the gill, the haemolymph returns to the heart by means of three wide non-valved openings (Bauchau, 1981). A large part of 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 absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels 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 presented around the stomach and in the onset of the maxillipeds. Lymphoid organ and haematopoietic tissue are not shown in Figure 1.3.

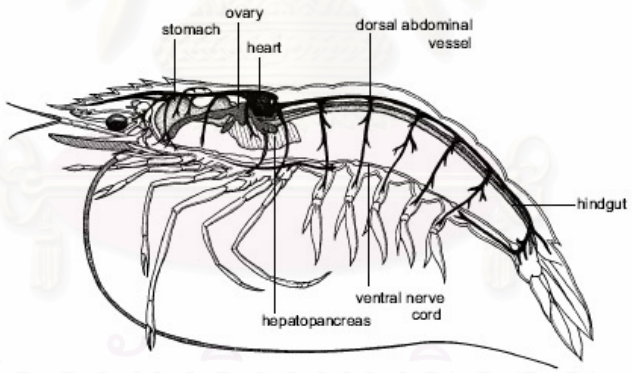


Figure 1.3 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990).

1.4 Distribution and life cycle

The giant black tiger shrimps are 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 various habitats (Figure 1.4). Juveniles prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinity, where mating and reproduction takes place. Females reproduce between 50,000-100,000 eggs per spawning (Rosenberry, 1997). The development of penaeid shrimps is complex. This complex life cycle begins with a larva hatching from the fertilized egg to the first stage, nauplius, metamorphoses to protozoa, mysis and post larval stages, sequentially (Figure 1.4). These stages require the developmental period of about 1-5 days, 5 days, 4-5 days, and 6-15 days, respectively (Solis, 1988). Shrimp larvae exhibit planktonic behavior. Swimming can be done using antennae in nauplii, antennae and thoracic appendages in protozoa and thoracic appendages in mysis larvae. The adult swims using the pleopods (abdominal appendages). Nauplii sustain on yolk granules with in their bodies while the feeding starts in protozoea and mysis. The protozoea feed on algae and metamorphose into myses. The myses feed on algae and zooplankton, having many characteristics of adult shrimp, and develop into megalopas, the stage commonly called postlarvae (PLs). After these stages, the postlarvae will develop into juvenile and sub-adults, tolerating the variant physio-chemical environment. Sub-adults migrate back to sea where they finally mature to mate and spawn. The life-span of penaeid shrimp are approximately 2 years (Solis, 1998: Anderson, 1993).

1.5Nutrition and growth

Organic compounds in the water stimulate the foraging activity of shrimp. Low concentrations of these compounds can be detected by cuticular hemosensory setae which are located at the anterior end of the

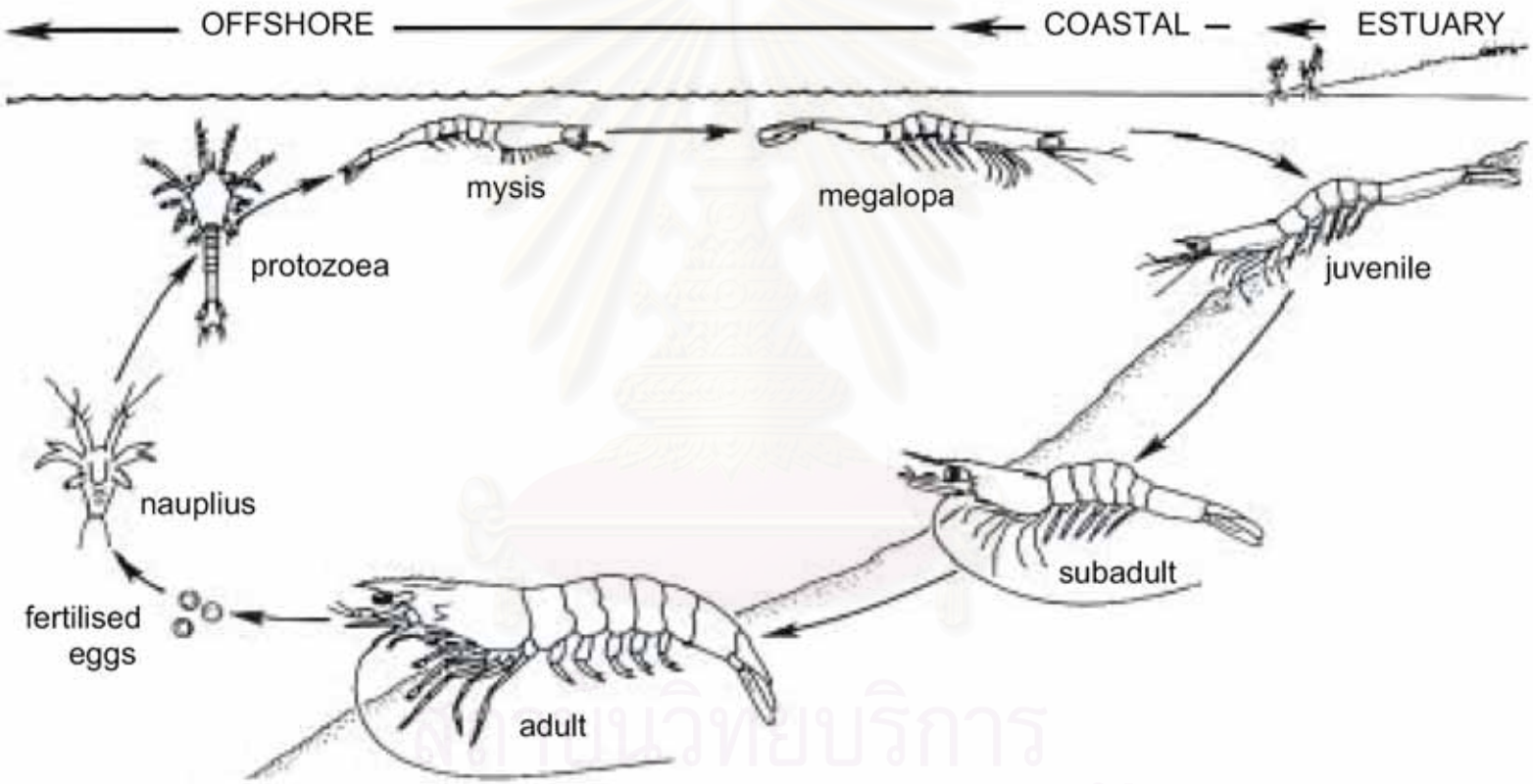


Figure 1.4 Life cycle of penaeid shrimp. Pictures are not in proportion to actual size.

body. Ranging from postlarval to adult shrimp can grasp food and pass to their mouth by using their pereiopods and maxillipeds. Shrimp slowly chew on the food by means of their mandibles and maxillae (Baily-Brock and Moss, 1992). Shrimp are omnivorous. They prefer small crabs, molluscs and small shrimp and fish (Motoh, 1984). When the food lack quality or become scarce, they will have the tendency to become cannibalistic.

The same as other invertebrates, crustacean such as shrimp or crab periodically lose their extracellular cuticle from the underlying epidermal layer to enable growth. The animal then rapidly leaves this rigid cuticle, takes up water and expands the new flexible exoskeleton. The exoskeleton is strengthened by using mineral and protein accumulation. This moulting or ecdysis process has several stages, varying in number and duration with species, temperature and growth phase. Immediately after moulting, the animals are quite vulnerable to pathogens and physical damage. Water is replaced by tissue during intermoult. This moulting process results in discontinuous size increases (Chang, 1992). Ecdysis is a complex process, in which all tissues are involved. This complex process includes lipid-reserved mobilization, cell division, new protein systhesis, and behavioral change.

1.6 Exploitation

Shrimp aquaculture is an important component to the economics of inter-tropical countries in South East Asia, Central and South America. In South East Asia, the main cultured shrimp species is *P. monodon*, Thailand has been regarded as a leader of *P. monodon* production for nearly a decade. The shrimp industry in Thailand provides not only employment to the workers, but also a significant source of revenue, and

a high quality food product (Lightner, 1998). However, Thailand has confronted with several problems as well as outbreak of diseases and local environment degradation. Since *P. monodon* farming still relies mainly on wild animals for supply of juveniles. The rapid growth of commercial shrimp operations may lead to overfishing of wild shrimp larvae and broodstock animals (Browdy, 1998).

The infectious disease outbreaks have become more serious problem, dramatically affecting the shrimp production. The causative agents of infectious diseases in shrimp are mainly viruses and bacteria; such as white-spot syndrome virus (WSSV), yellow-head virus (YHV) and luminescent bacteria, *Vibrio* species (Chou et al., 1995; Flegel et al., 1995; Jiravanichpaisal et al., 1994). These pathologies hamper particularly the larvae production and lead to mortalities and thus profitability problems (Bachère, 2000). Consequently, the prevention and control of diseases turned into a priority for an ecologically and economically viable shrimp production. However, mechanism and expression of genes responding to infection and immune systems are not well understood. Furthermore, shrimp aquaculture is also dependent on the selection of disease resistant animals. Therefore, more knowledges on shrimp immune system and genetics are required.

1.7 Shrimp diseases

Infectious disease problem becomes serious in shrimp industry because of increase in shrimp farming and lack of proper knowledge involving shrimp biology, farm management, and disease. Moreover, shrimp aquaculture is presently based on wild animals which are not completely acclimatized to the artificial conditions of shrimp hatcheries and farms where water quality, microbiological flora and nutrition are

vastly different from those in the natural habitat. These intensive artificial conditions lead to physiological disturbances and immunodeficiencies that increase sensitivity to pathogens.

The diseases of *P. monodon* are mainly caused by virus and bacteria as mentioned above. In Thailand, the outbreaks of infectious disease became the most serious problem since 1993. Yellow head disease spread in central and southern Thailand during 1993 to 1994 (Hasson et al., 1995), white spot disease during 1994 to 1996 (Flegel, 1997). Nowadays, luminous bacteria disease is increasingly considered to be the most serious problem for the shrimp culture. These pathogens cause serious diseases of larval and postlarval stages of *P. monodon* and lead to stock mortality. They also lead to the overfishing of wild shrimp larvae and an overexploitation of bloodstock.

1.7.1 Viral disease

The shrimp farming industry in Thailand encountered with a severe problem from viral infectious disease for over a decade. The important virus species that have been reported in *P. monodon* are White spot syndrome virus (WSSV) and Yellow-head virus (YHV) which cause white spot syndrome disease (WSS) and yellow-head disease (YH), respectively (Boonyaratpalin et al., 1993; Wongteerasupaya et al., 1995). The outbreak of these virus diseases causes great losses in the shrimp industry in several producing countries including Thailand.

White spot syndrome (WSS) disease

White spot syndrome (WSS) is a viral disease, which affect most of the commercially globally cultivated marine shrimp species (Chou et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Span and Lester, 1997). Lightner (1996) has called this virus white spot syndrome baculovirus

(WSSV) because its morphological characteristics are similar to insect baculovirus. However, phylogenetic analysis of ribonucleotide reductase and protein kinase genes revealed that WSSV does not share a common ancestor with baculovirus (van Hulten et al., 2000; van Hulten and Valk, 2001). This virus is an enveloped DNA virus of bacilliform to cylindrical morphology with an average size of 120 x 275 +/- 22 nm and has a tail-like projection at one end of particle (Kasornchandra et al., 1995; Wongteerasupaya et al., 1995) (Fig. 1.5). The viral genome contains double-stranded DNA of about 292 to 305 kb in length (Van Hulten et al., 2001; Yang et al., 2001).

White spots on the exoskeleton and epidermis are the most commonly observed clinical sign of WSS disease 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 shrimp that have white spots may survive indefinitely. In some cases, if the white spots appear together with lethargy, a pink to reddish-brown coloration, the gathering of affected shrimp 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 of the onset of the signs.

WSS disease can cause up to 100 % mortality, which a correspondingly devastating economic impact. In 1996, Lightner pointed out that no significant resistance to this disease had been reported for any species of shrimp, and this still remains true. WSSV is extremely virulent and has a wide host range (Lo et al., 1996b).

Diseases caused by viruses especially by white spot syndrome virus (WSSV) are the greatest challenge to worldwide shrimp aquaculture. The

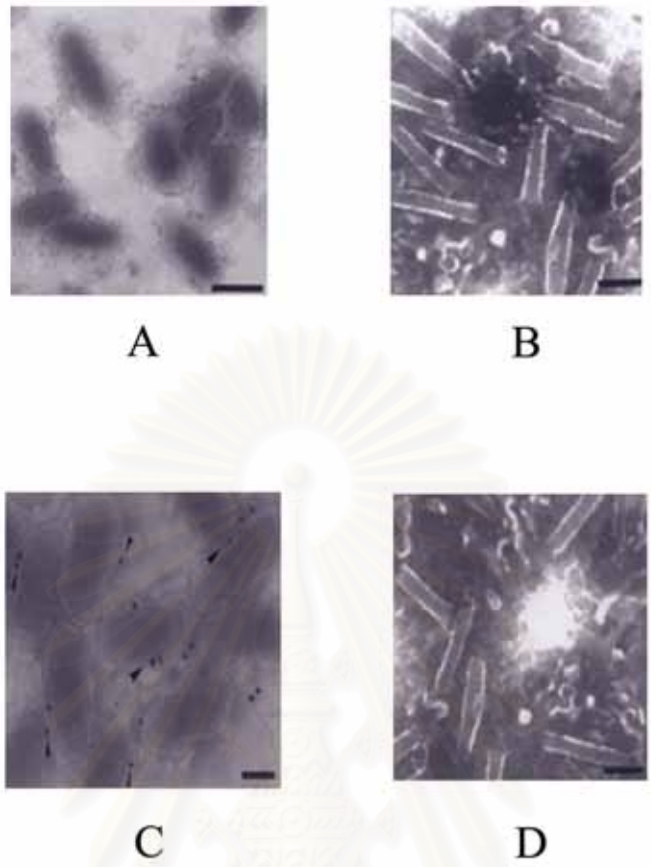


Figure 1.5 Immuno-electron microscopy of purified WSSV virions and nucleocapsids with anti-GST-P22 IgG followed by gold-labeled secondary antibody. (A) Intact WSSV Virions (scale bar, 185 nm); (B) nucleocapsids of WSSV (scale bar, 185 nm); (C) WSSV virions labeled with gold (scale bar, 238 nm); and (D) WSSV nucleocapsids labeled with gold (scale bar, 185 nm). Arrows indicate gold particles. The picture took from www.socgenmicrobial.org.uk/JGVDirect/18043/18043ft.htm.

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 virus-resistant shrimp *P. 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 in inhibiting virus-induced cytopathic effect in fish cell *in vitro*. (Lau et al., 2003)

Yellow-head (YH) disease

YH disease was known to infect and cause mass mortality in shrimp farming operations throughout South East Asian countries. In Thailand, the disease was first reported in 1990. YH disease 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). YHV is a pleomorphic, enveloped virus with single stranded RNA of positive polarity primarily localized in the cytoplasm of infected cells (Cowley et al., 1999). It may belong to the family coronaviruses (Figure 1.6).

YHV infected shrimp often exhibits light yellow coloration of the dorsal cephalothorax area and have a generally pale or bleached appearance (Limsuwan, 1991). At the onset of YHD, shrimp have been observed that food consumption was at an abnormally high rate for several days, then abruptly ceased feeding. A few moribund shrimp appear swimming slowly near the surface at the pond edges. After infection, mortality may reach as high as 100% of affected populations within 3-5 days from the onset of disease. In the black tiger shrimp, typical signs of YH disease include characteristic yellowing of the hepatopancreas and gill. YHV may occur

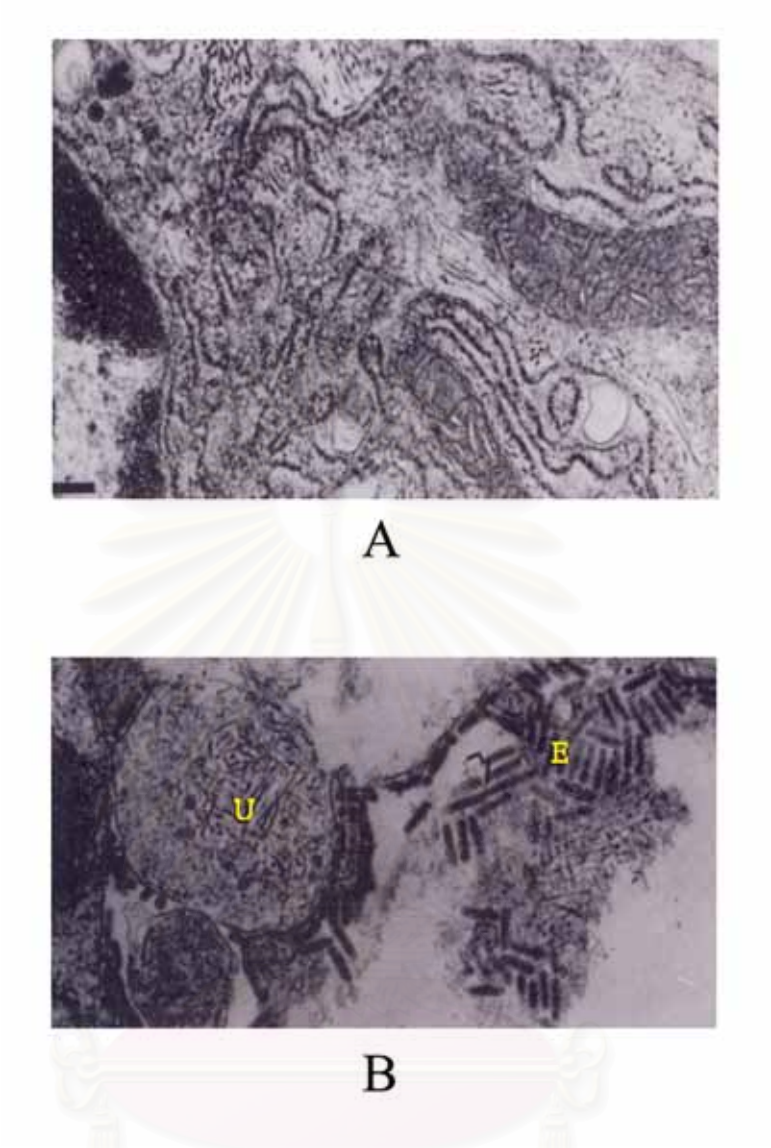


Figure 1.6 Transmission electron micrographs of hepatopancreatic interstitial cells from yellow-head specimens. (A) A viral infected cell. The viral material is sectioned transversely and longitudinally. It appears in two densities, probably indicating the presence (dense) or absence (less dense of capsid material (bar=200 nm). (B) Unenveloped virion (U) bell. The average length of the short virions was approximately 170 nm. Note the double length virion (arrow) (bar=200 nm) (Flegel and Sriurairatana, 1993).

as latent, asymptomatic infections in broodstock shrimp and may possibly transfer from these shrimps to their offspring in larval rearing facilities (Chantanachookin et al., 1993).

1.7.2 Bacterial disease

Vibrio species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). However, the bacteria causing the most serious diseases of the larval and postlarval stages of *P. monodon* are of the genus *Vibrio* which behave more like true pathogens than opportunist (Johnson, 1978; Lightner, 1983, 1988, 1992).

The luminescent bacterium, *Vibrio harveyi*, frequently related to outbreaks of luminous vibriosis in cultured *P. monodon* in hatcheries in many countries such as Australia, China, India, Indonesia, Thailand, the Philippines, and Taiwan (Vandenberghe et al., 1998). In Thailand, vibriosis is the main cause of production loss in penaeid shrimp farms (Nash et al., 1992). This bacterial disease causes mortality up to nearly 100 % of affected populations; larvae, post-larvae, juveniles, sub-adults and adults (Lightner, 1983). Luminescent vibriosis bacteria, *V. harveyi*, were claimed to be the most causative agent associated with shrimp mortality. *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 bacteria is able to emit a blue-green color light (Figure 1.7). 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 *V. harveyi* is widely known as luminous disease. The gross signs of localized infection in the cuticle or sub-cuticle are called shell disease or black or brown spot disease. These superficial infections can develop into systemic infections under some circumstances. These systemic infections can cause mortality. Other gross features of the infected shrimp are milky white body and appendages, weakness, disoriented swimming, lethargy and loss of appetite, eventually leading 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 *V. harveyi* showed strong luminescence in dim light.

