การตอบสนองโดยการต้านไวรัสและเป้าหมายทางชีวภาพของตัวยับยั้งซีรีนโปรตีเนสชนิดคาซาล SPI*Pm*2 จากกุ้งกุลาดำ *Penaeus monodon*

นางสาวศิริขวัญ พลประทีป

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2556

ANTIVIRAL RESPONSES AND BIOLOGICAL TARGETS OF A KAZAL-TYPE SERINE PROTEINASE INHIBITOR SPI*Pm*2 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*

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ศิริขวัญ พลประทีป : การตอบสนองโดยการต้านไวรัสและเป้าหมายทางชีวภาพของตัวยับยั้งซีรีนโปรตีเนสชนิดคาซาล SPI*Pm*2 จากกุ้งกุลาดำ *Penaeus monodon* (ANTIVIRAL RESPONSES AND BIOLOGICAL TARGETS OF A KAZAL-TYPE SERINE PROTEINASE INHIBITOR SPI*Pm*2 FROM THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.วิเชียร ริมพณิชยกิจ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.ดร.อัญชลี ทัศนาขจร, Prof. Chu-Fang Lo, Ph.D., 108 หน้า.

โรคตัวแดงดวงขาวเป็นโรคติดเชื้อไวรัสที่รุนแรงในกุ้งกุลาดำซึ่งก่อให้เกิดการตายอย่างสูง การเข้าใจระบบภูมิคุ้มกันของกุ้งจะนำไปสู่องค์ความรู้ในการป้องกันและแก้ไขปัญหาของโรคได้ ในกุ้งกุลาดำพบโปรตีนหลายชนิดมีการตอบสนองต่อการติดเชื้อไวรัส ตัวยับยั้งซีรีนโปรตีเนสชนิดคาซาลชนิดที่ 2 (SPI*Pm*2) เป็นโปรตีนในกุ้งกุลาดำที่มีการแสดงออกอย่างมากในเม็ดเลือดกุ้งและมีความเกี่ยวข้องกับระบบภูมิคุ้มกันแต่กำเนิดของกุ้งกุลาดำต่อการติดเชื้อไวรัสตัวแดงดวงขาว เทคนิค immunofluorescent และ immunogold labeling แสดงให้เห็นว่าในเม็ดเลือดกุ้งสุขภาพดี มีการแสดงออกของโปรตีน SPI*Pm*2 ซึ่งเก็บอยู่ในแกรนูลของเม็ดเลือดชนิดเซมิแกรนูลาร์และแกรนูลาร์ เมื่อมีการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้ง พบว่าจำนวนเม็ดเลือดที่ผลิตโปรตีนนี้ลดลงเหลือครึ่งหนึ่งของทั้งหมด ซึ่งเป็นไปตามคาดว่า การลดลงของ SPI*Pm*2 เนื่องมาจากโปรตีนนี้ถูกหลั่งจากเม็ดเลือดอย่างรวดเร็ว จึงทำให้มีการลดลงของโปรตีนนี้ในเม็ดเลือด เราได้ใช้โปรตีนรีคอมบิแนนท์ SPI*Pm*2 (rSPI*Pm*2) เพื่อศึกษาผลของโปรตีนในกุ้งที่ติดเชื้อตัวแดงดวงขาว การฉีด rSPI*Pm*2 ก่อนการติดเชื้อไวรัสส่งผลให้การตายของกุ้งช้าลง การใช้เซลล์ปฐมภูมิของกุ้งกุลาดำศึกษาการต้านไวรัสโดย neutralization และ protection พบว่า rSPI*Pm*2 และปริมาณ rSPI*Pm*2 ที่เพิ่มขึ้น สามารถ neutralize และป้องกันการติดเชื้อไวรัสในเซลล์เม็ดเลือดกุ้งได้ โดยส่งผลให้การแสดงออกของยีน VP28 ซึ่งเป็นยีนไวรัสในช่วง late phase ลดลง การต่อต้านไวรัสของ SPI*Pm*2 เนื่องมาจากการจับของ SPI*Pm*2 กับองค์ประกอบของตัวไวรัสและเยื่อหุ้มเซลล์เม็ดเลือดกุ้ง งานวิจัยนี้ยังได้ใช้เทคนิค yeast two hybrid screening เพื่อระบุโปรตีนเป้าหมายของ SPI*Pm*2 ในห้องสมุดดีเอ็นเอของเชื้อตัวแดงดวงขาว ทำให้พบ WSV477 ซึ่งมีรายงานว่ามีแอกทิวิตีของ ATP/GTPase เราได้ใช้เทคนิค In vitro pull down assay ในการยืนยันปฏิสัมพันธ์ระหว่างโปรตีน rSPI*Pm*2 และ rWSV477 เมื่อยับยั้งการแสดงออกของยีน WSV477 โดย dsRNA-WSV477 ในเซลล์เม็ดเลือดปฐมภูมิของกุ้งกุลาดำที่ติดเชื้อไวรัส พบการแสดงออกของ VP28 ซึ่งเป็นยีนในช่วง late phase ของไวรัสลดลงซึ่งแสดงถึงการลดลงของการติดเชื้อไวรัส