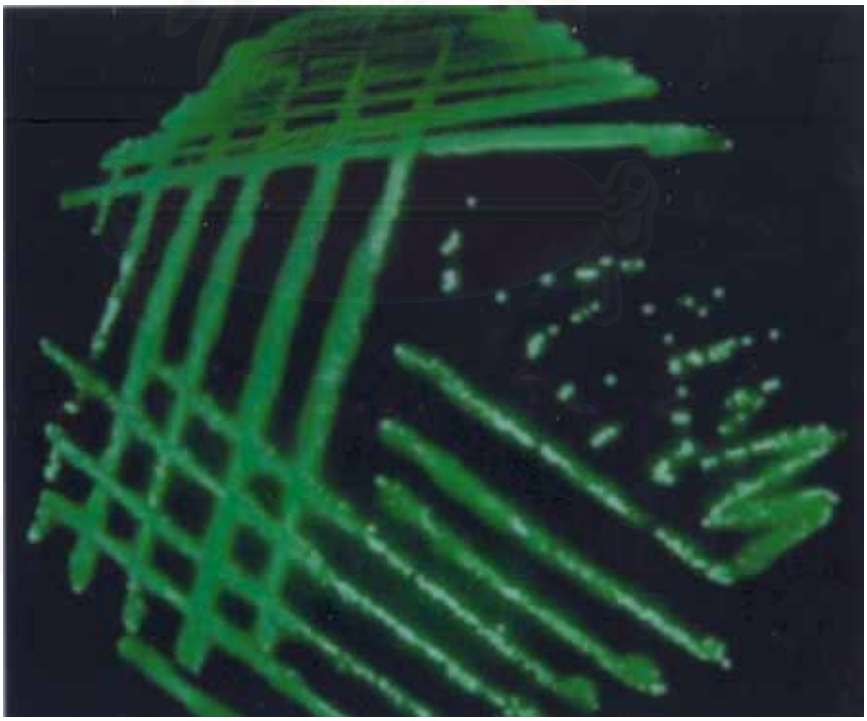


Figure 1.7 The blue-green color of light emission from *Vibrio harveyi*

1.8 Blood cells

Crustaceans have open circulations. The major defense systems of crustaceans are concentrated on haemolymph. The recognition molecules may interact with activate haemocytes, thus activating host defense mechanism. Haemocytes are the effectors of cellular immune response and also entail in humoral effector synthesis. The circulating haemocytes of crustacean are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Johansson and Soderhall, 1989; Soderhall and Cerenius, 1992). Crustaceans have three morphologically different haemocyte types: hyaline, semigranular, and granular cells. These different haemocyte types carry out different functions in immunity (Johansson et al., 2000).

Hyaline cells lacking cytoplasmic granules are the smallest group. They are 5-10% of the total haemocytes. The previous report indicates that, hyaline cells are involved in phagocytosis (Soderhall et al., 1986). Granular and semigranular haemocytes are oval, plate-shaped structure. Their longest dimension is 15-20 μm. Semigranular cells are the most abundant type of haemocyte and contain a variable number (1-40) of small (S) granules. These haemocytes are responding in some phagocytosis and encapsulation (Persson et al., 1987). Granular cells contain a large number of secretory large (L) granules. L-granules contain at least 24 proteins, a majority of which are clotting factor serpins, and various lectins. In contrast, S-granules contain at least six proteins with molecular masses of less than 30 kDa, in addition to an antimicrobial peptide tachyplesin and its analogues (Shigenaga et al., 1993; Muta et al., 1990).

1.9 Crustacean immune system

Crustacean immune system is non-specific immune system so-called the innate immunity (Söderhäll, 1998). The innate immune system is an ancient defense mechanism and can be found in all multicellular organisms. This system is the first line of defense that helps to limit infection at early stage (Janeway, 1998). Innate immunity is predicated on cellular and humoral reactions that are mainly related to their blood and haemolymph. Present knowledge of crustacean defense systems mainly concerns haemocyte activities such as phagocytosis and encapsulation, the hemolymph clotting reaction (Bachère et. al., 1995), and most of all, the prophenoloxidase activating cascade which has been particularly well-studied in crayfish (Soderhall et al., 1996). Briefly, the latter defense system leads to the production of quinones and intermediates in the biosynthesis of melanin. These compounds are associated with encapsulation of foreign organisms as well as with the production, by phagocytes, of reactive oxygen intermediates (Nappi and Vass, 1993). In crayfish, melanin was shown to display fungistatic properties, by acting as an inhibitor of both growth and proteinase activity of the fungus, (*Aphanomyces astaci*) (Soderhall and Ajaxon, 1982).

To prevent non-self invasion, innate immunity has many defense mechanisms including haemolymph coagulation, melanization, agglutination, phagocytosis, encapsulation, and protein recognition. Most mechanisms have been extensively studying in crustacean. According to function in immune system, associated proteins can be divided to several groups.

1.9.1 Haemolymph coagulation

Blood coagulation is not only forestalling bleeding, but preventing dissemination of infectious agents and immobilizing invading organisms as well. The shrimp coagulation system can also be activated by microbes or their surface components by inducing the release of a transglutrminase from hyaline haemocytes. This transglutaminase polymerizes the clotting protein (CP) (Plascencia et al., 2002). Clotting protein then has been studied in many organisms. Clotting proteins in *P. vannamei* shrimp have been studied. They are lipoglycoproteins having many subunits and playing key roles in coagulation (Montano-Perez et al. 1998). A very high-density lipoprotein (VHDL) purified from the hemolymph of the white shrimp *Penaeus vannamei* is shown to be identical to the clotting protein (CP) previously reported from the same organism based on size, subunits and N-terminal amino acid sequence. The 440-kDa protein, a homodimer of 200-kDa subunits, was identified in white shrimp *Penaeus vannamei* (Plascencia et al., 2002). The another protein responsible for clot_formation was also isolated from plasma of the white shrimp *Penaeus* v*annamei* by affinity chromatography. The clotting protein (CP) was found to be a lipoglycoprotein, composed of two 210-kDa subunits covalently bound by disulfide bridges (Pérez et al., 1999). Moreover, the size and amino acid composition of the clottable protein are similar to those of several other shrimps, prawns, lobster and crayfish, and their N-terminal amino acid sequences are 60–80% identical. Monosaccharide analysis of the clottable protein revealed the presence of mannose, glucosamine or *N*-acetylglucosamine and possibly glucose in this glycoprotein of about 5% sugar content in *Paeneus Monodon* (Yeh et al., 1998).

1.9.2 Prophenoloxidase system (pPO system)

The shrimp prophenoloxidase (pPO) system is located inside haemocyte granules and it is released under microbial stimulus. Outside the cell, the system becomes active by the effect of plasma calcium on the pPO activating enzyme (PPAE). This PPAE is a serine protease releasing a 17 kDa peptide and produces an active phenoloxidase (97 kDa) which in turn catalyzes the initial reaction for melanizaton (Gollas-Galvan et al., 1999). Melanization accomplished by means of many steps of enzymatic activation is a crucial immune system in crustacean (Aspan et al., 1995; Johansson et al., 1994). Prophephenol oxidase activating system is an enzymatic cascade reported in many invertebrates. The activation of the pPO system is brought about by an extremely low amount (pg/L) of microbial cell wall component such as lipopolysaccharide (LPS), (β1-3) glucan. These cell wall components can activate serine proteinase cascade enabling to polymerize melanin, thus causing deposition on the intruders. Because crustacean pPO system carries out in haemolymph, there are many studies having done in crustacean. pPO proteins are extracted from *P. monodon* (Sritunyalucksan et al., 2001), *L. japonicus* and *P. californiensis* haemocytes (Gollas-Galvan et al., 1999).

1.9.3 Pattern recognition proteins

Pattern recognition proteins have been isolated and characterized in several invertebrates. Some of them are haemagglutinin that have the ability to bind to specific carbohydrates expressed on some parts of bacterial cell wall or fungi cell wall, thereby causing infectious agent agglutination. Monodin glycoprotein extracted from black tiger shrimp plasma can stimulate *Vibrio vulnivicus* agglutination (Ratanapo and chulavatnatol, 1992).

Agglutinins and/or lectins having been regarded as having a putative role in non-self recognition in vertebrate and invertebrate immunity have important roles in agglutination, non-specific immune response. These ubiquitous proteins promoting cell agglutination by specifically binding to a variety of carbohydrates expressed on cell surfaces (Marques and Barracco, 2000). They can agglutinate microorganisms and enhance their phagocytosis by mediating binding between the haemocyte surface and a foreign body (opsonic role), and are apparently synthesised by invertebrate immune cells hemocytes. In contrast to immunoglobulins, the specificity of invertebrate agglutinins is restricted only to sugar residues (Cominetti et al., 2002).

1.9.4 Antimicrobial peptides

The production of antimicrobial peptides is a first-line host defense mechanism of innate immunity. Although, antimicrobial peptides are important to infectious diseases in crustaceans, few molecules displaying antimicrobial activities have been fully characterized in these invertebrates (Destoumieux et al., 2000). The penaeidins could be secreted or released from hemocytes by degranulation into the blood upon immune response stimulation. Although the shrimps used in these experiments have not been experimentally immune-challenged, it can be assumed that, under the intense conditions of stress generated by their harvest, some hemocyte activation has occurred leading to the release of the penaeidins into the blood circulation. The antimicrobial activity spectrum of penaeidins, established with yeast-expressed recombinant peptides, is rather large with antibacterial and antifungal properties (Destoumieux et al., 1999). The antibacterial activity is predominantly directed against gram-positive bacteria with different specificities in their mode of action, depending of the bacterial strain considered. Indeed, a

bactericidal effect of the penaeidins has been observed against the bacteria, *Bacillus megaterium*, whereas, the peptides display a bacteriostatic effect on *Micrococcus luteus*, or a slow bactericidal effect on the crustacean pathogenic strain, *A.* v*iridans*. In the experimental conditions used, the penaeidins had no effect on gram-negative bacteria such as the Vibrionaceae (Destoumieux et al., 1999). On the contrary, the peptides inhibit the growth of a large range of filamentous fungi, including *F. oxysporum*, pathogenic for shrimp. At a concentration lower than the minimum inhibitory concentration value MIC-5 mM, penaeidins cause reduced growth and elongation of the fungal hyphae leading to abnormal morphology, while at higher concentrations 10 mM, the peptides have a fungicidal effect on the *Fusarium* spores.

Another kind of immunity is peptides having gram negative resisting property, anti-lipopolysaccharide (LPS) factor. Anti-LPS factor discovered from horseshoe crab can neutralize gram negative bacterial endotoxin (Morita et al., 1996). Anti-LPS factor is a basic protein comprising 101 amino acids. Antimicrobial peptides are fascinating many groups of scientist because they can easily be produced in blood cell and released to resist to organism invasion. Although it has been report that antimicrobial peptides have been found in crustacean (Boman, 1995), until now, these peptide mechanism studies are quite limited. In horseshoe crab, there are some reports that blood cells are sensitive to LPS and β (1-3) glucan. These immunogens can activate blood cell to degranulate many substances relating in defense reaction (Iwanaka, 1998).

1.9.5 Proteinase inhibitors

Protein inhibitors of proteinases are ubiquitous. They are present in multiple forms in numerous tissues of animals and plants as well as in

microorganisms and play crucial roles in many biological processes (Michael and Ikunoshin, 1980). Their main physiological functions are to limit and to control proteinase activity at a site spatially and temporally away from the initial site of activation (Laskowski and Kato, 1980 and Roberts et al, 1995). The majority of proteinase inhibitors known and characterized so far are directed toward serine proteinase. Most of these interact with the proteinases according to a common mechanism.

Haemolymph of insects and other arthropods contains relatively high concentrations of serine proteinase inhibitors from several different gene families (Kanost and Jiang, 1996 and Palanoski and Wilusz, 1996). These inhibitors may exist in plasma or in haemocyte granules. According to their characteristics, serine proteinase inhibitors can be divided into several families including small disulfide-rich domains, α-macroglobulin, and serpin.

1.9.5.1 Inhibitors composed of small, disulfide-rich domains

Low molecular weight serine proteinase inhibitors from several gene families have been identified. These may occur as single, small proteins or in some cases as chains of inhibitor domains that are part of the same polypeptide. Some are plasma proteins and others are located within haemocytes. They have the common property of containing multiple disulfide bonds, which makes them stable. 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).

An inhibitor from the Kazal family was isolated from haemocytes of the crayfish. This protein contains four Kazal family domains and inhibits chymotrypsin and subtilisin (Johansson et al., 1994). This is the

only arthropod haemolymph protein reported so far containing Kazal domains, although a thrombin inhibitor that is a salivary protein from the blood sucking bug *Rhodnius prolixus* is composed of two Kazal domains (Friedrich et al., 1993). One Kazal domain contains 6 cysteines form 3 intradomain disulfide bonds. Many Kazal serine proteinase inhibitors from several multicellular organisms have been identified and characterized. Mammalian Kazal inhibitors contain one inhibitory domain except for the dog submandibular inhibitor which contains two domains. Several inhibitory domains are common in ovomucoid and ovoinhibitors from birds (Laskowski and Kato, 1980). From 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 anemone (Tschesche et al., 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 in crayfish (Johansson et al., 1994) and horseshoe crab (Nakamura et al., 1987). From a 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 acids. 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 inhibitors 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.

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).

1.9.5.2 α-macroglobulin

In comparison to the group of inhibitors described above, α-macroglobulins 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 traping the proteinase in a cavity formed by the α-macroglobulin dimer (Sottrup-Jensen, 1989). The change in conformation also leads to formation of covalent crosslinks between the thiol ester region of α-macroglobulin and lysine side chains of the proteinase, thereby resulting in irreversible inhibition of the proteinase, even though its active site is not affected.

α-macroglobulin have been identified and characterized in horseshoe crabs and crustaceans (Kanost and Jiang, 1996). A cDNA for *Limulus polyphemus* α-macroglobulin was cloned (Iwaki et al., 1996). The amino acid sequence of the horseshoe crab protein was 28-29 % identical to mammalian α-macroglobulins and included a conserved bait region, thiol ester site, and receptor binding domain. It is expressed in haemocytes and stored in the large granules.

1.9.5.3 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 formation of a very stable serpin-proteinase complex (Stone et al, 1997). Among arthropods, serpins from haemolymph have been purified, or their cDNAs clones, from two species of lepidopteran insects, a crayfish, and a horseshoe crab. Although the nature of the covalent serpin–proteinase complex has not been unequivocally established, it appears likely to be an acyl enzyme complex that represents a normal intermediate on the substrate pathway of a serine proteinase (Lawrence et al., 1995; Wilczynska 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 (Stratiko et al., 1997).

1.10 Roles of serine proteinase inhibitors in arthropod immunity

1.10.1 Protection against microbial proteinases

In many pathogenic fungi, proteinases enable them to penetrate the cuticle of their arthropod hosts. Proteinases can 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, *B. mori* FPI is active against proteinases from fungal pathogens (Eguchi et al., 1993). Moreover, inducible proteinase inhibitors may also play roles in antifungal defense (Vilcinskas and Wedde, 1997).

1.10.2 Regulation of endogenous proteinases

The function of proteinase inhibitors in arthropod haemolymph may lie in knowledge of the roles of serpins in vertebrate plasma. In vertebrates, blood vessel injury and microbial infection lead to activation of the blood coagulation and complement systems. Both of these systems for maintaining homeostasis employ cascades of serine proteinases to

amplify an initial signal (wounded tissue or the presence of microbial polysaccharides), thereby resulting in rapid and efficient responses (Whaley and Lemercier, 1993; O'Brien and McVey, 1993). Blood clotting process and complement can be harmful to the host organism if they are not locally limited. Consequently, the proteinases in these systems are tightly regulated by serpins that exist in plasma (Potempa et al., 1994). Such proteinase systems, regulated by inhibitors, may be a common evolutionary strategy in innate immune responses of animals.

1.10.3 Haemolymph coagulation

The haemolymph coagulation system in horseshoe crabs is the most thoroughly characterized of such pathways in invertebrates. The horseshoe crab coagulation system is composed of a complex pathway of high specificity serine proteinases, thus leading to proteolytic activation of a coagulogen and clot production. When exposed to bacterial lipopolysaccharide or β(1-3) glucan (from fungal cell walls), horseshoe crab haemocytes release clotting factors stored in membrane-bound granules. Almost of these factors are serine proteinase zymogens, and the rest are coagulogen, clotting protein precursors analogous to fibrinogen (Kawabata et al., 1996). These proteinase zymogens act as sensors for the presence of microorganisms in the haemolymph. Proteinase zymogens are activated to active proteinase called clotting enzyme. Clotting enzyme cleaves coagulogen to produce coagulin, which forms an insoluble gel-like clot. When degranulation occurs and the clotting factors are released, three serpins are also released from the granules. Each proteinase is paired with a serpin that may regulate its

activity *in vivo*, in a manner analogous to the vertebrate plasma serpins in regulating coagulation.

1.10.4 Phenoloxidase activation

Melanization, a response to wounding and infection in insects and crustaceans, involves serine proteinases. Wounding or infection leads to rapid activation of a proteinase that in turn activates a phenoloxidase zymogen (prophenoloxidase; pPO). Subsequent oxidation of phenols by phenol oxidase leads to production of quinones that polymerize to form melanin. The proteinases in this system have not been well characterized, but there are some evidences that activation of pPO, like blood clotting, involves a serine proteinase cascade (Söderhäll, 1996; Ashida and Brey, 1997). Like the horseshoe crab clotting system, pPO activation can be stimulated by LPS or β(1-3) glucans. Recognition of these microbial polysaccharides results in activation of a serine protainase that cleaves pPO to activate this enzyme. 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 (Kanost and Jiang, 1996). 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.

1.10.5 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 that eventually cleaves an inactive protein called spätzle, 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 spätzle to Toll initiates a

signal transduction pathway that leads to 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 regulating 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 to be a precursor, which can be cleaved by a proteinase to liberate the C-terminal peptide, which can then act on a target such as plasmatocytes. One would be expected that such a proteinase, and other proteinases involved in proteolytic activation of cytokines and had not been yet discovered, are regulated by proteinase inhibitors.

1.10.6 Other serine proteinases in haemolymph

Several serine proteinases that have been characterized by protein purification or by molecular cloning have not yet been assigned a function. A serine proteinase purified from plasma of *B. mori* exists as a zymogen that is a proteolytically activated upon exposure of plasma to β(1-3) glucan (Katsumi et al., 1995) and then is rapidly inactivated by a plasma serpin (Ashida and Sasaki, 1994). However, this enzyme does not appear to be part of prophenoloxidase activating pathway (Katsumi et al., 1995). Consequently, microbial polysaccharide exposure may stimulate more than one process involving activation of serine proteinase zymogens. Several cDNA clones with sequences which apparently encode non-digestive serine proteinases have been obtained from

D. melanogaster (Coustau et al., 1996) and from the mosquito *Anopheles gambiae*(Sidén-Kiamos et al., 1996; Dimopoulos et al.,1996), but their physiological roles are still not known. Furthermore, two different cDNAs that encode an amino-terminal clip domain and a carboxyl-terminal serine proteinase domain (similar to the structure of horseshoe crab proclotting enzyme) have been isolated from haemocytes of *M. sexta* (Jiang et al., 1998). This group of proteinases, with unearthed functions, hints at the existence of complex systems of proteinases and inhibitors regulating a variety of processes in haemolymph.

In a previous study, expressed sequence tags (ESTs) from the haemocyte of black tiger shrimp, *P. monodon*, were generated in order to identify gene associated with shrimp immunity (Supungul et al., 2001). Two haemocyte cDNA libraries were constructed from normal and *Vibrio harveyi* challenged shrimp *P. monodon*. Randomly selected cDNA clones with insert 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 % matched genes involved in defense reaction molecules, such as the components of the pPO system including pPO, pPO activating enzymes; the component of the clotting system including glutamine gammaglutamyl transferase, haemocyte protease; the antioxidative enzymes, peroxidase and catalase; antimicrobial peptides including anti-lipopolysaccharide factor, penaeidins, 11.5 kDa antibacterial peptides, lysozyme, and serine proteinase inhibitors.

Four full-length cDNA clones of serine proteinase inhibitors were isolated from the haemocyte cDNA libraries. They showed high homology to the four-domain Kazal proteinase inhibitor from crayfish, *Pacifastacus leniusculus*. The deduced amino acid of these cDNA clones

indicates variation in the number of Kazal domains. In this study, we aim to characterize the Kazal proteinase inhibitor of *P. monodon* by cloning and expression of the cDNA clone (SH415) in the *Escherichia coli* system. The clone, containing 5 Kazal domains, 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. Since the Kazal inhibitor that has been reported in the crayfish contains signal sequence, the signal peptide was predicted from the deduced amino acid of SH415 clone using signal peptide prediction program (Nielsen et al., 1997). A version of the gene expressing NH_2-terminal truncated protein was constructed to eliminate the signal peptide to obtain a mature protein. The gene product will be assayed for the inhibitory activity toward various types of serine proteinase.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

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

Automatic micropipettes P10, P100, P200, and P1000 (Gilson Medical Electrical S.A., France)

Cytospin (Cytospin 3: SHANDON)

–20 °C Freezer (Whirlpool)

–80 °C Freezer (Revco)

Hybridization oven (Hybrid, USA)

Incubator 37 °C (Memmert)

Larminar flow: Dwyer Mark II Model # 25 (Dwyer instruments, USA)

Microcentrifuge tubes 0.5 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

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

PCR thin wall microcentrifuge tubes 0.2 ml (Perkin Elmer)

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

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

Poly-L-lysine coated slide (O. Kindler GmbH & Co)

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

Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Touch mixer Model # 232 (Fisher Scientific, USA)

Transilluminator 2011 Macrovue (LKB)

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

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

2.1.2 Chemicals and Reagents

Absolute ethanol, C_2H_5OH (BDH)

Absolute methanol, CH_3OH (Merck)

Acetic anhydride (Sigma)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide (Pharmacia)

Agarose (Sekem)

Ammonium acetate (Ajax chemicals)

Ammonium persulfate (USB)

Amplicillin (BioBasic)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

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

Bromophenol blue (BDH)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue R-250 (BDH)

Dextran sulfate (BioBasic Inc.)

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

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

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

Dithiothreitol (Pharmacia)

Ethidium bromide (Sigma)

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

Ficoll^{TM} 400 (Amersham)

Formaldehyde (BDH)

Formamide (Acros)

Glacial acetic acid (Merck)

Glucose, $C_6H_{12}O_6$ (Ajax chemicals)

Glycerol (Scharlau)

Glycine (Scharlau)

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

Isoamylalcohol (Merck)

Isopropanol (Merck)

Lithium chloride, LiCl (Sigma)

Levamisole (Sigma)

2-mercaptoethanol (Scharlau)

N, N', methylenebisacrylamide (Fluka)

Nitroblue tetrazolium (NBT) (Roche)

Normal sheep serum (Roche)

Paraformaldehyde (Sigma)

Phenol crystals, C_6H_5OH (Carlo Erba)

RNase A (Sigma Chemical Co., USA)

Salmon sperm DNA (Sigma)

Sodium acetate (Merck)

Sodium citrate (Carlo Erba)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

Sucrose, $C_{12}H_{22}O_{11}$ (Fluka)

Transfer RNA (tRNA) from *E. coli* (Sigma)

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

Triethanolamine (Unilab)

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

Tryptic soy broth (Difco)

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

2.1.3 Enzymes

Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus, USA)

Bam HI (Biolabs)

Kpn I (Biolabs)

RNase-free DNase I (Promega)

Sal I (Biolabs)

T_4 DNA ligase (Biolabs)

T_3 RNA polymerase (Roche)

T_7 RNA polymerase (Roche)

Xba I (Biolabs)

2.1.4 Bacterial strains

Escherichia coli strain JM109 (Genotype: *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_K-, m_K+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *laq*I^qZΔM15])

Vibrio harveyi 1526

2.2 Animals

Juvenile shrimps (approximately 3 month-old) were perchased from local shrimp farms. They were separated into 2 groups; the first group was the normal shrimps and the another was *P. monodon* experimentally infected with *V. harveyi* 1526. They were kept in aquaria

in aerated water at the salinity of 15 ppt. and at the ambient temperature ($28 \pm 4^{o}C$) for at least 1 day before used in the experiments.

2.3 Construction of recombinant pTrcHis 2C

2.3.1 Preparation of Serine proteinase inhibitor (SPI) gene

5'- terminal truncated SPI gene was constructed by Polymerase Chain Reaction (PCR). The pBlueScript SK plasmids containing SPI gene were used as PCR template. The PCR amplifications were run for 30 cycles of 60s at $94^{o}C$, 120s at $56^{o}C$ and 60s at $72^{o}C$. All reactions were performed using standard conditions: in a final volume of 15 μl, 25 ng of DNA template was added in the presence of 0.45 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.45 unit of *Taq* DNA polymerase. The amplified products were analyzed by agarose gel electrophoresis. The size of the products was compared with standard GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas).

The following primers (BIOBASIC Inc.) were used for PCR amplifications. Restriction sites are underlined.

5'-CCATGGATCCGGGCTACGGAAAAGGGGGGAAAATCC-3'

Deleted signal sequence forward primer with *Bam* HI site

5'-ATGGTCGACTAGGTACAGTCTGCGACCACAGATTCC-3'

Reverse primer with *Sal* I site

The amplified SPI gene was cloned into pGEM-T easy vector. The transformants were selected using amplicillin agar plates. The recombinant plasmids were isolated and cut with *Bam* HI and *Sal* I. The restriction enzyme digestion was analyzed by 1.2 % agarose gel electrophoresis. The band in size of approximately 750 bp was purified using Nucleospin gel extraction kit (MACHEREY-NAGEL) as describe below:

After electrophoresis, the agarose gel containing 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^{o}C until the gel pieces had been dissolved for 5-10 minutes. The sample was then briefly vortexed every 2-3 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 x g for 1 minute. The flowthrough was discarded. Five hundred microlitres of NT2 buffer was add into the column and centrifuged as described above. Six hundred microlitres of NT3 buffer was added into the column and centrifuged. The flowthrough was discarded. Two hundred microlitres of NT3 buffer was added into the column and centrifuged at 11,000 x g for 2 minutes to remove NT3 buffer quantitatively. The column was placed into a clean 1.5 ml microcentrifuge tube. Fourty 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 x g for 1 minute. The flowthrough that was the DNA containing fraction was then stored at 4^{o}C until used.

2.3.2 Plasmid DNA preparation

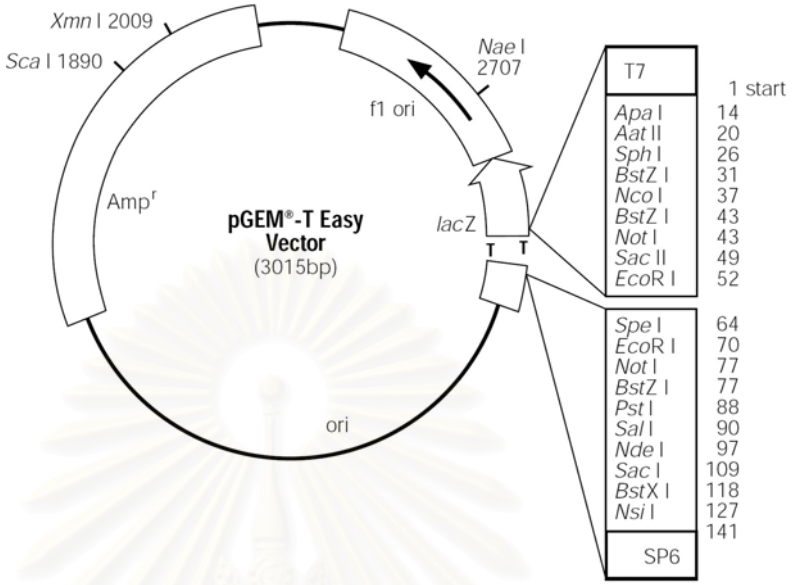
The single colony containing recombinant plasmid was inoculated into 1.5 ml of LB broth containing 50 μg/ml of amplicillin. Then the culture was grown overnight at 37^{o}C with shaking at 250 rpm. The culture was transferred to 1.5-ml microcentrifuge tube and centrifuge at 800 x g for 5 minutes. The supernatant was discarded. The pellets were resuspended in 100 μl of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose, and 0.5 % (w/v) lysozyme). The reaction was mixed by vigorous vortex and placed on ice for 30-60 minutes. Two

hundred microlitres of freshly prepared solution II (0.2 N NaOH and 1% (w/v) SDS) was added for cell lysis and DNA denaturation and mixed gently. After incubating on ice for 10 minutes, the mixture was added with 150 μl of solution III (3 M sodium acetate, pH 4.8) for renaturation. The reaction was mixed gently and placed on ice for 30 minutes. The tube was centrifuged at 10,000 x g for 10 minutes to separate cell debris. The supernatant was transferred to a new 1.5-ml microcentrifuge tube. An equal volume of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) was added, mixed gently and centrifuged at 10,000 x g for 10 minutes. The upper aqueous phase was transferred to a new microcentrifuge tube. The plasmid DNA was precipitated by adding 2 volumes of cold absolute ethanol, then mixed well and kept at –80^{o}C for at least 1 hour. The mixture was centrifuged at 10,000 x g for 10 minutes. The plasmid DNA was washed with 70 % (v/v) ethanol, air-dried, and then dissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0).

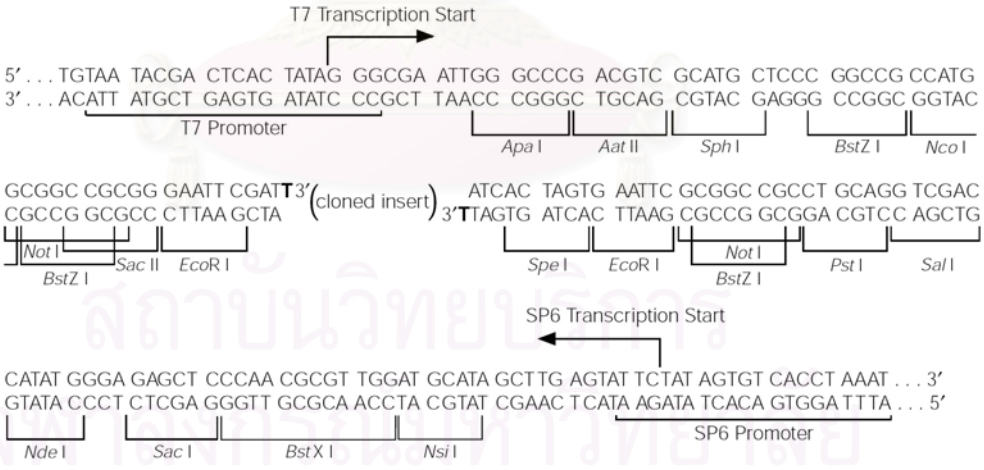
The expression vector used in this experiment was pTrcHis 2C. The expression of the gene of interest was under *trc* (*trp*-*lac*) promoter. The *trc* promoter contained the -35 region of the *trp* promoter together with -10 region of the *lac* promoter. As shown in Figure 2.2, the vector also comprised *lac* operator (*lac*O), *rrn*B antitermination region (antiterm), bacteriophage gene 10 ribosome binding site (g10 RBS), minicistron, initiation ATG, multiple cloning sites (MCS), *myc* epitope coding region (*myc*), polyhistidine region (6xHis), terminal sequence (term), amplicillin resistance gene (β-lactamase), pBR322 origin, and *lac*I^{q}, (the gene encoding Lac repressor). The restriction sites used to clone the serine proteinase inhibitor gene into the vector was *Bam* HI and *Sal* I (Figure 2.2). The clone containing pTrcHis 2C vector was selected by using amplicillin resistance property.

2.3.3 Restriction enzyme digestion

Because both of the amplified SPI gene and the expression vector pTrcHis 2C contained *Bam* HI and *Sal* I restriction sites, *Bam* HI and *Sal* I were used for sequential digestion. Conditions for the first enzymatic digestion of the amplified SPI gene or pTrcHis 2C comprised 600 ng of the DNA, 1 x *Bam* HI reaction buffer (10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT), 4 units of *Bam* HI (SibEnzyme), and 400 μg/ml BSA in a 40 μl reaction volume. The reaction was incubated at 37^{o}C for 4 hours. After incubation, 10 μl of the reaction were mixed with 5 μl of loading dye. The mixture was loaded onto 1.2% agarose gel and electrophoresed in 1 x TBE buffer at 100 volts. Sizes of DNA were visualized under a UV transiluminator by comparing with a standard GeneRuler^{TM} 100 bp DNA Ladder Plus (Fermentas) (for the amplified SPI gene) or λ/*Hin* dIII marker (for pTrcHis 2C). The rest of the reaction was further cut with *Sal* I by adding 1x *Sal*I reaction buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT) and 4 units of *Sal* I (SibEnzyme) in a 40 μl reaction volume. The reaction was incubated at 37^{o}C for 4 hours. The reaction was then purified by phenol-chloroform extraction. After ethanol precipitation, the DNA pellet was dissolved in 10 μl of steriled distilled water. Two microlitres of the DNA solution was mixed with 5 μl of loading dye and 8 μl of TE buffer. The mixture was loaded onto 1.2 % agarose gel and electrophoresed in 1xTBE buffer at 100 volts. DNA concentration was estimated under the UV transilluminator by comparing with 100 bp ladder and λ/*Hin* dIII marker. The digested pTrcHis 2C and amplified SPI gene were used in ligation.



[a]

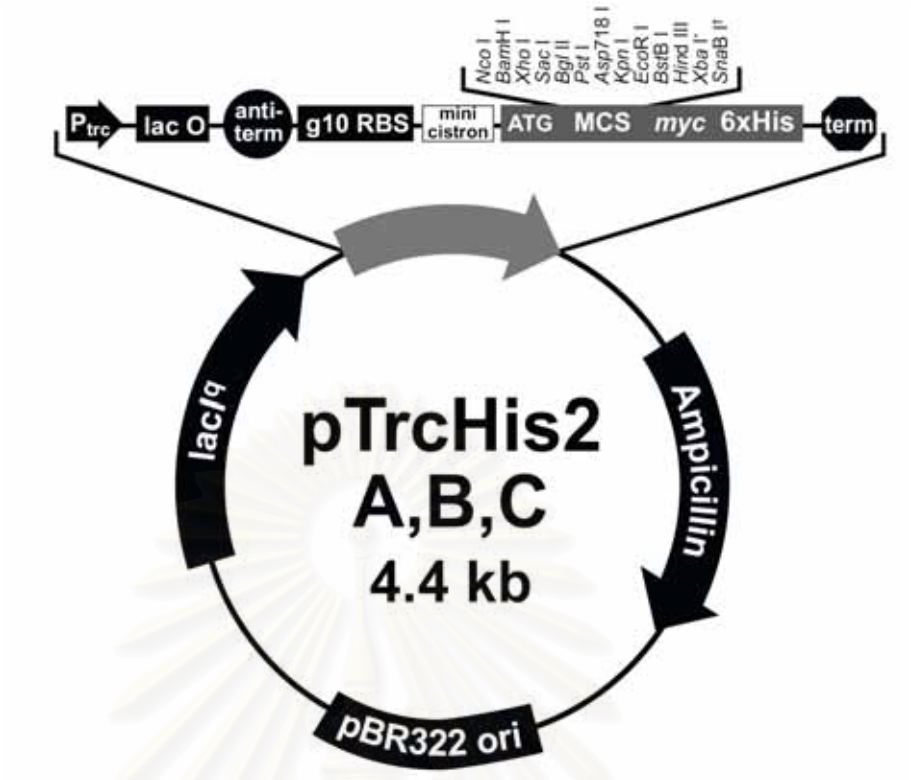


[b]

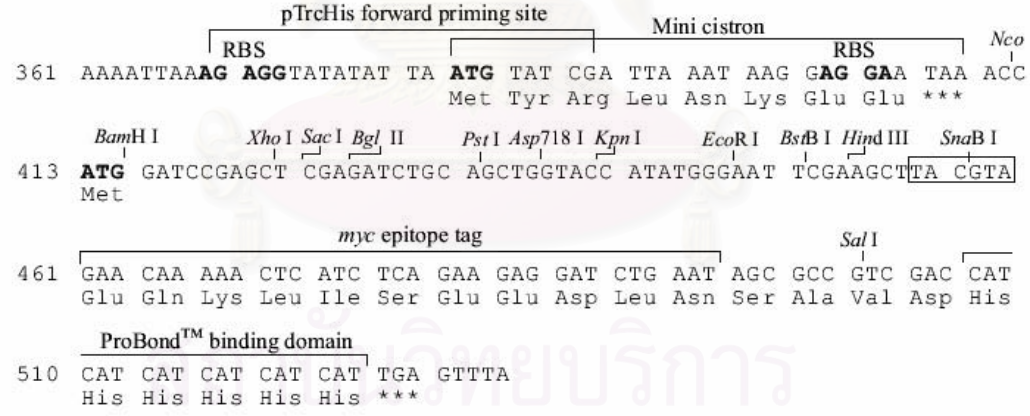
Figure 2.1 pGEM®-T easy vector map and multiple cloning site sequences

[a] Map of pGEM®-T easy vector

[b] Sequences in and around the pGEM®-T easy multiple cloning site



[a]



[b]

Figure 2.2 pTrcHis 2C vector map and multiple cloning site sequences

[a] Map of pTrcHis 2C expression vector

[b] Sequences in and around the pTrcHis 2C multiple cloning sites

2.3.4 Ligation

A suitable molecular ratio between vector and inserted DNA in the mixture of cohesive-end ligation is usually 1:3. The 15 μl of ligation reaction was composed of 1.5 μl of 10 x T_4 DNA ligase buffer, 1 μl of 10 mM ATP, 3 units of T_4 DNA ligase, 150 ng of amplified SPI gene and 50 ng of pTrcHis 2C which were previously cut with *Bam* HI and *Sal* I. Steriled distilled water was added to make the final volume of 15 μl. The mixture was mixed, briefly spun for 30 seconds and incubated at 16^{o}C for overnight. One microlitre of the ligation mixture was transformed into a competent *E. coli* strain JM109.

2.3.5 Competent cell preparation

Since the pTrcHis 2C expression vector should be propagated in recombinant deficient (*rec*A), endonuclease A-deficient (*end*A) *E. coli* strain, *E. coli* strain JM109 was used in this experiment

A single colony of *E. coli* strain JM 109 was cultured as the starter in 10 ml of LB broth (1 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, and 1 % (w/v) NaCl) and incubated at 37^{o}C with shaking at 250 rpm for overnight. Ten millilitres of the microbial starter was inoculated into 1,000 ml of LB broth and incubated at 37^{o}C with shaking at 250 rpm for 3-5 hours until the optical density at 600 nm (OD_{600}) of the cells reached 0.5-0.8. Cells were then chilled on ice for 15-30 minutes and harvested by centrifugation at 5,000 x g for 10 minutes at 4^{o}C.

2.3.6 Electrotransformation

The competent cells were gently thawed on ice. Fourty microlitres of the cell suspension were mixed with 1 μl of ligation mixture, mixed well and placed on ice for 1 minute. The mixture was transformed by

electroporation in an ice-cold 0.2 cm cuvette with setting the apparatus as follows; 25 μF of the Gene pulser, 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^{o}C with shaking at 250 rpm for 1 hour. The cell suspension was spread on the LB agar plates containing 50 μg/ml amplicillin and incubated at 37^{o}C for overnight. After incubation, colonies were randomly selected for plasmid DNA isolation.

2.3.7 Detection of the recombinant plasmid

The recombinant plasmid which contained SPI gene was detected with 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 (100 bp ladder, Biolabs). The clone containing DNA fragment in size of approximately 750 bp was selected. DNA sequencing was used to confirm the correction of the vector and inserted DNA junction.