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

สาขาวิชา.....ชีวเคมี..... ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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ลายมือชื่อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: KAZAL-TYPE SERINE PROTEINASE INHIBITOR/ WHITE SPOT SYNDROME VIRUS/ BLACK TIGER SHRIMP/ *Penaeus monodon*

SIRIKWAN PONPRATEEP: ANTIVIRAL RESPONSES AND BIOLOGICAL TARGETS OF A KAZAL-TYPE SERINE PROTEINASE INHIBITOR SPI*Pm*2 FROM THE BLACK TIGER SHRIMP *Penaeus monodon* ADVISOR: ASSOC. PROF. VICHIEN RIMPHANITCHAYAKIT, Ph.D., CO-ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., PROF. CHU-FANG LO, Ph.D., 108 pp.

White spot syndrome (WSS) is a severe viral disease in *Penaeus monodon* which causes high mortality. The understanding of shrimp immunity will lead to the knowledge for prevention and solving the disease. In black tiger shrimp, several proteins are expressed in response to viral infection. Among others, a Kazal-type serine proteinase SPI*Pm*2 is abundantly expressed in the hemocytes and shown to be involved in innate immune response against white spot syndrome virus (WSSV). In the healthy hemocyte, the immunofluorescent and immunogold labeling techniques showed that the SPI*Pm*2 was expressed and stored in the granule of semi-granular and granular hemocytes. Immunocytochemical study showed that, after WSSV infection, the percentage of SPI*Pm*2-producing hemocytes was reduced by about half. Expectably, the SPI*Pm*2 was secreted readily from these hemocytes after WSSV challenge. The recombinant SPI*Pm*2 (rSPI*Pm*2) was used to investigate the effect of SPI*Pm*2 on viral infection in shrimp. Injection of rSPI*Pm*2 prior to WSSV injection prolonged the mortality rate of WSSV-infected shrimp. By using shrimp primary hemocyte cell culture, the antiviral activity was neutralization and protection. The rSPI*Pm*2 temporarily and dose-dependently neutralizes the WSSV and protects the hemocytes from viral infection judging from the substantially less expression of WSSV late gene VP28. The antiviral activity was very likely due to the binding of SPI*Pm*2 to the components of viral particle and hemocyte cell membrane. The yeast two-hybrid screening had identified a viral target protein of SPI*Pm*2 in the WSSV cDNA library, namely WSV477. The WSV477 was reported to have ATP/GTPase activity. In vitro pull down assay confirmed the protein-protein interaction between rSPI*Pm*2 and rWSV477. In WSSV-infected culture, silencing of WSV477 by dsRNA-WSV477 reduced the expression of viral late gene VP28 and, hence, the reduction of viral infectivity.

Department ..... Biochemistry ..... Student's Signature .....

Field of Study ..... Biochemistry ..... Advisor's Signature .....

Academic Year ..... 2013..... Co-advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

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

ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
sec	second
SPI	serine proteinase inhibitor
μg	microgram
μl	microlitre
μM	micromolar
UTR	untranslated region
WSSV	white spot syndrome virus

CHAPTER I

INTRODUCTION

1.1 Shrimp aquaculture in Thailand

Shrimp farming has been a multi-billion dollar industry. The industry produces high most economically impact in several countries in Asia, especially China and Thailand because their shrimp production shares up to 75% of the world shrimp trade (Source: FAO databases, 2007). Thailand has been the world leader in exporting shrimp products since 1990s, and used to be the biggest exporter of the black tiger shrimp, *Penaeus monodon* (Wyban, 2007). The rapid expansion of shrimp farming gave rise to several problems on culture management of shrimp broodstocks, farm wastes and the outbreaks of pandemic diseases (Mohan et al., 1998).

The infectious diseases from white spot syndrome (WSSV), taura syndrome (TSV) and yellow head (YHV) viruses and the luminescent bacterium *Vibrio harveyi* cause main losses in the major shrimp production farms (Flegel, 2006). Subsequently, the production of the black tiger shrimp in Thailand has been substantially dropped while the white shrimp, *Litopenaeus vannamei*, has then become an alternative shrimp species owing to its specific pathogen resistance, high growth rate and high survival rate.

Nevertheless, the black tiger shrimp; *Penaeus monodon* is still more advantageous because they are fast growing and tolerant to a wide range of salinity. Moreover, they are native species in Thailand. Therefore, the research and

improvement of *P. monodon* are important in order to maintain the farming industry of black tiger shrimp. For the black tiger shrimp farming, the management of the outbreak of diseases is very important. The understanding of shrimp immunity provides one of the most effective ways to help control the diseases, and consequently conserve the black tiger shrimp farming.

1.2 Infectious diseases in shrimp

Infectious diseases cause the serious impact on shrimp aquaculture and lead to the high mortality rate of shrimp. The major causes of shrimp diseases are mainly by virus and bacteria. The viral pathogens such as White Spot Syndrome Virus (WSSV), Infectious hypodermal and Hematopoietic Necrosis Virus (IHHNV), Yellow Head Virus (YHV) and Taura Syndrome Virus (TSV) are much more pandemic than the bacteria, but the infectious disease from bacteria, genus Vibrio, can also cause 100% cumulative mortality (Alvarez et al., 1998; Flegel, 2006).

White spot disease or syndrome (WSS or WSD) is one of the highly virulent viral diseases in penaeid shrimp caused by white spot syndrome virus infection. This disease leads to the mortality rate of 100% within days (Lightner, 1996). The virus has a wide host range of decapod crustaceans and more than 93 species of arthropods have been reported as hosts or carriers of WSSV (Lo et al., 1996; Flegel, 1997; Flegel and Alday-Sanz, 1998).

White spot syndrome virus (WSSV) is a double-stranded DNA (dsDNA) virus. The intact virion is a large (80-120 × 250-380 nm) enveloped rod-shaped particle (Wang, et al., 1995; Durand et al., 1997; Inouye et al., 1994, van Hulten et al.,

2001) (Fig 1.1). The WSSV genome size has been reported to be about 300 kb. A total of 531 putative open reading frames (ORFs) have been identified of which 181 ORFs are likely to encode the functional proteins (OIE, 2003).