2.4 Expression of recombinant serine proteinase inhibitor

The clone containing recombinant pTrcHis 2C was inoculated in 2 ml of LB medium including 50 μg/ml of amplicillin. The culture was grown overnight at 37^{o}C in a shaking incubator at 250 rpm. Two hundred microlitres of the culture were added to 10 ml of LB medium containing 50 μg/ml of amplicillin and incubated at 37^{o}C with vigorous shaking until OD_{600} reached 0.6. A 1 ml aliquot of culture cells was collected at this

time for the zero time point sample. The collected cells were centrifuged at 12,000xg for 30 seconds at 4°C. The supernatant was aspirated and the cells were stored at –20°C until used. One hundred millimolar of IPTG was then added to the culture to make final concentration to be 1 mM for expression induction. The culture was incubated at 37°C with vigorous shaking. Expression culture was collected for a volume of 1 ml aliquot every hour for 5 hours. The collected cells were centrifuged as above. *E. coli* strain JM 109 containing the parent pTrcHis 2C was used as control. It was grown and induced at the same conditions as above. Protein expression was analyzed by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

2.5 SDS-Polyacrylide Gel Electrophoresis (SDS-PAGE) analysis

After induction, each time point collected culture of both of parent pTrcHis 2C and SPI-pTrcHis 2C transformants were analyzed on 10 % SDS-PAGE (Sambrook et al., 1989). Each time point pellet was resuspended in 100 μl of 1x SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol, and 14.4 mM 2-mercaptoethanol), boiled for 5 minutes to denature proteins, and briefly centrifuged. Five microlitres of each sample were separated on 10% SDS-PAGE gels.

The 10% separating gel (for two 10 cm x 8 cm x 75 mm gels, need 10 ml) was prepared by mixing 4.17 ml of deionized water, 3.33 ml of 30% mixed acrylamide solution (29 % (w/v) of acrylamide and 1 % (w/v) of N,N', methylenebisacrylamide), 2.5 ml of separating gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4 % (w/v) of SDS), 60 μl of 10 % (w/v) ammonium persulfate, and 10 μl of TEMED. When the TEMED was added, the separating gel solution was rapidly swirled and poured into the gap

between the glass plates. Deionized water was carefully overlaid on the top of the separate gel solution. The gels were placed in a vertical position for 60 minutes at room temperature for complete polymerization. The overlaid water was poured off. The stacking gel was immediately prepared and poured onto the surface of polymerized separating gel.

The 5% stacking gel (for two 10 cm x 8 cm x 75 mm gels, need 5 ml) was prepared by mixing 3.1 ml of deionized water, 1.84 ml of 30 % mixed acrylamide solution (29 % (w/v) of acrylamide and 1% (w/v) of N,N', methylenebisacrylamide), 1 ml of stacking gel buffer (0.5 M Tris-HCl, pH 6.8 and 0.4 % (w/v) SDS), 30 μl of 10 % (w/v) ammonium persulfate, and 5 μl of TEMED. The mixture was rapidly swirled and poured directly onto the surface of polymerized separating gel. The comb was then carefully inserted into the stacking gel solution to avoid trapping air bubble. The gels were placed in a vertical position at room temperature for 30 minutes. The combs were carefully removed. Unpolymerized acrylamide was removed by washing the wells with deionized water. The gels were placed into the electrophoresis chamber. The top and bottom reservoirs were filled up with 1x Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8.3, and 0.1 % (w/v) SDS). The samples and prestained protein ladder (Fermentas) were loaded into the wells. The loaded gels were electrophoresed at 36 mA (18 mA/gel) until the bromophenol blue reached to the ends of the separating gels. The gels were stained with Coomassie gel staining solution (0.1 % (w/v) Coomassie brilliant blue R-250, 45 % (v/v) methanol, and 10 % (v/v) glacial acetic acid) at room temperature for at least 4 hours with gentle shaking. After staining, the gels were destained by drenching in gel destaining solution (10 % (v/v) methanol and 10 %

(v/v) glacial acetic acid) with gentle shaking. The destaining solution was changed three or four times for 24 hours.

2.6 Serine proteinase inhibitory activity

2.6.1 SPI activity gelatin/SDS-PAGE assay

Twenty microlitres of 3 hour-collected culture of both pTrcHis 2C and SPI-pTrcHis 2C *E. coli* lysate were electrophoresed on 10 % SDS-PAGE containing 0.1 % gelatin at 18 mA/gel. Two micrograms of Aprotinin, a serine proteinase inhibitor, were used as positive control. Five micrograms of BSA were used as negative control. After electrophoresis, the prestained protein ladder lane was cut out of the gel and soaked in destaining solution. The rest of the gel was incubated overnight in 2% triton x-100 in 0.1 M sodium phosphate, pH 7.8 at room temperature with gentle agitation. The gel was incubated in 37^{o}C-warmed 0.1 M sodium phosphate, pH 7.8 at 37^{o}C for 30 minutes. Fourty micrograms per millilitre of serine proteinase (trypsin, chymotrypsin, or subtilisin) were added into the buffer and incubated at 37^{o}C for 4 hours. The gel was rinsed with deionized water and stained by Coomassie gel staining solution for overnight. The gel was soaked in gel destaining solution for 24 hours with three or four changes of the destaining solution. The sizes of non-hydrolyzed gelatin band were estimated by comparison with prestained protein ladder.

2.6.2 Serine proteinase inhibitory spectrum assay

The inhibitory activity of recombinant SPI toward serine proteinases was tested as follows (modified from Hergenhahn et al, 1987). A 10 μl portion of the *E.coli* lysate was preincubated with appropriate amount of serine proteinase (0.08 μg for Trypsin, 0.05 μg for Chymotrypsin, 0.3 μg for Subtilisin, and 0.6 μg for Elastase) in 0.1 M

Tris/HCl, pH 8.0 at 30^{o}C for 10 minutes. Residual serine proteinase activity was determined by addition of chromogenic substrate and incubated at 30^{o}C for 15 minutes. The reaction was terminated by adding equal volume of 50 % (v/v) acetic acid. The absorbance was measured at 405 nm. In this experiment, the activities of trypsin and subtilisin (substrate N-benzoyl-PHE-VAL-ARG *p*-nitroanilide), chymotrypsin (substrate N-succinyl-ALA-ALA-PRO-PHE *p*-nitroanilide) and elastase (substrate N-succinyl-ALA-ALA-ALA *p*-nitroanilide) were assayed.

2.7 Protein measurement

Protein concentration was determined by the method of Bradford (1976). This method utilizes the binding of Coomassie Brilliant Blue G 250 dye to proteins. The dye has both blue and red form. When the dye binds to proteins, the red form is converted to the blue one and the maximum absorption of the dye shifts from 465 to 595 nm. This method is an accurate determination for the sample containing 2.5-20 μg protein. Protein solution (maximum 100 μl) was pipetted into tube. Distilled water was added to make a total volume of 100 μl. Then 1 ml of Bradford working buffer (5 % ethanol, 9 % phosphoric acid, and 35 mg Coomassie Brilliant Blue G 250) was added and mixed. After 2 minutes but before 1 hour, the absorbance at 595 nm was read. Bovine serum albumin (BSA) was used as standard protein. The protein concentration of the *E. coli* cell lysate was determined by comparison with the standard BSA.

2.8 SPI gene expression analysis using *in situ* hybridization

2.8.1 Preparation of *Vibrio harveyi* infected shrimp (modified from Roque et al., 1998)

A single colony of *Vibrio harveyi* 1526 (kindly provided by Charoenpokphand Group of companies) was inoculated in tryptic soy

broth (TSB) (DIFCO, Becton Dickinson France S.A.) supplemented with 1% (w/v) sodium chloride at 30^{o}C for 8 to 10 hours. The culture was then diluted 1:1000 with sterile normal saline solution (0.85 % (w/v) NaCl). The titer of the diluted culture was monitored by the plate count method in tryptic soy agar (TSA) supplemented with 2 % (w/v) NaCl (modified from Austin, 1988). One hundred microlitres of the 10^{6} CFU/ml diluted culture were injected into the fourth abdominal segment muscle. One the other hand, the normal shrimp was injected with 100 μl of 0.85 % (w/v) NaCl.

After injection, the haemolymph was collected at 0, 6, 24, 48, and 72 hours. The shrimp were tested to confirm the infection by streak the suspension of hepatopancreas on TSA plates supplemented with 2 % (w/v) NaCl and incubate at 30^{o}C for overnight. Colonies of *V. harveyi* 1526 from the infected shrimp showed luminescence in the dark.

2.8.2 Haemocyte preparation

The shrimp haemolymph was collected from the ventral sinus located at the base of the first abdominal segment, using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe containing 500 μl of anticoagulant (Modified Alsever Solution (MAS): 27 mM Sodium citrate, 336 mM NaCl, 115 mM glucose, and 9 mM EDTA, pH 7.0). The haemolymph was immediately centrifuged at 800xg at 4^{o}C for 10 minutes to separate the haemocytes from plasma. The supernatant was eliminated. The haemocytes were resuspended in freshly prepared ice-cold 0.1 M phosphate buffer, pH 7.4 containing 10% (w/v) sucrose, and 4% (w/v) paraformaldehyde. The resuspended haemocytes were incubated on ice for 10 minutes for fixation. After centrifugation as above, the haemocyte pellets were washed twice in Tris buffer saline solution (TBS) (0.1 M

Tris-HCl, pH 7.4, 0.9 % NaCl) to eliminate plasma proteins. The pellets were resuspended in TBS. The haemocyte concentration of the suspension was determined by using a haemocytometer. Using the cytospin, the $2x10^5$ haemocytes were centrifuged onto the poly-L-lysine coated slide at 1,000 x g for 5 minutes. The haemocytes were dried at room temperature for 2 to 3 minutes. The slide was stored at -20^{o}C until used.

2.8.3 Riboprobe preparation

The pBlueScriptSK plasmids containing SPI cDNA were linearized by restriction enzyme digestion. The plasmids were digested with *Kpn*I to produce antisense probe for SPI *in situ* hybridization. The plasmids were digested with *Xho*I to produce sense probe. One hundred microlitres of linearized plasmids were precipitated by adding 50 μl of 3 M sodium acetate, 250 μl of -20^{o}C-cold absolute ethanol and incubated at -80^{o}C for at least 1 hour. The mixture was centrifuged at 13,000xg for 20 minutes at 4^{o}C, air-dried the pellet, and suspended in 20 μl DEPC-treated water. The linearlized plasmids were quantified on agarose gel electrophoresis using λ/*Hin* dIII as standard DNA marker.

Digoxigenin (DIG)-labeled probes were prepared by *in vitro* transcription using T7 polymerase for sense probe and T3 polymerase for antisense probe. The reaction composed of 1 μg of purified, linearized plasmid DNA, 2 μl of 10x concentrated DIG RNA labeling mix (10 mM each of ATP, CTP, and GTP, 6.5 mM UTP, 3.5 mM DIG-UTP; pH 7.5), 2 μl of 10 x concentrated transcription buffer (400 mM Tris-HCl, pH 8.0, 60 mM $MgCl_2$, 100 mM Dithiothreitol (DTT), and 20 mM spermidine), 40 units of T7 or T3 RNA polymerase. The DEPC-treated water was added to make a total reaction volume of 20 μl. The components were mixed and centrifuged briefly. The reaction was incubated at 37^{o}C for 2

hours. Two units of DNase I, RNase-free were added to the tube and incubated at 37^{o}C for 15 minutes. One microlitre of 0.5 M EDTA, pH 8.0 was added to the tube to stop the polymerase reaction. The labeled RNA transcript was precipitated by adding 10 μl of 4 M LiCl, 5 μl of 10 mg/ml tRNA, and 300 μl –20^{o}C- prechilled absolute ethanol and mixed well. The reaction was incubated at –80^{o}C for at least 1 hour. The reaction was centrifuged at 13,000 x g for 20 minutes at 4^{o}C. The supernatant was discarded. The pellet was washed with 50 μl ice-cold 70 % (v/v) ethanol. The reaction was centrifuged at 13,000 x g for 15 minutes at 4^{o}C. The supernatant was discarded. The pellet was dried for 15 minutes on ice and dissolved in 100 μl DEPC-treated water at 37^{o}C for 30 minutes.

2.8.4 Prehybridization treatments

The haemocyte coated slides were incubated in 0.2 M Tris-HCl, pH 7.4 twice for 5 minutes each and in 0.1 M glycine in 0.2 M Tris-HCl, pH 7.4 for 10 minutes. The cells were rinsed with PBS (0.1 M phosphate buffer, pH 7.4 containing 0.9 % NaCl) for 5 minutes. The cells were incubated in 4 % paraformaldehyde in PB containing 5 mM $MgCl_2$ for 15 minutes and rinsed with PBS for 5 minutes. The cells were incubated in 0.1 M triethanolamine, pH 8.0 containing 0.25 % anhydre acetic for 10 minutes. The cells were dehydrated in 30 %, 70 %, and 100 % graded ethanol for 5 minutes each. The cells were dried at room temperature for at least 2 hours.

2.8.5 Riboprobe hybridization

DIG-labeled riboprobes (40-100 ng per slide) were diluted in 2 x SSC containing 50 % formamide, 10 % dextran sulfate, 10x denhart's solution, 0.5 mg/ml tRNA from *E. coli*, 100 mM dithiothreitol, and 0.5 mg/ml salmon sperm DNA. The diluted DIG-labeled riboprobes was

denatured at 55^{o}C for 10 minutes and placed on ice for 5 minutes. The riboprobes were dropped onto the slide. The hybridization solution covered cells was covered by a glass slip. The slides were incubated overnight at 55^{o}C in adequate humid chamber (1 x SSC and 30 % formamide). The cells were washed twice for 15 minutes with 2 x SSC with shaking in a plat form shaker. The cells were treated with 20 μg/ml RNase A in 2 x SSC at 37^{o}C for 30 minutes and consecutively rinsed twice for 10 minutes with 1 x SSC supplemented with 0.07 % 2-mercaptoethanol at room temperature, 0.5 x SSC supplemented with 0.07 % 2-mercaptoethanol for 10 minutes at room temperature, 0.1 x SSC supplemented with 0.07 % 2-mercaptoethanol for 10 minutes at room temperature and twice for 30 minutes with 55^{o}C-0.1 x SSC supplemented with 0.07 % 2-mercaptoethanol at 55^{o}C in the water bath. The last solution with the slides was leave on ice for 1 hour.

2.8.6 Riboprobe detection

The slides were washed in 0.1 x SSC for 5 minutes and rinsed twice in TBS (0.1 M Tris-HCl, 0.9 % NaCl, pH 7.4) containing 0.05 % of triton x-100 for 5 minutes. The slides were pre-incubated in TBS supplemented with 1 % of normal sheep serum and 0.05 % of triton x-100 for 20 minutes. Five hundred microlitres of antibody solution (1/1000 diluted alkaline phosphatase-conjugated sheep anti-DIG antibody in TBS containing 1 % of normal sheep serum and 0.05 % of triton x-100) were added onto each slide and incubated overnight at room temperature in a humid chamber. After incubation, the slides were washed 3 times in TBS for 10 minutes each and 2 times in 0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, and 0.9 % NaCl for 5 minutes each. Five hundred microlitres of detection solution (375 μg/ml of NBT (Nitroblue tetrazolium diluted in Dimethyl formamide), 188 μg/ml of BCIP (5-bromo-4-chloro-indolyl

phosphate), and 1 mM of levamisole prepared in 0.1 M Tris-HCl, pH 9.5, 0.9 % NaCl, and 50 mM $MgCl_2$) were added onto the slides and incubated in the dark at room temperature. The reaction should be monitored until optimal development. For SPI detection, the optimal development was observed within 2 hours. The slides were washed by TBS for 10 minutes for stop the reaction and rinsed briefly with distilled water. The slides were mounted with a mixture of (1:9) TBS:glycerol and stored at 4^oC.

2.8.7 Controls

Sense riboprobe and RNase-treatment antisense riboprobe were used as negative controls. For RNase-treatment antisense riboprobe, 10 μg/ml of RNase A was added onto the slide and incubated at 37^oC for 30 minutes. The slide was then washed 3 times with TBS for 5 minutes each before proceeding to the hybridization step.

CHAPTER III

RESULTS

3.1 EST homologous of serine proteinase inhibitors in *P. monodon*

EST analysis of the haemocyte cDNA libraries of *P. monodon* (Supangul et al., 2002) identified 6 putative serine proteinase inhibitors by the BLASTX program. They showed 50 to 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 nucleotide sequences of these clones were confirmed by repeating the sequencing. Because in the EST analysis, all cDNA clones were single passed sequenced at the 5' direction, errors on the sequences may occur. From the sequence data, the nucleotide sequences of each clone were compared with the sequences obtained from ESTs. The differences of nucleotide sequence were observed at the end of sequences. All amino acid sequences were aligned with the Kazal domains of crayfish (Figure 3.1). Amino acid sequence alignment of these clones show conserved residues which are homologous to the serine proteinase inhibitor from crayfish. Corresponding Kazal domains of putative *P. monodon* serine proteinase inhibitors can be demonstrated by aligning to those from crayfish (Johansson et al., 1994). Each clone differs from the others in the number of the Kazal domain (Figure 3.2). Three of four clones—sh415, sh1064, and sh1069—are highly similar to each other. Whereas clone sh610 is different from the three clones. As shown in Figure 3.2, the reactive P1 residue of Kazal domains has high variation. This reactive P1 site is

importance for proteinase inhibitory specificity. P_1 residues of Kazal domains of *P. monodon* serine proteinase inhibitors consisted of threonine, alanine, glutamate, lysine, and leucine. The three similar clones, sh415, sh1064, and sh1069, consist of threonine, alanine, glutamate, and lysine at P_1 sites. The sh610 clone consists of glutamate, threonine, and leucine at P_1 sites. Each complete Kazal domain contains 6 cysteine residues. The deduced amino acids of the two clones, sh415 and sh610, show complete 5 and 4 Kazal domains, respectively (Figure 3.2). In other clones; sh1069 and sh1064 contain 3 complete and 2 incomplete Kazal domains. The fourth Kazal domain of clone sh1064 lacks cysteine at the fifth and the sixth positions. Only one clone, sh1064, had more Kazal domains than those obtained from the EST sequence, which had only 2 Kazal domains.