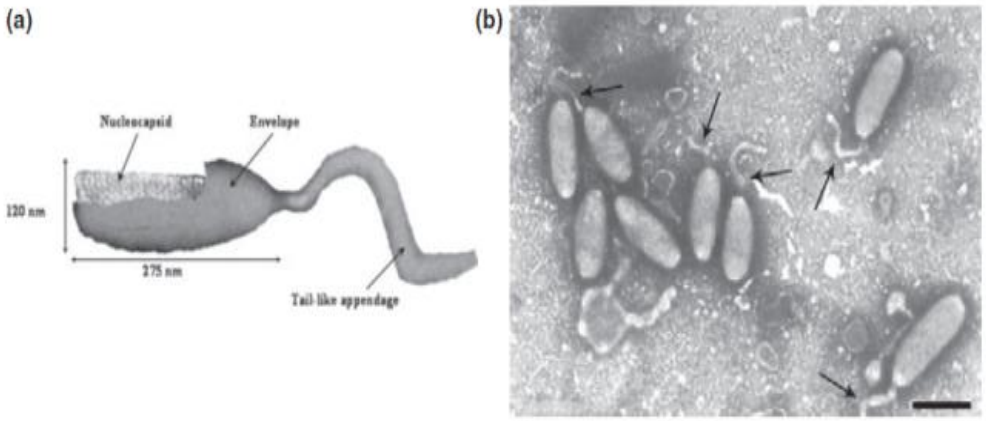

Figure 1.1 White spot syndrome virus (WSSV) virions. Morphology of WSSV (a) and electron micrograph of intact virions (b) (bar = 250 nm) (Durand et al., 1996).

However, the mechanism of WSSV infection and propagation still remains unclear although several proteins of WSSV have been identified and functional characterized. More than 40 WSSV proteins have been characterized (Table 1.1) including non-structural and structural proteins (reviewed by Escobedo-Bonilla et al., 2008). The non-structural proteins of WSSV that are probably involved in viral propagation are VP9 (Liu et al., 2006), WSV021 (Zhu et al., 2007), WSV477 (Han et al., 2007). Moreover, at least 38 structural proteins have been localized in the intact

WSSV virion. (Huang et al., 2002a; Tsai et al., 2004; Li et al., 2005a; Xie et al., 2006).

Table 1.1 List of characterized white spot syndrome virus (WSSV) proteins (Escobedo-Bonilla et al., 2008).

Protein name	Genbank accession number	Size (aminoacid residues)	Apparent size (kDa)	Putative function	Location in WSSV virion (references)
VP9	2GJIA	79	9	Transcriptional	Non-structural
VP11	AAL89262	433	11	Unknown	Not determined
VP12A (VP95)	AF402996	95	11	Structural	Tegument
VP12B (VP68)	AF411464	68	7	Structural	Envelope
VP13A	AAL89207	100	13	Energy metabolism	Not determined
VP13B (VP16)	AAL89245	117	13	Structural	Envelope
VP14	AAL89217	97	11	Structural	Envelope
VP15	AAL89137	80	15	DNA binding protein	Nucleocapsid/core
VP19	AAL89341	121	19	Structural	Envelope
WSV021	AAL33025	200	23	Regulation virus replication	Non-structural
VP22 (VP184)	AAL89227	891	100	Unknown	Not determined
VP24 (VP208)	DQ902656	208	24	Structural	Nucleocapsid
VP26	EF534253	204	26	Structural	Tegument
VP28	EF534254	204	28	Structural	Envelope
VP31	AY897235	261	31	Cell attachment	Envelope
VP32	AAL89121	278	32	Structural	Envelope
VP35	AY325896	228	26	Structural	Nucleocapsid
VP36A	AAL89002	297	36	Cell attachment	Tegument
VP33 (VP281)	EF534251	281	32	Cell attachment	Envelope
VP38A	AAL89182	309	35	Structural	Envelope
VP38B	AAL89317	321	38	Endonuclease	Not determined
VP39A	AAL89230	419	39	Structural	Tegument
VP39B	AY884234	283	32	Structural	Envelope
VP41A (VP292)	AF411636	292	33	Structural	Envelope
VP41B (VP300)	AF403003	300	34	Structural	Envelope
VP51A	AAL89162	486	51	Structural	Envelope
VP51B (VP384)	AAL89179	384	46	Structural	Envelope
VP51C (VP466)	AAL89232	466	50	Structural	Nucleocapsid
VP53A (VP150)	AAL88935	1301	144	Structural	Envelope
VP53B	AAL89039	968	53	Signal transduction pathway	Not determined
VP53C	AAL89192	489	53	Unknown	Not determined
VP55 (VP448)	AAL88919	448	55	Unknown	Not determined
VP60A (VP56)	AAL89249	465	60	Structural	Envelope
VP60B (VP544)	AAL89342	544	60	Adenovirus fibre-like protein	Nucleocapsid
VP75	AAL89256	786	75	Structural	Nucleocapsid
VP76 (VP73)	AAL89143	675	76	Class 1 cytokine receptor	Nucleocapsid
VP90	AAL89251	856	96	Structural	Envelope
VP95	AAL89370	800	89	Structural	Tegument
VP110	AAL88960	972	110	Cell attachment	Envelope
VP124	AAL89139	1194	124	Structural	Envelope
VP136A	AAL89194	1219	136	Cell attachment	Nucleocapsid
VP136B	AAL89392	1243	136	Unknown	Not determined
VP180 (VP1684)	AAL88920	1684	169	Collagen-like protein	Envelope
VP187	AAL89132	1606	174	Structural	Envelope
VP190	AAL33291	1565	174	Structural	Nucleocapsid
WSV477	DQ121373	208	30	DNA replication	Non-structural
VP664	AAL89287	6077	664	Cell attachment	Nucleocapsid
VP800	AAL02264	800	90	Unknown	Not determined