In this study, the complete five-Kazal domain clone, sh415, was selected for cloning and expression. 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 (Figures 3.3, 3.4). A signal sequence of 18 amino acids was predicted using SignalP VI.1 software (Nielsen et al., 1997). The sh610 cDNA clone consists of an open reading frame of 669 base pairs coding for a protein of 222 amino acids with 17-amino acid signal sequence. The sh1069 cDNA clone consists of an open reading frame of 669 base pairs coding for a protein of 222 amino acids with 18-amino acid signal sequence. The last full-length cDNA clone of serine proteinase inhibitor, sh1064, consists of an open reading frame of 861 base pairs coding for a protein of 246 amino acids with 18-amino acid signal sequence (Figures 3.5, 3.6, and 3.7).

```
Sh415        MANK---VALLTLLAVAVAVSGYGKGGKIRLCAKHCTTISPVCGSDGKTYDSRCHLENAA
Sh1069       MANK---VALLTLLAVAVAVSGYGKGGKIRLCAKHCTTISPVCGSDGKTYDS--------
Sh1064       MANK---VALLTLLAVAVAVSGYGKGGKIRLCAKHCTTISPVCGSDGKTYDSRCHLENAA
Crayfish     MM-----LSLLTWITTLLVVVASTAARCPSICP---LNYKPVCGSDLKTYGNSCQLNAAI
Sh610        MLLCKITLIHLLLQGFAVFNDANSDHDCIGYCP---EVYDPVCASNGWTYNNDCELQAMI
             *      :  *      :   .         *.      .***.*:  **..


Sh415        CGGVSVTFHHAGPCP--PPKRCPGICPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSK
Sh1069       --------------------RCPGLCPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSK
Sh1064       CGGVSVTFHHAGPCP--PPKRCPGLCPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSK
Crayfish     CRNPSLKKLYDGPCIDKPQPQCPSICPLDYNPVCGTDGKTYSNLCALR-IEACNNPHLNL
Sh610        KCQGWNITKTHDQAC-----ECLKACPTTFAPVCGSDNKTYLNECVFE-VASCWDHSLDK
                                .*   **  : ****::.*** * * :.   :* .  :.


Sh415        KHDGRCGCNP----IVACPEIYAPVCGSDGKTYDNDCYFQAAVCKNPDLKKVRDGNCDCT
Sh1069       KHDGRCGCNP----NVACPEIYAPVCGSDGKTYDNDCYFQAAVCKNPDLKKVRDGNCDCT
Sh1064       KHDGRCGCNP----NVACPEIYAPVCGSDGKTYDNDCYFQAAVCKNPDLKKVRDGNCDCT
Crayfish     RVDYQGECRPKNQCRNGCTLQYDPKCGTDGKTYSNLCDLEVAACNNPQLNLKVAYKGECK
Sh610        ASEGACGWGIH--CLQYCPEVYDPVCGSNGQTYTNECELQAAIQCRG-LQIAKRHDQACE
               :              *.  * * **::*:** * * ::.*   .  *:     .  *


Sh415        PLIGCP----KNYRPVCGSDGVTYNNDCFFKVAQCKN-PALVKVSDTRCECNHVCTEEYY
Sh1069       PLIGCP----KNYRPVCGSDGVTYNNDCFFKVAQCKN-PALVKVSDTRCECNHVCTEEYY
Sh1064       PLIGCP----KNYRPVCGSDGVTYNNDCFFKVASVQEPPRSSKSLILAVNCNHVCTERIT
Crayfish     QQNQCPTICTQQYDPVCGTDGKTYGNSCELGVAACNN-PQLNLKIAYKGACNFPQQQT--
Sh610        CHATCP----LIHDPVCGTDDRTYYNECFFTKASCWDRSILKKKNGPCDRKLEIPSGD--
                 **      : ****:*. ** *.* :  *   : .


Sh415        PVCGSNGVTYSNICLLNNAACLDSSIYKVSDGICGRRLYL
Sh1069       PVVRKQWCHGIRTFVC------------------------
Sh1064       TPCAGKPWVVTYSNICLLE--------------------
Sh610        ----------------------------------------
             ----------------------------------------
```

Figure 3.1 Amino acid sequence alignment of the serine proteinase inhibitors from *Penaeus monodon* (clones sh415, sh1069, sh1064, and sh610) and crayfish, *Pacifastacus leniusculus.* The asterisks indicate the residues are identical. Dots indicate that one of the following weaker groups (.) and stronger groups (:) is fully conserved. Cysteine residues are in bold. The sequences were aligned using Clustal X.

CRAYFISH

```
AAR
       *
CPSICPLNYKPVCGSDLKTYGNSCQLNAAICRNPSLKKLYDGP--CIDKP
CPSICPLDYNPVCGTDGKTYSNLCALRIEACNNPHLNLRVDYQGECRP
CRNGCTLQYDPKCGTDGKTYSNLCDLEVAACNNPQLNLKVAYKGECKQ
CPTICTQQYDPVCGTDGKTYGNSCELGVAACNNPQLNNKIAYKGACNFPQQQT
```

SH415

```
MANKVALLTLLAVAVAVSGYGKGGKIRL
        *
CAKH--CTTIS-PVCGSDGKTYDSRCHLENAA-CGGVSVTFHHAGPCPPPKR
CPGI--CPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSKKHDGRCG
CNPIVACPEIYAPVCGSDGKTYDNDCYFQAAV-CKNPDLKKVRDGNCD
CTPLIGCPKNYRPVCGSDGVTYNNDCFFKVAQ-CKNPALVKVSDTRCE
CNHV--CTEEYYPVCGSNGVTYSNICLLNNAA-CLDSSIYKVSDGICGRRLYLZ
```

SH610

```
MLLCKITLIHLLLQGFAVFNDANSDHD
      *
CIGYCPEVYDPVCASNGWTYNNDCELQAMIKCQGWNITKTHDQACE
CLKACPTTFAPVCGSDNKTYLNECVFEVAS-CWDHSLDKASEGACGWGIH
CLQYCPEVYDPVCGSNGQTYTNECELQAAIQCRGLQIAKRHDQACE
CHATCPLIHDPVCGTDDRTYYNECFFTKAS-CWDRSILKKKNGPCDRKLEIPSGD
```

SH1069

```
MANKVALLTLLAVAVAVSGYGKGGKIRL
        *
CAKH--CTTIS-PVCGSDGKTYDSR
CPGL--CPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSKKHDGRCG
CNPNVACPEIYAPVCGSDGKTYDNDCYFQAAV-CKNPDLKKVRDGNCD
CTPLIGCPKNYRPVCGSDGVTYNNDCFFKVAQ-CKNPALVKVSDTRCE
CNHV--CTEEYYPVVRKQWCHGIRTFVC
```

SH1064

```
MANKVALLTLLAVAVAVSGYGKGGKIRL
        *
CAKH--CTTIS-PVCGSDGKTYDSRCHLENAA-CGGVSVTFHHAGPCPPPKR
CPGL--CPAVYAPVCGTNGKTYSNLCQLENDRTCNGAFVSKKHDGRCG
CNPNVACPEIYAPVCGSDGKTYDNDCYFQAAV-CKNPDLKKVRDGNCD
CTPLIGCPKNYRPVCGSDGVTYNNDCFFKVASVQEPPRSSKSLILAVN
CNHV--CTERITTPCAGKPWVVTYSNICLLE
```

Figure 3.2 Alignment of the Kazal domains of the crayfish and *P.monodon* serine proteinase inhibitors. Highly conserved amino acid residues in the Kazal domains are in bold. Asterisks are P1 residues.

CGCGGCCGCTCAAGAGAGGAGTCGTCACAGATATACCAGAATCCGGAGTCAATACTTGTGATCTGAAAA
CCAAACGAAG**ATG**GCCAACAAAGTGGCACTCTTGACCCTTCTTGCAGTGGCCGTTGCAGTCTCTGGCTA
CGGAAAAGGGGGGAAAATCCGCCTCTGCGCCAAACACTGTACGACCATCTCCCCTGTGTGTGGCTCTGA
TGGAAAAACTTATGACAGCCGATGCCACCTGGAGAATGCTGCCTGTGGTGGCGTGAGTGTCACTTTCCA
CCATGCCGGACCCTGCCCTCCCCCAAAGAGATGTCCAGGAATATGCCCCGCGGTATATGCCCCTGTGTG
CGGGACCAACGGGAAAACTTACTCGAACTTATGCCAACTTGAGAATGACAGAACCTGCAACGGTGCTTT
CGTTTCCAAGAAGCACGATGGACGTTGTGGTTGCAACCCCATTGTCGCGTGCCCTGAGATCTATGCTCC
CGTGTGTGGCAGTGATGGCAAGACTTATGATAACGACTGCTATTTCCAGGCAGCTGTTTGCAAGAATCC
AGATCTTAAGAAGGTTCGAGACGGTAACTGCGACTGCACTCCTCTCATCGGCTGTCCCAAGAACTACAG
GCCTGTGTGTGGCAGCGACGGTGTAACTTACAACAACGACTGCTTCTTCAAGGTTGCTCAGTGCAAGAA
CCCCGCGCTCGTCAAAGTCTCTGATACTCGCTGTGAATGCAACCACGTCTGTACTGAAGAATATTACCC
CGTGTGCGGAAGCAATGGTGTCACGTATTCGAACATTTGTCTGTTGAATAATGCAGCGTGTTTAGATTC
CTCCATTTACAAGGTTTCGGACGGAATCTGTGGTCGCAGACTGTACCTA**TAA**GAAGGGATATTAAAGAG
AGATGTGGACTTGCAGTATTTTTCTTTTTCTCTCTTTTATCAAAATTTACTCTTTCGTATGATGTGATC
GCTTTAATCAACAAAGGAAATTAACTTGTAATTCTTATTTCGTATATAACACCATACGATGAATTTAAC
CAGTGATTGAATTTAACTGTAACTCTTATTTCGTATATGCATACCATATGATAAGATTTAATCAACAAA
TGACATTAATTGTAGCTTATTTTGTTTGATAAATATACCATATGATGAGAAAAAAAAAATTAGCTCATT
GGATTTTGTTAATACTACTGAAGACCCTATTTTGACAAAATCTTTGAATGGAAGTAAAATGATAATAAG
AAATAATAGATCAGATTATAACAGAAACAGGAGAAAATTTTAGAACTTA**AATAAA**GGGCAATATATTAT
CCGATGAACGATTATGACAATTTTCTGCATCTGTTTGTATATTAAATTGTAATGTCATGCTTTCTAACG
CTTTTTCTTTATCATGGTATCAGTAAAACAATGATTTATAATGCTGTTATATTTGTTATTTATATATCA
TTATAACGATATATTCCAAAGCACTACAGTCATTTTCAATGTTGATCAATTTTTTTTCTTTCTTTGATG
TCACTTCACTGAATATATGGATAATGCAAAATCAAAAAAAAAAAAAAAAGCGGCCGCGAATT

Figure 3.3 Nucleotide sequence of the cDNA clone, sh415. The clone contains a 1,579 bp insert fragment with an open reading frame (ORF) of 801 bp encoding 266 amino acids with the putative initiation methionine codon (ATG) beginning at nucleotide 79 and the stop codon (TAA) beginning at nucleotide 877. The start and stop codon are in bold. The ORF is underlined. The putative poly A addition site (AATAAA) is highlighted in black.

SH415

```
1      ATGGCCAACAAAGTGGCACTCTTGACCCTTCTTGCAGTGGCCGTTGCAGTCTCTGGCTAC
        M  A  N  K  V  A  L  L  T  L  L  A  V  A  V  A  V  S  G  Y
61     GGAAAAGGGGGGAAAATCCGCCTCTGCGCCAAACACTGTACGACCATCTCCCCTGTGTGT
        G  K  G  G  K  I  R  L  C  A  K  H  C  T  T  I  S  P  V  C
121    GGCTCTGATGGAAAAACTTATGACAGCCGATGCCACCTGGAGAATGCTGCCTGTGGTGGC
        G  S  D  G  K  T  Y  D  S  R  C  H  L  E  N  A  A  C  G  G
181    GTGAGTGTCACTTTCCACCATGCCGGACCCTGCCCTCCCCCAAAGAGATGTCCAGGAATA
        V  S  V  T  F  H  H  A  G  P  C  P  P  P  K  R  C  P  G  I
241    TGCCCCGCGGTATATGCCCCTGTGTGCGGGACCAACGGGAAAACTTACTCGAACTTATGC
        C  P  A  V  Y  A  P  V  C  G  T  N  G  K  T  Y  S  N  L  C
301    CAACTTGAGAATGACAGAACCTGCAACGGTGCTTTCGTTTCCAAGAAGCACGATGGACGT
        Q  L  E  N  D  R  T  C  N  G  A  F  V  S  K  K  H  D  G  R
361    TGTGGTTGCAACCCCATTGTCGCGTGCCCTGAGATCTATGCTCCCGTGTGTGGCAGTGAT
        C  G  C  N  P  I  V  A  C  P  E  I  Y  A  P  V  C  G  S  D
421    GGCAAGACTTATGATAACGACTGCTATTTCCAGGCAGCTGTTTGCAAGAATCCAGATCTT
        G  K  T  Y  D  N  D  C  Y  F  Q  A  A  V  C  K  N  P  D  L
481    AAGAAGGTTCGAGACGGTAACTGCGACTGCACTCCTCTCATCGGCTGTCCCAAGAACTAC
        K  K  V  R  D  G  N  C  D  C  T  P  L  I  G  C  P  K  N  Y
541    AGGCCTGTGTGTGGCAGCGACGGTGTAACTTACAACAACGACTGCTTCTTCAAGGTTGCT
        R  P  V  C  G  S  D  G  V  T  Y  N  N  D  C  F  F  K  V  A
601    CAGTGCAAGAACCCCGCGCTCGTCAAAGTCTCTGATACTCGCTGTGAATGCAACCACGTC
        Q  C  K  N  P  A  L  V  K  V  S  D  T  R  C  E  C  N  H  V
661    TGTACTGAAGAATATTACCCCGTGTGCGGAAGCAATGGTGTCACGTATTCGAACATTTGT
        C  T  E  E  Y  Y  P  V  C  G  S  N  G  V  T  Y  S  N  I  C
721    CTGTTGAATAATGCAGCGTGTTTAGATTCCTCCATTTACAAGGTTTCGGACGGAATCTGT
        L  L  N  N  A  A  C  L  D  S  S  I  Y  K  V  S  D  G  I  C
781    GGTCGCAGACTGTACCTATAA
        G  R  R  L  Y  L  *
```

Figure 3.4 Nucleotide and amino acid sequences of the ORF of cDNA clone, sh415, encoding a serine proteinase inhibitor from the black tiger shrimp *P. monodon*. Amino acids are shown as single letter abbreviation. Start and stop codons are in bold. The putative signal peptide is underlined.

SH610

```
1     ATGTTGTTGTGCAAGATTACTCTTATCCATCTCCTGTTGCAAGGATTTGCTGTCTTTAAT
       M  L  L  C  K  I  T  L  I  H  L  L  L  Q  G  F  A  V  F  N

61    GACGCCAACTCCGATCATGATTGTATCGGCTACTGTCCTGAAGTGTATGATCCTGTGTGT
       D  A  N  S  D  H  D  C  I  G  Y  C  P  E  V  Y  D  P  V  C

121   GCCAGTAACGGCTGGACTTACAACAACGACTGCGAACTACAGGCTATGATAAAGTGCCAG
          A  S  N  G  W  T  Y  N  N  D  C  E  L  Q  A  M  I  K  C  Q

181   GGATGGAATATCACCAAGACACACGACCAAGCATGTGAATGCCTCAAGGCCTGCCCCACG
       G  W  N  I  T  K  T  H  D  Q  A  C  E  C  L  K  A  C  P  T

241   ACCTTTGCCCCTGTGTGTGGGTCAGACAACAAGACCTATCTCAACGAGTGTGTCTTCGAG
       T  F  A  P  V  C  G  S  D  N  K  T  Y  L  N  E  C  V  F  E

301   GTGGCTTCTTGCTGGGATCATTCGCTCGACAAGGCGTCTGAAGGAGCTTGTGGCTGGGGT
       V  A  S  C  W  D  H  S  L  D  K  A  S  E  G  A  C  G  W  G

361   ATCCATTGCCTGCAGTACTGCCCTGAGGTATACGACCCTGTGTGTGGAAGCAACGGCCAG
       I  H  C  L  Q  Y  C  P  E  V  Y  D  P  V  C  G  S  N  G  Q

421   ACTTACACGAACGAATGCGAGTTGCAGGCTGCCATACAGTGTCGTGGGTTGCAGATTGCA
       T  Y  T  N  E  C  E  L  Q  A  A  I  Q  C  R  G  L  Q  I  A

481   AAGAGGCACGACCAGGCTTGTGAGTGCCACGCCACTTGCCCCCTGATCCACGACCCTGTT
       K  R  H  D  Q  A  C  E  C  H  A  T  C  P  L  I  H  D  P  V

541   TGTGGCACTGACGATAGGACTTACTACAACGAGTGCTTCTTCACTAAAGCTTCTTGTTGG
       C  G  T  D  D  R  T  Y  Y  N  E  C  F  F  T  K  A  S  C  W

601   GATAGGTCCATTTTGAAGAAGAAAAACGGACCTTGTGACAGGAAACTGGAAATACCTTCT
       D  R  S  I  L  K  K  K  N  G  P  C  D  R  K  L  E  I  P  S

661   GGAGATTAA
       G  D  *
```

Figure 3.5 Nucleotide and amino acid sequences of the ORF of cDNA clone, sh610, encoding a serine proteinase inhibitor from the black tiger shrimp *P. monodon.* Amino acids are shown as single letter abbreviation. Start and stop codons are in bold. The putative signal peptide is underlined.

SH1064

```
1     ATGGCCAACAAAGTGGCACTCTTGACCCTTCTTGCAGTGGCCGTTGCAGTCTCTGGCTAC
       M  A  N  K  V  A  L  L  T  L  L  A  V  A  V  A  V  S  G  Y

61    GGAAAGGGGGGGAAAATCCGCCTCTGCGCCAAACACTGTACGACCATCTCCCCTGTGTGT
       G  K  G  G  K  I  R  L  C  A  K  H  C  T  T  I  S  P  V  C

121   GGCTCTGATGGAAAAACGTATGACAGCCGATGCCACCTGGAGAATGCTGCCTGTGGTGGC
       G  S  D  G  K  T  Y  D  S  R  C  H  L  E  N  A  A  C  G  G

181   GTGAGTGTCACTTTCCACCATGCCGGACCCTGCCCTCCCCCAAAGAGATGCCCAGGATTA
       V  S  V  T  F  H  H  A  G  P  C  P  P  P  K  R  C  P  G  L

241   TGCCCCGCAGTATATGCCCCTGTGTGCGGGACCAACGGGAAAACTTACTCGAACTTATGC
       C  P  A  V  Y  A  P  V  C  G  T  N  G  K  T  Y  S  N  L  C

301   CAACTTGAGAATGACAGAACCTGCAACGGTGCTTTCGTTTCCAAGAAGCACGATGGACGT
       Q  L  E  N  D  R  T  C  N  G  A  F  V  S  K  K  H  D  G  R

361   TGTGGTTGCAACCCCAATGTTGCGTGCCCTGAGATCTATGCTCCCGTGTGTGGCAGTGAT
       C  G  C  N  P  N  V  A  C  P  E  I  Y  A  P  V  C  G  S  D

421   GGCAAGACTTATGATAACGACTGCTATTTCCAGGCAGCTGTTTGCAAGAATCCAGATCTT
       G  K  T  Y  D  N  D  C  Y  F  Q  A  A  V  C  K  N  P  D  L

481   AAGAAGGTTCGAGACGGTAACTGCGACTGCACTCCTCTCATCGGCTGTCCCAAGAACTAC
       K  K  V  R  D  G  N  C  D  C  T  P  L  I  G  C  P  K  N  Y

541   AGGCCTGTGTGTGGCAGCGACGGTGTAACTTACAACAACGACTGCTTCTTCAAGGTTGCC
       R  P  V  C  G  S  D  G  V  T  Y  N  N  D  C  F  F  K  V  A

601   TCAGTGCAAGAACCCCCGCGCTCGTCAAAGTCTCTGATACTCGCTGTGAATTGCAACCAC
       S  V  Q  E  P  P  R  S  S  K  S  L  I  L  A  V  N  C  N  H

661   GTCTGTACTGAAAGAATAACTACCCCGTGTGCCGGAAAGCCATGGGTTGTCACGTATTCG
       V  C  T  E  R  I  T  T  P  C  A  G  K  P  W  V  V  T  Y  S

721   AACATTTGTCTGTTGGAATAA
       N  I  C  L  L  E  *
```

Figure 3.6 Nucleotide and amino acid sequences of the ORF of cDNA clone, sh1064, encoding a serine proteinase inhibitor from the black tiger shrimp *P. monodon*. Amino acids are shown as single letter abbreviation. Start and stop codons are in bold. The putative signal peptide is underlined.