Escobedo-Bonilla et al. (2008) have also reviewed the morphogenesis of WSSV. The morphogenesis is directly related to the development of cellular lesions (Fig. 1.2).

Stage 1: The host cells are invaded by WSSV particles.

Stage 2: WSSV virion attaches to a susceptible cell using its envelope proteins binding with the cell attachment motifs.

Stage 3: WSSV recognizes and enters into the cell.

Stage 4: WSSV envelope probably fuses with the endosome allowing the naked nucleocapsid to transport the viral DNA into the nucleus of the host cell in a similar way as baculoviruses.

Stage 5: The naked WSSV nucleocapsid binds to the nuclear membrane and releases the genome into the nucleus.

Stage 6: The replication of WSSV genome proceeds.

Stage 7: The early virogenic stroma appears consisting of the viral material in the nucleus. Accumulation of the cellular chromatin is observed near the nuclear membrane and the rough endoplasmic reticulum (RER) becomes enlarged and active.

Stage 8: The dense chromatin of host cell is observed (shaded area) in contrast to the less dense virogenic stroma. The vesicles are probably formed with membranous material found in the ring zone as in baculoviruses. The virogenic stroma is stored the viral nucleosome that contained nucleocapsid proteins.

Stage 9: The assembly of new WSSV virions within an electron-dense inclusion is observed. The nucleocapsids are filled into the empty envelopes. The nuclear membranes of the host cell are disrupted.

Stage 10: The intact WSSVs are released from the disrupted cells to begin the new cycle in other susceptible cells.

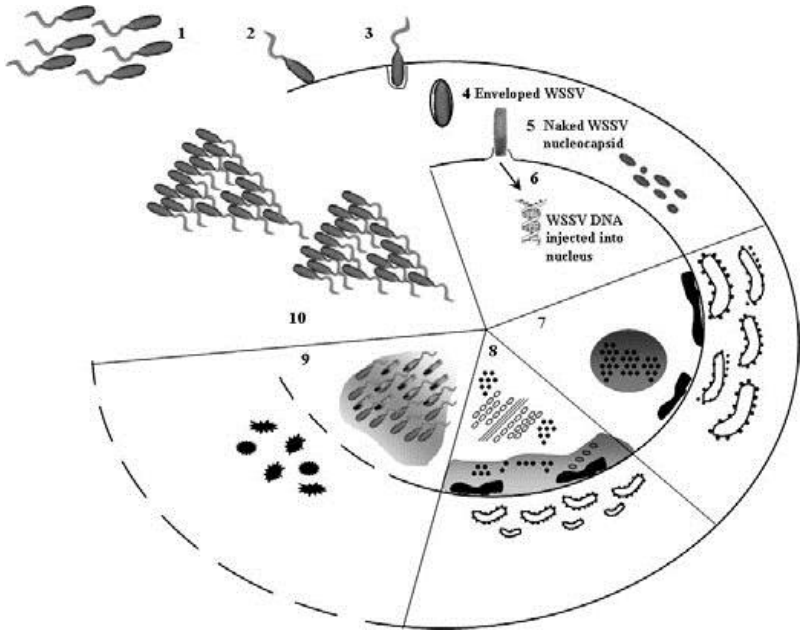

Figure 1.2 A proposed model of the morphogenesis of white spot syndrome virus (WSSV) (Escobedo-Bonilla et al., 2008).