SH1069

```
1     ATGGCCAACAAAGTGGCACTCTTGACCCTTCTTGCAGTGGCCGTTGCAGTCTCTGGCTAC
       M  A  N  K  V  A  L  L  T  L  L  A  V  A  V  A  V  S  G  Y

61    GGAAAGGGGGGGAAAATCCGACTCTGCGCCAAACACTGTACGACCATCTCCCCTGTGTGT
       G  K  G  G  K  I  R  L  C  A  K  H  C  T  T  I  S  P  V  C

121   GGCTCTGATGGAAAAACGTATGACAGCCGATGCCCAGGATTATGCCCCGCGGTATATGCC
       G  S  D  G  K  T  Y  D  S  R  C  P  G  L  C  P  A  V  Y  A

181   CCTGTGTGCGGGACCAACGGGAAAACTTACTCGAACTTATGCCAACTTGAGAATGACAGA
       P  V  C  G  T  N  G  K  T  Y  S  N  L  C  Q  L  E  N  D  R

241   ACCTGCAACGGTGCTTTCGTTTCCAAGAAGCACGATGGACGTTGTGGTTGCAACCCCAAT
       T  C  N  G  A  F  V  S  K  K  H  D  G  R  C  G  C  N  P  N

301   GTTGCGTGCCCTGAGATCTATGCTCCCGTGTGTGGCAGTGATGGCAAGACTTATGATAAC
       V  A  C  P  E  I  Y  A  P  V  C  G  S  D  G  K  T  Y  D  N

361   GACTGCTATTTCCAGGCAGCTGTTTGCAAGAATCCAGATCTTAAGAAGGTTCGAGACGGT
       D  C  Y  F  Q  A  A  V  C  K  N  P  D  L  K  K  V  R  D  G

421   AACTGCGACTGCACTCCTCTCATCGGCTGTCCCAAGAACTACAGGCCTGTGTGTGGCAGC
       N  C  D  C  T  P  L  I  G  C  P  K  N  Y  R  P  V  C  G  S

481   GACGGTGTAACTTACAACAACGACTGCTTCTTCAAGGTTGCTCAGTGCAAGAACCCCGCG
       D  G  V  T  Y  N  N  D  C  F  F  K  V  A  Q  C  K  N  P  A

541   CTCGTCAAAGTCTCTGATACTCGCTGTGAATGCAACCACGTCTGTACTGAAGAATATTAC
       L  V  K  V  S  D  T  R  C  E  C  N  H  V  C  T  E  E  Y  Y

601   CCCGTTGTGCGGAAGCAATGGTGTCACGGTATTCGAACATTTGTCTGTTGA
       P  V  V  R  K  Q  W  C  H  G  I  R  T  F  V  C  *
```

Figure 3.7 Nucleotide and amino acid sequences of the ORF of cDNA clone, sh1069, encoding a serine proteinase inhibitor from the black tiger shrimp *P. monodon*. Amino acids are shown as single letter abbreviation. Start and stop codons are in bold. The putative signal peptide is underlined.

3.2 Recombinant expression of Serine proteinase inhibitor (SPI) in *E. coli* expression system

3.2.1 Construction of recombinant pTrcHis 2C

3.2.1.1 Preparation of SPI gene

The 5'-terminal truncated SPI gene was constructed by PCR amplification using psh415 as a template and oligonucleotide primers incorperating 5' *Bam* HI (5'-CCATGGATCCGGGCTACGGAAAAGGG GGGAAAATCC-3') and 3' *Sal* I (5'-ATGGTCGACTAGGTAC AGTCTGCGACCACAGATTCC-3') cleavage sites. These allowed directional cloning into the expression vector. The resulting PCR products were run on a 1.2 % agarose gel and a specific 750 bp fragment of expected size was observed (Figure 3.8).

3.2.1.2 Cloning of serine proteinase inhibitor gene into pGEM®-T easy vector

PCR products were purified and ligated into pGEM®-T easy vector. After ligation, the reaction mixture was transformed to *E. coli* strain JM109. The recombinant clone was first selected with LB agar plate containing amplicillin, X-Gal, and IPTG. White colonies were randomly selected to culture in LB medium containing 50 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 3.9). The clone containing the inserted DNA in size of approximately 750 base pairs was selected for recombinant pGEM®-T/SPI augmentation. After digestion and electrophoresis, the 750-bp inserted DNA was purified from the agarose gel for further ligation to pTrcHis 2C.

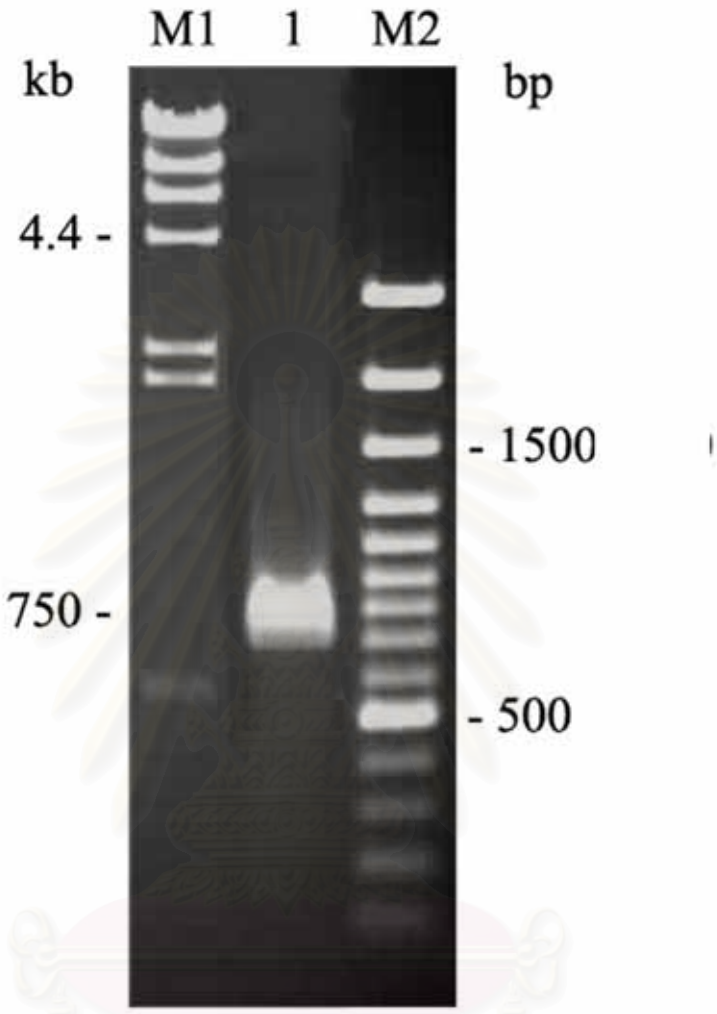


Figure 3.8 Ethidium bromide staining of NH_2-terminal truncated serine proteinase inhibitor gene amplified by PCR. The PCR products were run on a 1.2 % agarose gel at 100 volts for 1 hour.

Lane M1: DNA marker (λDNA/*Hin* dIII)

Lane M2: Standard DNA ladder (100 bp marker)

Lane 1: amplified 5'-terminal truncated serine proteinase inhibitor gene products

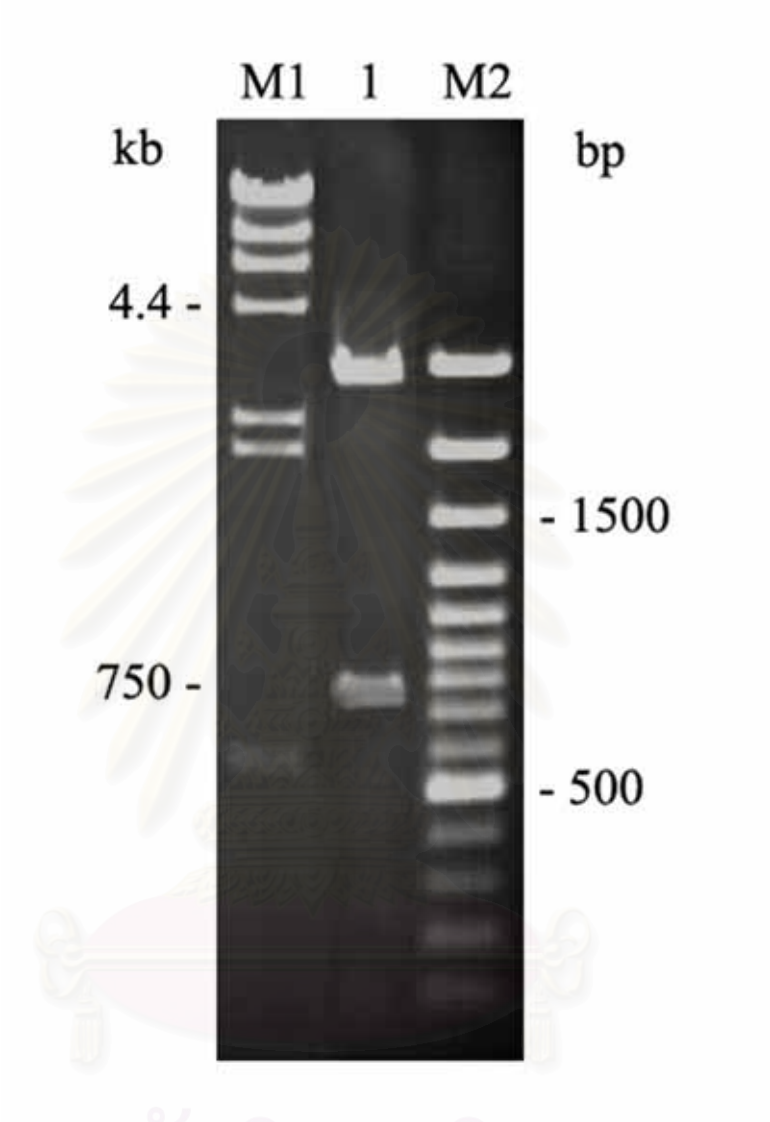


Figure 3.9 Ethidium bromide staining of the recombinant pGEM®-T/SPI digested with restriction enzyme *Bam* HI and *Sal* I. The DNA was run on 1.2 % agarose gel at 100 volts for 1 hour.

Lane M: DNA marker (λDNA/*Hin* dIII)

Lane m: Standard DNA ladder (100 bp marker)

Lane 1: digested pGEM-T/SPI

3.2.1.3 Subcloning of serine proteinase inhibitor gene into pTrcHis 2C

Bam HI and *Sal* I digested pTrcHis 2C vectors were purified, ligated with the eluted 5'-terminal truncated serine proteinase inhibitor gene fragment, and transformed into *E. coli* strain JM109. The recombinant clones were first selected with LB/amplicillin agar plates and further selected according to the presence of the inserted fragment using restriction enzyme digestion (Figure 3.10). A positive clone was selected and its recombinant plasmid was sequenced to confirm the correction of the junction between vector and inserted DNA. The sequences are shown in Figure 3.11. The recombinant proteins obtained from pTrcHis 2C/SPI contained 3 extra amino acids; methionine, aspartic acid, and proline, at N-terminus. The serine proteinase inhibitor portion starts at the fourth residue (glycine). At C-terminus, valine, aspartic acid, and six histidines were obtained from the pTrcHis 2C vector as extra amino acids. This clone was used for serine proteinase inhibitor expression.

3.2.2 Expression and analysis of recombinant serine proteinase inhibitor

The clone containing recombinant pTrcHis 2C was inoculated and grown in LB medium containing 50μg/ml amplicillin. Expression of the serine proteinase inhibitor in the transformants was induced with 1 mM IPTG for 0 to 5 hour. After induction step, the cells were collected and lysed. *E. coli* strain JM109 containing the parental pTrcHis 2C was used as control. It was grown and induced at the same condition. Both groups of cell lysate, pTrcHis 2C and pTrcHis 2C/SPI, were electrophoresed on 10 % SDS-PAGE. An approximately 35 kDa protein band was detected in

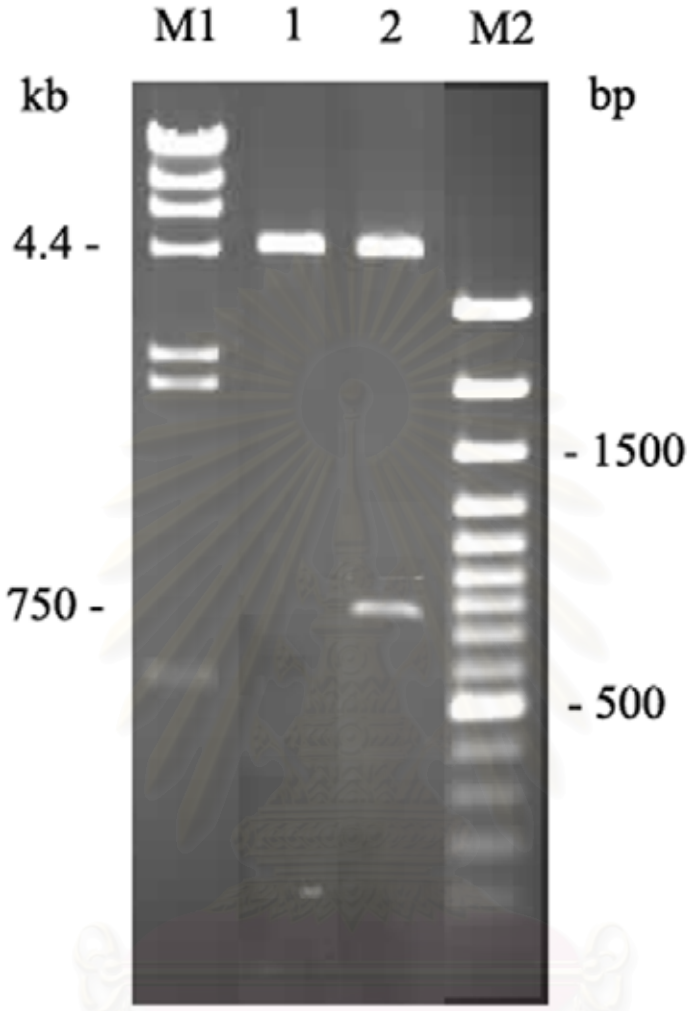


Figure 3.10 Ethidium bromide staining of recombinant pTrcHis 2C vector digested with restriction enzyme *Bam* HI and *Sal* I. The DNA fragment was analyzed on a 1.2 % agarose gel, electrophoresed at 100 volts for 1 hour.

Lane M1: DNA marker (λDNA/*Hin* dIII)

Lane M2: Standard DNA ladder (100 bp ladder)

Lane 1: *Bam* HI and *Sal* I digested pTrcHis 2C

Lane 2: *Bam* HI and *Sal* I digested recombinant pTrcHis 2C/SPI

```
1    ATGGATCCGGGCTACGGAAAAGGGGGGAAAATCCGCCTCTGCGCCAAACACTGTACGACC
     M  D  P  G  Y  G  K  G  G  K  I  R  L  C  A  K  H  C  T  T
61   ATCTCCCCTGTGTGTGGCTCTGATGGAAAAACTTATGACAGCCGATGCCACCTGGAGAAT
     I  S  P  V  C  G  S  D  G  K  T  Y  D  S  R  C  H  L  E  N
121  GCTGCCTGTGGTGGCGTGAGTGTCACTTTCCACCATGCCGGACCCTGCCCTCCCCCAAAG
     A  A  C  G  G  V  S  V  T  F  H  H  A  G  P  C  P  P  P  K
181  AGATGTCCAGGAATATGCCCCGCGGTATATGCCCCTGTGTGCGGGACCAACGGGAAAACT
     R  C  P  G  I  C  P  A  V  Y  A  P  V  C  G  T  N  G  K  T
241  TACTCGAACTTATGCCAACTTGAGAATGACAGAACCTGCAACGGTGCTTTCGTTTCCAAG
     Y  S  N  L  C  Q  L  E  N  D  R  T  C  N  G  A  F  V  S  K
301  AAGCACGATGGACGTTGTGGTTGCAACCCCATTGTCGCGTGCCCTGAGATCTATGCTCCC
     K  H  D  G  R  C  G  C  N  P  I  V  A  C  P  E  I  Y  A  P
361  GTGTGTGGCAGTGATGGCAAGACTTATGATAACGACTGCTATTTCCAGGCAGCTGTTTGC
     V  C  G  S  D  G  K  T  Y  D  N  D  C  Y  F  Q  A  A  V  C
421  AAGAATCCAGATCTTAAGAAGGTTCGAGACGGTAACTGCGACTGCACTCCTCTCATCGGC
     K  N  P  D  L  K  K  V  R  D  G  N  C  D  C  T  P  L  I  G
481  TGTCCCAAGAACTACAGGCCTGTGTGTGGCAGCGACGGTGTAACTTACAACAACGACTGC
     C  P  K  N  Y  R  P  V  C  G  S  D  G  V  T  Y  N  N  D  C
541  TTCTTCAAGGTTGCTCAGTGCAAGAACCCCGCGCTCGTCAAAGTCTCTGATACTCGCTGT
     F  F  K  V  A  Q  C  K  N  P  A  L  V  K  V  S  D  T  R  C
601  GAATGCAACCACGTCTGTACTGAAGAATATTACCCCGTGTGCGGAAGCAATGGTGTCACG
     E  C  N  H  V  C  T  E  E  Y  Y  P  V  C  G  S  N  G  V  T
661  TATTCGAACATTTGTCTGTTGAATAATGCAGCGTGTTTAGATTCCTCCATTTACAAGGTT
     Y  S  N  I  C  L  L  N  N  A  A  C  L  D  S  S  I  Y  K  V
721  TCGGACGGAATCTGTGGTCGCAGACTGTACCTAGTCGACCATCATCATCATCATCATTGA
     S  D  G  I  C  G  R  R  L  Y  L  V  D  H  H  H  H  H  H  *
```

Figure 3.11 Nucleotide and deduced amino acid sequence of recombinant pTrcHis 2C containing NH_2-terminal truncated serine proteinase inhibitor gene. Amino acids are shown as single letter abbreviation. The nucleotides shown underlined correspond to 5' *Bam* HI and 3' *Sal* I sites. The nucleotides and amino acids obtained from the serine proteinase inhibitor gene are in bold.

both groups. Comparing to control group, 35 kDa band of pTrcHis 2C/SPI has more intensity at 2 to 3 hours after induction (Figure 3.12). At 3 hours after induction, the most band intensity difference was observed among various induction times. Therefore, the cell lysate at this time then will be used for serine proteinase inhibitory activity assay.

3.3 Serine proteinase inhibitory activity

3.3.1 Serine proteinase inhibitory activity gelatin/SDS-PAGE assay

To ensure that the 35-kDa band observed by SDS-PAGE analysis is the recombinant serine proteinase inhibitor, the inhibitory activity of the crude *E. coli* lysate was tested using serine proteinase inhibitor activity gelatin/SDS-PAGE assay. In this experiment, aprotinin, a serine proteinase inhibitor was used as positive control. After eletrophoresis on 10% SDS-polyacrylamide gel containing 0.1% gelatin, the prestained protein ladder lane was cut out of the gel and soaked in destaining solution. The rest of the gel was incubated overnight in 2 % triton x-100 solution for protein refolding. After refolding step, the gel was inclubated in serine proteinase solution for 4 hour at 37^{o}C to degrade any protein but not serine proteinase inhibitor band. Bands of undegraded blue-stained gelatin indicate the presence of serine proteinase inhibitors. Gels incubated with trypsin, chymotrypsin, and subtilisin showed a single blue band at 35 kDa (Figure 3.13 A, B, C). The result suggested that the protein contain inhibitory activity against trypsin, chymotypsin, and subtilisin (Figure 3.13). No remaining band was observed in the parental pTrcHis 2C crude lysate. These results suggested that the 35-kDa protein should be recombinant serine proteinase inhibitor.

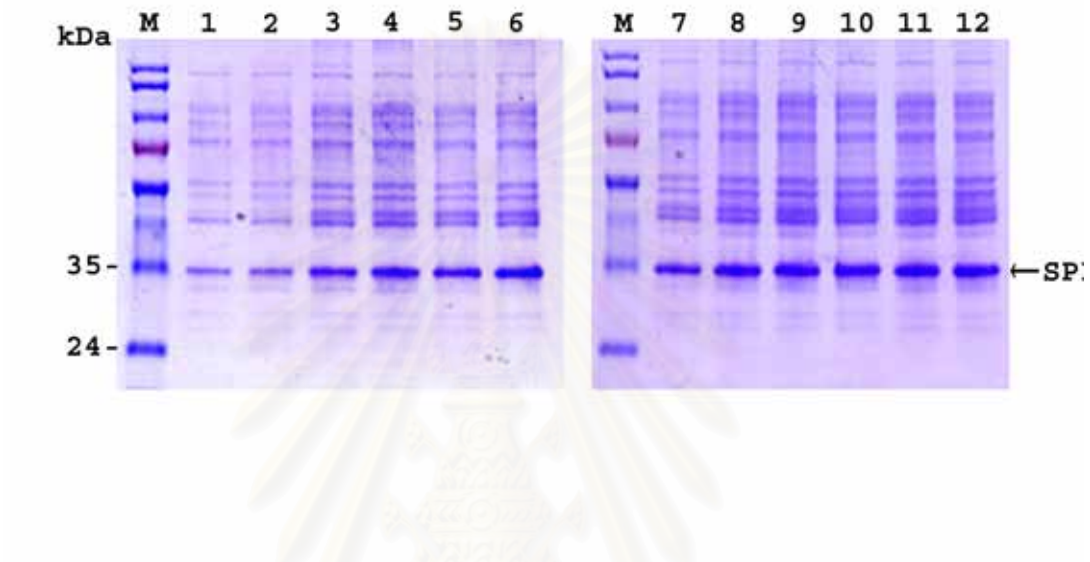


Figure 3.12 SDS-PAGE analysis of the recombinant serine proteinase inhibitor in *E.coli*. Lanes 1, 3, 5, 7, 9 and 11; the lysate of *E.coli* containing the parent pTrcHis2C after induction with 1 mM IPTG for 0, 1, 2, 3, 4 and 5 hours, respectively. Lanes 2, 4, 6, 8 and 10; the lysate of *E.coli* containing the SPI-pTrcHis2C after induction for 0, 1, 2, 3, 4 and 5 hours, respectively. Lane M; Prestained protein ladder (Fermentas). Arrow indicates SPI expected band.