1.3 Crustacean immunity

Shrimp and crustacean alike lack adaptive immune system. The defense mechanism depends completely on the innate immune system. The innate immune system divided into the cellular mediated and humoral mediated response. Hemocytes are the main effective tissue, which play the important role to both type of responses against the invading pathogens. In crustacean, the hemocytes are classified into three

groups; hyaline cell (agranular), semigranular cell (small granular) and granular cell (large granular) (Bauchau, 1980; Tsing et al., 1989). The functions of different hemocytes in the immune defense system are previously reported. The hyaline hemocytes are potent phagocytic cells and involved in initiation of blood clotting while generally functions in apoptosis, encapsulation, melanization and nodulation are generated by the granular hemocytes (Pech and Strand, 2000; Sung et al., 1998).

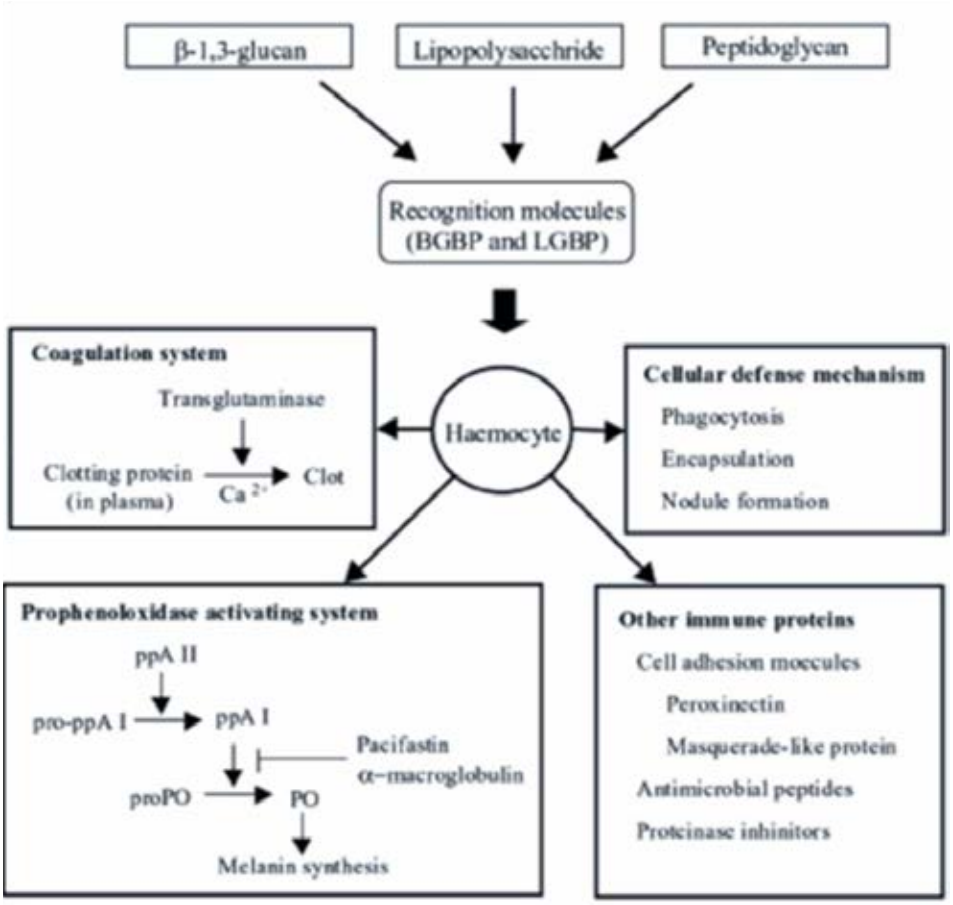

Figure 1.3 Schematic overview of crustacean defense responses (Janeway Jr, 1998).

1.3.1 Cellular-mediated defense system

Three main cell-mediated defense mechanisms are known, namely phagocytosis, encapsulation and nodule formation (Millar and Ratcliffe, 1994). These mechanisms are performed by the hemocytes.