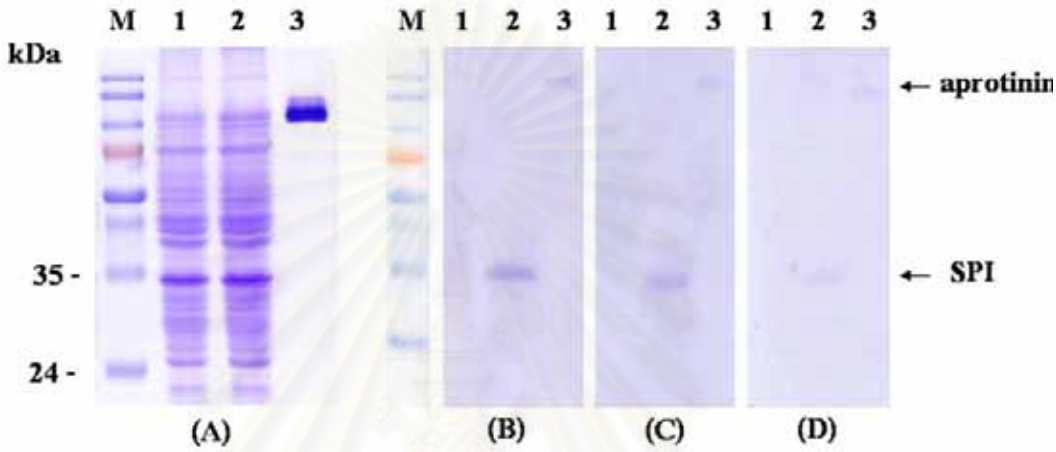


Figure 3.13 Serine proteinase inhibitor activity assay. Crude *E. coli* lysate was run under reducing conditions. (A) Lane 1; the lysate of *E. coli* containing the parent pTrcHis 2C. Lane 2; the lysate of *E. coli* containing SPI-pTrcHis 2C. Lane 3; aprotinin as positive control. After electrophoresis, the gel was incubated in 2% triton X-100 for protein refolding. Then the gel was incubated in trypsin (B), chymotrypsin (C), and subtilisin-containing solution (D), respectively. Lane M; Prestained protein ladder (Fermentas).

3.3.2 Serine proteinase inhibitory spectrum assay

The inhibitory activity of recombinant serine proteinase inhibitor toward serine proteinases was tested by preincubation of crude *E. coli* lysate and serine proteinase. The residual serine proteinase activity was determined by addition of chromogenic substrate which can be cleaved by the serine proteinase and released *p*-nitroanilide. Absorbance of the chromophore *p*-nitroanilide produced was measured at 405 nm. Therefore, inhibition of serine proteinase hydrolysis of chromogenic substrate can be measured by following the change in absorbance at 405 nm. The 31.6 μg total protein of crude *E. coli* lysate was used in the experiment. A decrease in absorbance was observed when the *E. coli* lysate was preincubated with trypsin (89 %), chymotrypsin (70 %), and subtilisin (8 %) but not elastase (Table 3.1) suggesting that the protein is trypsin/ chymotrypsin/ subtilisin inhibitor.

3.4 Serine proteinase inhibitor gene expression analysis using *in situ* hybridization

3.4.1 Preparation of *Vibrio harveyi* infected shrimp

Sub-adult *P. monodon* were infected with 10^6 cfu/ml of *V. harveyi* 1526 as described in 2.5.1. Haemolymph was collected from the ventral sinus of each shrimp using MAS as an anticoagulant at 0, 6, 24, 48, and 72 hours after injection.

3.4.2 Haemocyte preparation

After collection, haemocytes were prepared by centrifugation of the haemolymph and treated as described in 2.5.2. The haemocyte quantity of the suspension was determined by using a haemocytometer. The average total numbers of haemocytes per animal at 0, 6, 24, 48, and

72 h after infection are 1,200±81 x 10^5, 210±76 x 10^5, 590±160 x 10^5, 1,100±96 x 10^5, and 1,200±105 x 10^5, respectively (Table 3.2). The standard deviation and mean values were calculated by using Oneway ANOVA program. Approximately 2 x 10^5 haemocytes per slide was used for hybridization.

3.4.3 *In situ* hybridization of *P. monodon* haemocytes

DIG-labeled riboprobes were prepared from linearized psh415 as described in 2.8.3. Hybridization solution containing 40-100 ng of DIG-labeled riboprobes was incubated with the prehybridized haemocytes. After hybridization, the haemocytes were treated with alkaline phosphatase-conjugated sheep anti-DIG antibody. The detection solution was added onto the haemocytes and incubated until the purple color appeared in antisense probe treated haemocytes. Positive cells, which had stronger purple staining when compared with sense and RNase-treated antisense probe hybridized cells, were counted. Numbers of positive cells in terms of percent were calculated from the number of purple stained cells divided by total cells.

The strong purple stained normal shrimp haemocytes (Figure 3.14 A) suggested that the serine proteinase gene expression took place at the haemocytes. The haemocytes of three normal shrimp were hybridized and the positive cells were detected. The average numbers of the positive cells were 42.7±3.5 % (Table 3.2). Among various times of shrimp infection, the haemocytes of 6 h-infected shrimp showed the least strong purple staining (Figure 3.14 B). At 24 h after the infection, the stronger purple staining was detected (Figure 3.14 C). The results suggested that mRNA level of serine proteinase inhibitor decreased at 6 h and recovered at 24 h after the infection. The stronger staining was also detected at 48 and 72 h

Table 3.1 Inhibition of some serine proteinases by the inhibitor

The enzymes were assayed with chromogenic substrates as described above. The activity was monitored as the release of *p*-nitroaniline at 405 nm. The 31.6 μg of *E.coli* lysate was used in the experiment. The experiments were performed in duplicate.

Proteinase	A_{405} without the inhibitor	A_{405} with the inhibitor	Inhibition (%)
Trypsin (0.08 μg/assay)	0.82	0.11	89
Chymotrypsin (0.05 μg/assay)	0.40	0.12	70
Subtilisin (0.09 μg/assay)	0.85	0.78	8
Elastase (0.6 μg/assay)	0.62	0.72	0

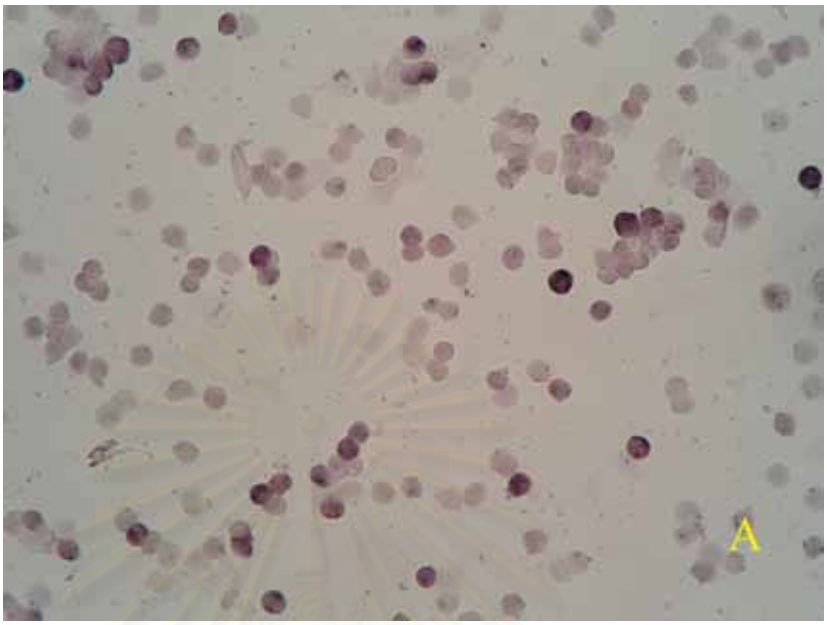
Table 3.2 *In situ* hybridization of *P. monodon* haemocytes

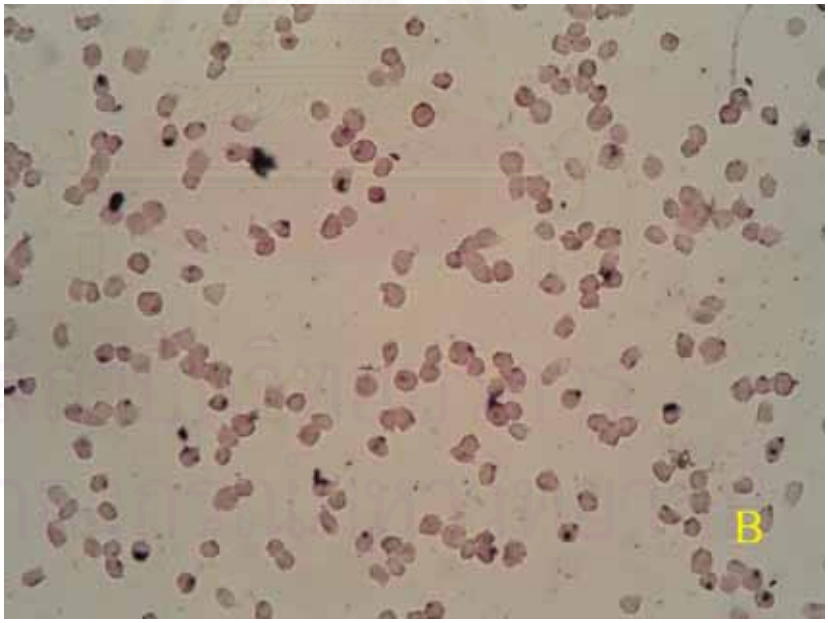
Hour after infection	0	6	24	48	72
Average total haemocyte number (x 10^5)	1,200±81	210±76	590±160	1,100±96	1,200±105
Positive cells (%)	42.7±3.5[a]	35.9±3.5[a]	56.5±6.7[b]	46.1±2.6[a]	47.3±7.6[a]

Mean (±S.D.) THC (total haemocyte count) and positive cells of *P. monodon* at different time *V. harveyi* infection. Values with different letters are significantly different ($P<0.001$).

after the infection same as normal shrimp haemocytes (Figure 3.14 D, E, and A). The average numbers of the positive cells at 6, 24, 48, and 72 hours after infection were 35.9±3.5 %, 56.5±6.7 %, 46.1±2.6 %, and 45.7±8.4 %, respectively (Table 3.2). The SPI mRNA-positive haemocyte number significantly increases at 24 h post-infection (P<0.001). Whereas the positive haemocyte number does not significantly decrease at 6 h post-infection (P>0.001). There was no significant difference in the positive haemocyte numbers of normal, 48 h, and 72 h post-infection. The statistical values were determined by using Turkey's honestly significant difference test. These results suggested that serine proteinase inhibitor expressed haemocytes are the most abundant at 24 h after infection.

Figure 3.14 *In situ* hybridization of *P. monodon* haemocytes with antisense SPI riboprobes. (A) normal shrimp haemocytes (B)-(E) infected shrimp haemocytes at 6, 24, 48, and 72 h post-infection, respectively (F) RNase-treated shrimp haemocytes (G) Control: shrimp haemocytes hybridized with sense SPI riboprobes.


A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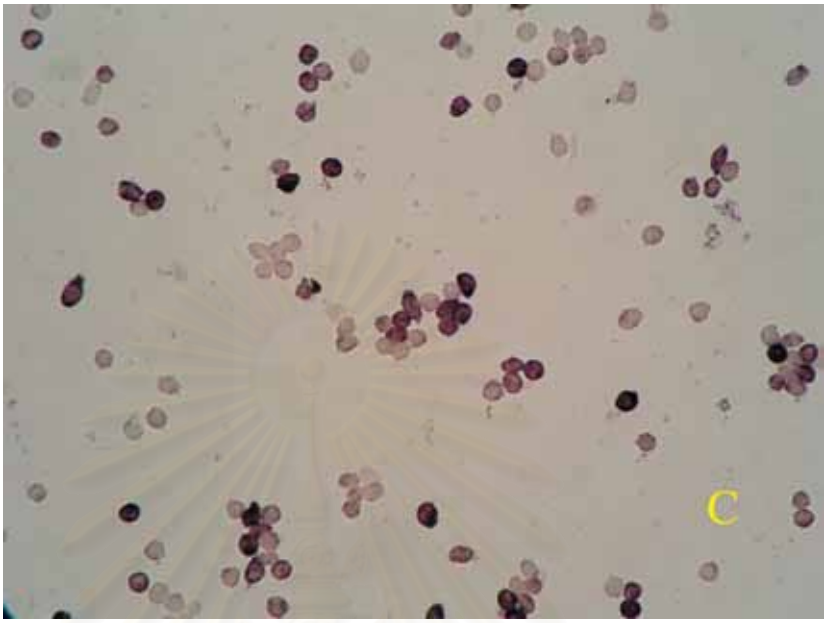
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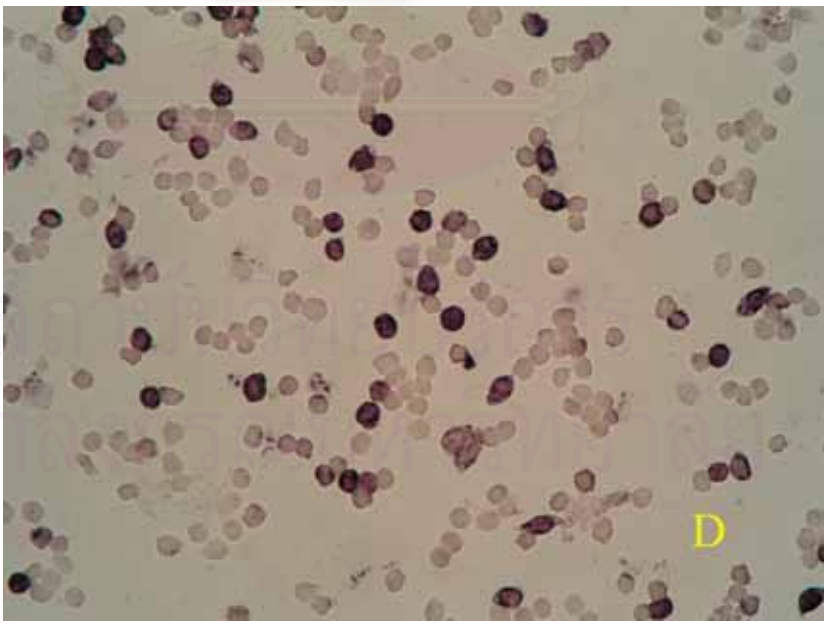
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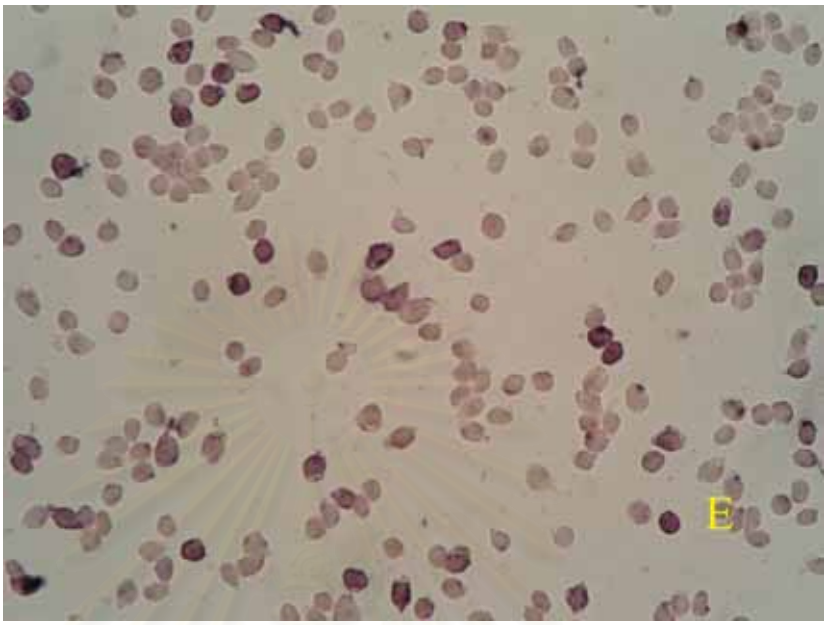
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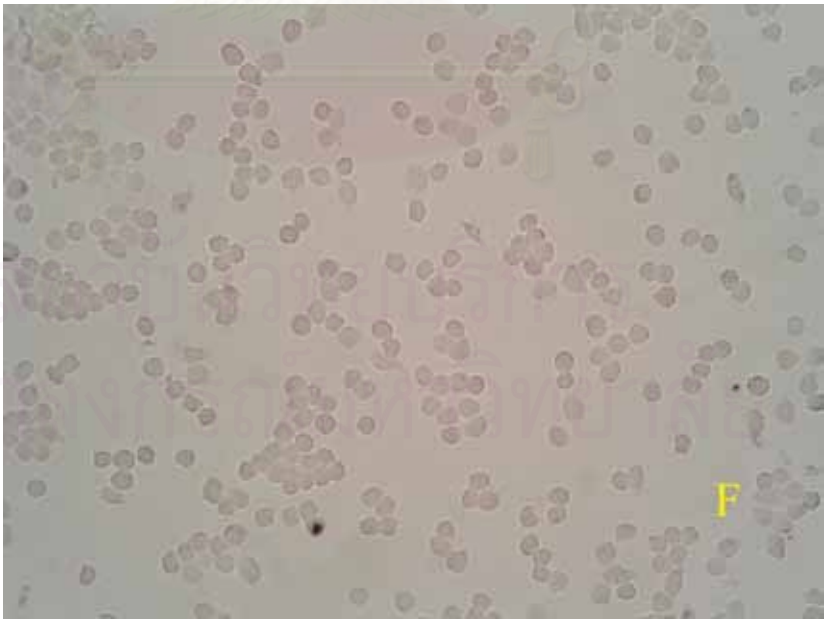
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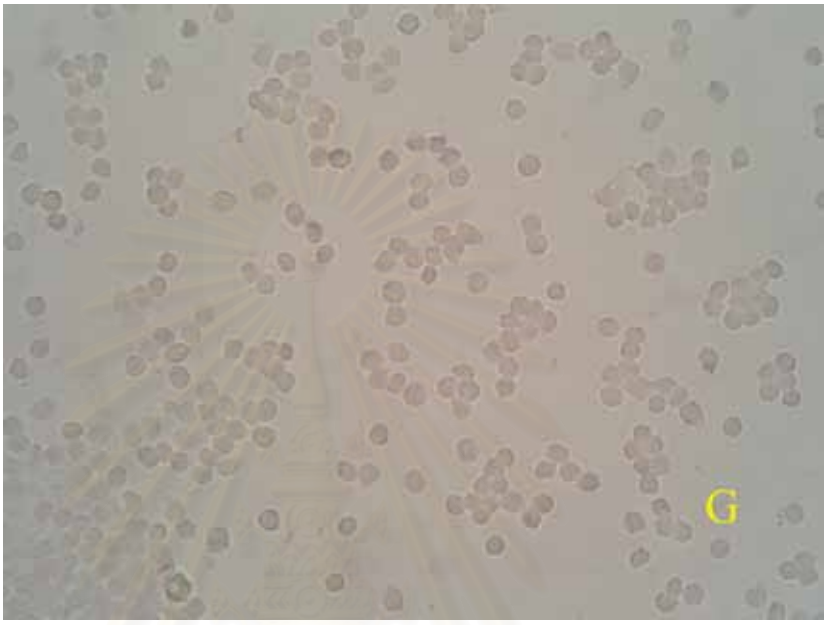

CHAPTER IV

DISCUSSIONS

The infectious outbreaks have become more serious problem, dramatically affecting the shrimp production. Shrimp disease prevention requires knowledge in shrimp immune system. However, the shrimp immune system is not clearly understood. To comprehend the immune system, biomolecules involved in immunity must be studied. Supungul et al (2002) used Expressed Sequenced Tags (ESTs) analysis to identify cDNAs encoding immune related proteins from the black tiger shrimp, *P. monodon,* haemocytes. One hundred and fifteen cDNA clones representing 34 different genes were putative immune genes. Six of these cDNA clones were identified as putative serine proteinase inhibitors by using BLASTX program. They showed 50-58 % homology to a Kazal serine proteinase inhibitor derived from the crayfish *Pacifastacus leniusculus*.

Since all of members identified by the proteinase cascade for the innate immunity of arthropods belong to the serine proteinase family (Adachi et al., 2003). This serine proteinase family should also involve in the proteinase cascade for crustacean, one subphylum of arthropod, immunity. The immune reactions excessively activated by serine proteinases can be harmful to host animal, so these serine proteinases must be tightly controlled by serine proteinase inhibitors. This experiment focused on the serine proteinase inhibitor characterization. Four of 6 putative serine proteinase inhibitor cDNA clones obtained from cDNA shrimp haemocyte libraries were full-length genes. All full-length serine proteinase inhibitor cDNA clones were repeatedly sequenced to confirm sequencing data obtained from ESTs. The differences at the end of

sequences were found. Whereas other parts of nucleotide sequences were similar. These differences in nucleotide sequences may caused by sequencing error that always be found at the end of sequences. Each cDNA clone contained an open reading frame (ORF) with difference in length. All known Kazal inhibitors are secreted proteins containing signal peptide (Scott et al., 1987), including Kazal inhibitor found in the crayfish. The signal sequences of all SPI cDNA clones were predicted using SignalP VI.1 software (Nielsen et al., 1997). Deduced amino acids of ORF of each cDNA clone had putative signal peptide with 17 to 18 amino acid residues. From multiple sequence alignment (Fig. 3.1) and alignment of the Kazal domains of SPI (Figure 3.2), three of four clones—sh415, sh1064, and sh1069—are highly similar to each other. Whereas clone sh610 is different from the three clones. These suggested that the three clones— sh415, sh1064, and sh1069—are allelic variances; which encode from the same locus. Whereas sh610 clone encodes from the different locus. The inhibitory specificity of a domain depends on the reactive amino acid (P_1), which varies widely among the Kazal inhibitors (Ødum et al., 1999). The P_1 residue is the third amino acid residue which comes after the second cysteine of each Kazal domain. From previous studies, if Kazal domain contains arginine or lysine residue at P_1 site, the inhibitor tends to inhibit trypsin. In the same manner, if P_1 residue is tyrosine, methionine, leucine or phenylalanine; the inhibitor is most likely to inhibit chymotrypsin, subtilisin, and/or elastase. Kazal inhibitors with P_1 leucine and methionine are strong inhibitors of elastase (Laskowski et al., 1980). If Kazal domain contains alanine or serine, the inhibitor is supposed to inhibit elastase (Laskowski et al., 1980; Scott et al., 1987, and Saxena et al., 1997). Each domain can function independently; therefore, one Kazal inhibitor can inhibit more than one type of serine

proteinase depending on its P_1 residues. In crayfish, P_1 position in three Kazal domains are leucine and the rest is glutamine. The putative inhibitory activity of the Kazal inhibitor from the crayfish was supposed to inhibit chymotrypsin, subtilisin and/or elastase. Ascribing to independently inhibitory function, the three crayfish Kazal domains contain leucine at P_1 sites. Consequently, one inhibitor is anticipated to bind to 3 inhibited serine proteinase molecules. However, Johansson et al. (1994) reported that the Kazal inhibitor binds 1:1 to chymotrypsin and this inhibitor can inhibit chymotrypsin and subtilisin, but not trypsin, elastase or thrombin. These implied that not all Kazal domains are active to inhibit serine proteinases. As shown in Figure 3.2, P_1 residues of Kazal domains of *P. monodon* serine proteinase inhibitors consisted of threonine, alanine, glutamate, lysine, and leucine. The three similar clones, sh415, sh1064, and sh1069, consist of threonine, alanine, glutamate, and lysine at P_1 sites. All five Kazal domains of sh415 clone are complete domains. However, two of five Kazal domains of sh1064 and sh1069 clones are incomplete domains. The sh610 clone consists of glutamate, threonine, and leucine at P_1 sites. Since sh415 clone is the first full-length gene, which contains complete Kazal domains, obtained from the shrimp haemocyte libraries. The sh415 clone was used in this study. The putative activity of sh415 serine proteinase inhibitor was predicted from the P_1 sites. Since the P_1 residue consists of threonine, alanine, glutamate, and lysine, the inhibitory activity against trypsin and/or elastase was predicted. Since cDNA of this clone has both 5’ and very long 3’-untranslated regions and active form of SPI found in crustacean haemocytes contained no signal sequences (Johansson et al., 1994), the 5’-terminal truncated serine proteinase inhibitor gene was constructed by using PCR amplification. An advantage of this method is the orientation of gene of interest can be directed by using different restriction sites

incorporated to the designed primers and ligated into expression vector at the same sites. Another advantage based on PCR amplification technique, the primers can be designed to cover only the interested gene while the gene flanking regions were eliminated.

The *Pichia pastoris* yeast expression system was used to express this SPI gene. However, no expression of the recombinant protein was observed on SDS-PAGE analysis and inhibitory activity was not detected using inhibitory spectrum assay (data not shown). The *E. coli* expression system was widely used to express this protein from several sources including insect *Rhodnius prolixus*, tachyzoite *Toxoplasma gondii*, chicken, and human (Friedrich et al., 1993; Pszenny et al., 2000; DeKoster et al., 1997; Mägert et al., 1999; Lauber et al., 2001; 2003). Thus, the *E. coli* expression system was selected to express this *P. monodon* SPI. Advantages of this expression system are simple, cost effective, and time saving. The low expression level of recombinant SPI was detected by using SDS-PAGE analysis. This might be resulted from either vector type or codon usage difference between *E. coli* and *P. monodon.* According to other reports, they used the T7 RNA polymerase-based expression vector; pET, to overexpress multiple Kazal-type inhibitors (DeKoster et al., 1997 and Lauber et al., 2001, 2003), instead of *trc* promoter-based expression vector, pTrcHis 2C, that was used in this experiment. We assumed that specific promoter type may influence on expression level. In addition to promoter type, codon usage may be the limitation of expression. From the evidence, CCC and AUA are more utilized in *P. monodon* than in *E. coli.* Consequently, *E. coli* would not be able to afford tRNA to translation process, resulting in low expression level. However, the inhibitory activities of the recombinant SPI against trypsin, chymotrypsin and subtilisin were observed by using SPI activity

gelatin/SDS-PAGE assay. From the results, the most undegraded gelatin blue band intensity was observed in trypsin incubation, suggesting that the recombinant SPI was strong inhibitor of trypsin. In the same way, chymotrypsin and subtilisin were also inhibited by this recombinant SPI. The least band intensity in subtilisin incubation suggested that the recombinant SPI was weak inhibitor of subtilisin. The inhibitory activity of the recombinant SPI was determined by using SPI spectrum assay. From this method, we found that the recombinant SPI can inhibit trypsin, chymotrypsin, and subtilisin, but not elastase. Corresponding to SPI activity gelatin/SDS-PAGE assay, the recombinant SPI can strongly inhibit trypsin activity and also chymotrysin. Whereas subtilisin was slightly inhibited by the recombinant SPI. Corresponding to P_1 reactive site as described above, the recombinant SPI had predicted inhibitory activity against trypsin and elastase. From the inhibitory activity assay, the recombinant SPI can inhibit trypsin but not elastase. As described in Agerberth and Johansson's reports (1989; 1994), the Kazal domain may lack predicted activity against serine proteinase. In porcine, the Kazal inhibitor lacks virtually all trypsin inhibitory activity in spite of the presence of an arginyl residue at the P_1 site of the putative proteinase binding loop, which would be expected to confer trypsin binding specificity. The Kazal inhibitor from crayfish has only one active P_1 leucine residue as observed from 1:1 binding of the inhibitor to chymotrypsin. Like these Kazal inhibitors, the recombinant SPI of this study lacks elastase inhibitory activity. This may caused by inactive alanine P_1 residue. In addition to P_1 residue, amino acid residues located around P_1 residue are also important to inhibitory activity of SPI. For instance, reactive site with proline in the P_1', the fourth residue apart from the second cysteine residue, are not active as inhibitor. In some cases, bulky side chain of amino acid residue located around P_1 residue may

cause steric hindrance and obstruction during access to the active site of serine proteinase (Tanaka et al., 1999). These affect binding of SPI to its serine proteinase, causing decrease in inhibitory activity of inhibitor. In this study, the inhibitory activity against chymotrypsin and subtilisin was also observed in the recombinant SPI. However, previous studies so far lacked information about threonine and glutamate as P_1 residue.

Although the biological function of the *P. monodon* Kazal inhibitors in the blood cells is not known. The inhibitory activity against trypsin implies that this Kazal inhibitor might be useful for regulation of prophenoloxidase (pPO) activating system. Because the pPO activating system of shrimp can be activated by trypsin-like serine proteinase cascade (Perazzolo et al., 1997; 2002). In *P. monodon*, several putative trypsin cleavage sites were detected close to the N-terminus of shrimp pPO, one of those sites was predicted to be a cleavage site for activation of shrimp pPO (Sritunyalucksana et al., 1999). This system is one of defence reaction of shrimp against microbial invasion. Moreover, there is an evidence indicating that the serine/cysteine proteinases in the haemocytes are involved in the activation of hemocyanin conversion into a phenoloxidase-like enzyme (Adachi et al., 2003). This might also be regulated by the serine proteinase inhibitor. The recombinant SPI also had inhibitory activity against subtilisin. This suggested that its biological function might be to inhibit microbial proteinases. However, subtilisin acvitity was slightly inhibited by this SPI suggesting that this biological function might not be a major role of this Kazal inhibitor. Nevertheless, understanding of Kazal SPI role in shrimp immunity needs more studies. Expression technique must be developed for higher recombinant SPI production and purification. The purified recombinant SPI will be tested

for pPO activity inhibition. The inhibitory activity against shrimp pathogen proteinases should also be tested.

To determine the change in SPI transcripts in haemocytes of *Vibrio harveyi* challenged *P. monodon*, *in situ* hybridization was performed. In this experiment, RNA probes or riboprobes were used. Two groups of shrimp haemocytes, normal and *V. harveyi* infected groups, were studied. Shrimp were challenged by injection of live *V. harveyi* and haemolymph was collected from three individual animals at different times (0, 6, 24, 48, and 72 h) following injection. A strong decrease in average total haemocyte counts (THC) was observed in the first 24 h after injection, with the strongest difference at 6 h between challenged and nonchallenged animals. The number of total haemocytes returned to levels observed for unchallenged animals at 48 and 72 h. The reducing in THC after infection and haemocyte recovery correspond to many reports; the number of crustacean free haemocytes can vary and can, for instance, decrease dramatically during an infection (Smith and Söderhäll; 1983a; Smith et al., 1984; Persson et al., 1987b; Lorenzon et al., 1999). Injection of a β-1,3-glucan caused a severe loss of haemocytes, followed by a rapid recovery in *P. leniusculus* (Söderhäll et al., 2003). After bacterial and fungal infections, *L. vannamei* THC significantly decreased at 6 h after injection (Muñoz et al., 2002). In Taura syndrome virus-infected pacific white shrimp, *L. vannamei*, THC decreased significantly when compared with untreated control values (Song et al., 2003). Significant reductions in THC were observed in WSSV-infected shrimp, *Penaeus indicus* (Yoganandhan et al., 2003). In the black tiger shrimp, *Penaeus monodon*, THC rapidly droped following injection of foreign material (Van de Braak et al., 2002). In crustaceans, three classes of haemocytes; the hyaline cells, the semigranular cells, and the granular cells, which all play

a key role in immobilizing or destroying invasive microorganisms, are observed within the haemolymph (Söderhäll and Cerenius, 1998; Johansson et al., 2000). The decrease in THC is attributed to different defence activities. Haemocyte migration to the injection site, as observed in Van de Braak's study (2002) and Muñoz's study (2002), accounted for a reduced cell concentration in the haemolymph. In addition, haemocytes aggregated into non-circulating clumps after acute bacterial infection (Johnson, 1976). Phagocytic haemocytes, hyaline cells, may leave the circulation after phagocytosis and enter the heart, connective tissue, gills or other haemal sinuses (Cornick and Stewart, 1968; Factor and Beekman, 1990). Semigranular haemocytes were also the major phagocytic cells in ridgeback prawn, *Sicyonia ingentis* (Hose and Martin, 1989; Martin et al., 1996) and might follow Cornick and Factor's observations. In the black tiger shrimp, haemocytes seem to settle first, mainly in the lymphoid organ, and phagocytose (Van de Braak et al., 2002), corresponding to the theory of Johnson (1987) that fixed phagocytes in most crustaceans are derived from circulating cells. Moreover, haemocyte lysis caused by exocytosis, activation of pPO and haemolymph coagulation might result in the reduction of circulating haemocytes of infected shrimp (Johansson and Söderhäll, 1985; Aono and Mori, 1996). Haemocyte deficiency, which is induced during an infection in invertebrate (Johansson and Söderhäll, 1988), constitutes a serious threat to the health of the animal. Thus, new haemocytes need to be compensatorily and proportionally produced from haematopoietic tissue (Johansson et al., 2000).

From *in situ* hybridization, strong purple positive staining of normal shrimp haemocytes indicated that expression of Kazal inhibitor localized in the haemocytes. However, there is gradient staining among the positive normal shrimp haemocytes. Since the exact localization of the

shrimp Kazal inhibitor in the blood cells is not known. The results obtained from *in situ* hybridization will be discussed into 2 directions; all cell types can express the Kazal inhibitor and only some cell types can express the Kazal inhibitor. In the case that all cell types can express the Kazal inhibitor, the gradient staining might be attributable to difference in expression level of each haemocyte. The haemocytes that were not stained might be abounded in Kazal inhibitors within these haemocytes, thus making them unnecessary to transcribe. In the case that only some cell types can express the Kazal inhibitor analogous to Johansson's report (2000). Each cell type is active in difference defence reaction, for example; in crayfish, the hyaline cells are chiefly involved in phagocytosis, the semigranular cells are the cells active in encapsulation. Storage and release of the pPO system and cytotoxicity were participated in the semigranular and granular cells. In shrimp, penaeidins are solely present in granular and semigranular cells, and are absent from the hyaline cell population, devoid of granules (Muñoz et al., 2002). In this case, the gradient staining referred to difference in SPI expression level of the expressed haemocytes and the non-staining haemocytes were the cell type that was no SPI expression.

At 6 h after infection, there were no strong staining haemocytes, indicating reduction in the high expressed type of haemocytes ―if SPI are present in specific cell types― or the low SPI expression level within all haemocytes, if SPI are present in all cell types. In previous semi-quantitative RT-PCR analysis study, amount of serine protease inhibitor mRNA slightly decrease at 3 and 6 hours after *V. harveyi* infection (Supungul, 2002). The more efficacy method; such as real time PCR should be done to corroborate these observations. At this time, irregular shape and size of haemocytes also were observed. The morphological

changes may be related to an active immune response which is mediated by the exocytosis of immune proteins in association with the pPO, thus resulting in cell size change. The pPO activation can trigger further cell degranulation and ultimately cell death (Söderhäll and Smith, 1986; Cárdenas et al., 2000).

At 24, 48, and 72 h after infection, the SPI mRNA-positive haemocytes were also observed as described in normal shrimp haemocytes, indicating that SPI expressed haemocytes were produced to compensate the reduction of circulating haemocytes during infection.

Consideration in all timings, SPI mRNA-positive haemocyte number significantly increases at 24 h after infection, and return to initial levels after 48 h post-infection. We assume that ― if SPI are expressed in granular and semigranular cell types ― significantly increase in SPI mRNA-positive haemocyte at 24 h after infection might be attributed to SPI up-regulation that reflects an induced proliferation process responding to infection stress (Muñoz et al., 2002). One another possible to describe the increase in the positive haemocyte number at 24 h post-infection; it might be attributed to SPI production for serine proteinase cascade regulation, as mentioned above. SPI mRNA-positive haemocyte number return to the same as unchallenged number after 48 h post-infection. However, 3 and 12 h *V. harveyi* infected haemocytes should be further investigated for manifest observing tendency of pathological changes in both of THC and SPI mRNA-positive haemocytes.

CHAPTER V

CONCLUSIONS

Understanding in shrimp immunity is necessary for infectious disease prevention. Whereas knowledge in shrimp immune system is still not clearly known. Therefore, studying of molecules related to shrimp immune system will be useful for more understanding in shrimp immunity. Serine proteinase inhibitors (SPI) considered to have role in crustacean immunity were chosen to study in terms of protein expression, testing SPI activity, and observing pathological changes in cellular mRNA level. From *P. monodon* haemocyte cDNA libraries, cDNA coding immune related proteins were identified by using Expressed Sequence Tags (ESTs) analysis. Six cDNA clones coding putative Kazal serine proteinase inhibitor were identified. Four of these cDNA clones were full-length genes. They were repeatedly sequenced to confirm sequence corrections. The putative signal peptides of all SPI cDNA clones were predicted. Sh415 and sh610 clones contain 5 and 4 complete Kazal domains, respectively. Sh1064 and sh1069 clones contain 3 complete and 2 incomplete Kazal domains. One clone, sh415, which had 5 complete Kazal domains containing 4 different amino acids at P_1 residue of each domain, was used for recombinant protein expression. The *E. coli* expression system was used in this study. Low expression level of the recombinant SPI was detected by SDS-PAGE. Inhibitory activity against trypsin, chymotrypsin, and subtilisin was observed in the recombinant SPI. These suggested that this SPI might play a role in regulation of pPO system which is activated by trypsin-like serine proteinase. This SPI might also inhibit microbial proteinases because it had subtilisin inhibitory activity. However, slight inhibitory activity against subtilisin indicated that the microbial proteinase inhibition was

not a major role of this SPI. The biological activity of Kazal SPI responded to shrimp immune system should be investigated further.

From *in situ* hybridization technique, we found that the Kazal serine proteinase inhibitor located in the haemocytes. The reduction of total circulating haemocyte counts (THC) at early post-infection was common observation in shrimp and crustacean. The decrease in THC is attributed to different defence activities which cause many phenomenons; such as haemocyte migration to the injection site, haemocyte aggregation, leaving of circulation of phagocytic haemocytes, and haemocyte lysis. After reduction of circulating haemocytes, the new haemocytes will be compensatorily produced from haematopoietic tissue to maintain shrimp homeostasis. The Kazal SPI had different expression level among the SPI mRNA-positive haemocytes. The reduction in SPI expression level was observed at 6 h post-infection. Because whether SPI are expressed in all circulating haemocytes or not, this reduction might be resulting in decrease in SPI high expression haemocytes. The expression of the Kazal SPI responded to microbial challenge. This corresponded to the result obtained from RT-PCR analysis, but SPI mRNA level detected by RT-PCR was not significantly decreased (Supungul, 2002). Irregular shape and size of haemocytes at 6 h post-infection may be related to an active immune response, mediated by the exocytosis of immune proteins in association with the pPO, thus resulting in cellular morphology change. Significantly increase in SPI mRNA-positive haemocyte number at 24 h post-infection reflects an induced proliferation process responding to infection stress, corresponding to increasing trend in THC at this time. The SPI mRNA-positive haemocyte number returned to levels observed for unchallenged haemocytes after 48 h post-infection. The early stage of

infected haemocytes, 3 and 12 h, should be investigated for observing tendency of pathological changes.

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