Phagocytosis is a common process shared by several organisms. It consists of 3 major steps including foreign particle attachment, internalization and destruction (Jeon et al., 2010). In case of the big foreign matter, the hemocytes form multiple layers surrounding the foreign particle to prevent spreading of the pathogen and, hence, named the process as encapsulation (Gillespie et al., 1997). Another mechanism of entrapment of invading pathogen is called nodule formation. The entrapped foreign matter is in the center of the forming nodules. Subsequently, the invading pathogens are destroyed by secretory humoral defense molecules from the cell such as oxygen species produced by the prophenoloxidase activating system (Jiravanichpaisal et al., 2006).

1.3.2 Humoral defense system

1.3.2.1 Pattern recognition proteins

Invading of pathogenic microorganisms such as bacteria, fungi and virus, stimulates the host cell immune responses. The surface components of the foreign matter, called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acids (LTA) of Gram-positive bacteria, peptidoglycan of microbial cell wall, β-1,3-glucan of fungi, mannans of yeasts, glycolipids of mycobacteria, and double-stranded RNA of viruses, can be recognized by the first line factor, pattern recognition proteins (PRPs), of the

host cells (Hoffmann et al., 1999). The interaction can trigger the cascades of immune responses in host cells to destroy the pathogenic microorganism. Several pattern recognition proteins have been reported such as LPS binding proteins (LBPs), β-1,3-glucan-binding proteins (βGBPs), peptidoglycan recognition protein, lectins, and hemolin (Lee and Söderhäll, 2002).

1.3.2.2 Prophenoloxidase (proPO) activating system

One of the important innate immune response in crustaceans is the prophenoloxidase (proPO) activating system, which comprises of many proteases participating in the activating cascade to finally generate the cytotoxic products, oxygen species and melanin. The process to generate the melanin as end product is called melanization. It is reported the proPO acitivating system is involved in phagocytosis, encapsulation and cell adhesion (Söderhäll and Cerenius, 1998).

To generate the melanin product, pathogen-associated molecular patterns (PAMPs) of pathogenic microorganism firstly stimulated via pattern recognition proteins the prophenoloxidase-activating factors (ppA, PPAEs, PPAFs, PPAPs). This activation leads to a cascade of stepwise activation of proteinases in the proPO system, and eventually produces prophenoloxidase (PO) (Ariki et al., 2004). The system is controlled by serine protease inhibitors. The PO, then, catalyzes the conversion of monophenols to diphenols by hydroxylation reaction and oxidation of diphenols to quinones. The quinones non-enzymatically polymerize themselves to form insoluble melanin.

In the melanization steps, reaction intermediates and insoluble melanin can restrain the pathogen growth (Söderhäll and Ajaxon, 1982). In addition,

the production of melanin serves in wound healing, sclerotisation and encapsulation of foreign particles as well (Theopold, 2004). In the black tiger shrimp, four members of the proPO activating system, *Pm*proPO1, *Pm*proPO2, *Pm*PPAE1 and *Pm*PPAE2 have been identified. Gene silencing (RNAi) of these genes show significantly reduction of the total PO activity and higher mortality rate of the *V. harveyi*-challenged shrimp (Amparyup et al., 2009; Charoensapsri et al., 2009; Charoensapsri et al., 2011).

1.3.2.3 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are found in several organisms from vertebrate, invertebrate to microorganisms. AMPs are evolutionarily conserved in innate immunity. Some of these peptides have a broad spectrum killing activity against pathogenic microorganisms such as Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses. Actually, these molecules have a small size approximately less than 200 amino acid residues having cationic net charge and amphipathic structure. Owing to its small size, the AMPs can be rapidly synthesized in response to infection and readily translocated to the wound sites. In the process, they can attach and insert themselves into the membrane bilayer forming pores and resulting in the leakage of cellular components. To date, the pore forming mechanism can be classified to 3 distinct models as follows; barrel-stave, carpet and toroidal-pore models (Brogden, 2005).

Several AMPs have been discovered and their biological functions have been studied in penaeid shrimp (Tassanakajon et al., 2010) including penaeidins, crustins, lysozymes, anti-lipopolysaccharide factors, hemocyanin and stylicin. In *L.*

vannamei and *P. setferus*, penaeidins exhibit antimicrobial activity against Gram-positive bacteria and fungi (Destoumieux et al., 1997; Gross et al., 2001). Several isoforms of crustins, like the 11.5 kDa, are characterized from *Carcinus maenas*, *L. vannamei*, *L. setiferus* and *P. monodon* and possess antimicrobial activity and antiviral activity (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004; Supungul et al., 2004). The anti-lipopolysaccharide factor isoform 3 (ALF3) from *P. monodon* has a broad spectrum antimicrobial activity (Somboonwiwat et al., 2005). The ALF3 has also been characterized the anti-viral property against WSSV in *P. monodon* (Tarntada et al., 2009).

1.3.2.4 Blood coagulation system

In innate immune defense system, blood coagulation serves an important role in preventing hemolymph bleeding at the wound sites as well as obstructing pathogen dissemination throughout the body (Martin et al., 1991). The cross-linking reaction of coagulation-associated proteins in plasma is catalyzed by hemocyte-derived factors (Cerenius and Söderhäll, 2010).

In invertebrate, after stimulation of pathogen-associated molecular patterns (PAMPs) through pattern recognition proteins, hemocyte derived factors or enzymes involved in the clotting are released via exocytosis and, subsequently, converted to proteolytically active forms. These enzymes are responsible for the activation of proclotting enzyme to clotting enzyme which can transform coagulogen to coagulin. The coagulin polymerize itself by non-covalent bond to form a large insoluble gel to immobilize the pathogenic microorganisms (Cerenius and Söderhäll, 2010; Kawabata et al., 2009).

In the black tiger shrimp *P. monodon*, there are three isoforms of Tranglutaminase in the black tiger shrimp, the *Pm*STG I (Huang et al., 2004) and two members of *Pm*STG II (Chen et al., 2005; Yeh et al., 2006). These *Pm*STG IIs exert the polymerization activity using clotable proteins as a substrate; whilst *Pm*STG I lacks the blood coagulation activity.

1.3.2.5 Apoptosis

Apoptosis plays an important role in cellular development, differentiation and cell-mediated immune response. Apoptosis is a process of programmed cell death which may result from biochemical changes such as cellular blebbing and shrinkage, DNA fragmentation, and chromatin condensation leading to cell morphology disorder, and finally cell death (Kerr et al., 1977; Thompson, 1995). In crustacean, apoptosis plays a crucial role in the antiviral immunity (Liu et al., 2009). The DNA fragmentation and chromatin condensation are observed in tissues of the black tiger shrimp *P. monodon* infected with either WSSV or YHV (Khanobdee et al., 2002; Sahtout et al., 2001).

1.3.2.6 Proteinases and their inhibitors

Proteinases can be classified by their active-site catalytic residue into main proteinase families as follows: i) metalloproteinase, ii) serine proteinase, iii) cysteine proteinase, iv) threonine proteinase, v) aspartic proteinase, and vi) unidentified proteinase families. In arthropods, serine proteinases are supposed to be one of the most vital elements in the immune system. The relatively high concentration of proteinase were found in the hemolymph (Kanost, 1999; Tyndall et al., 2005; van Eijk et al., 2003).

To maintain the homeostasis, the activity of the proteinases is regulated by the cognate proteinase inhibitors. Proteinase inhibitors can be classified into 2 major groups according to their inhibitory mechanisms, active site inhibitors and alpha-2-macroglobulin (A2M). The inhibitors directly interact with proteinases at the active site are called active site inhibitors, while the A2M possesses a distinctly unique mechanism to inhibit the proteinase activity (Armstrong, 2001; Laskowski and Kato, 1980). In arthropod, several proteinase inhibitors are identified, for example, serpins, alpha-2-macroglobulin (A2M), Kunitz, Kazal, and pacifastin (Kanost, 1999).

1.4 Serine proteinase inhibitors

Serine proteinase inhibitors (SPIs) are ubiquitous and essential for homeostasis. In several multicellular organisms, they are important factors involving in controlling the various proteinase-mediated biological processes, such as the complement system, blood coagulation, melanization, apoptosis, etc. (Iwanaga et al., 2005; Jiravanichpaisal et al., 2006). They perform their function in the humoral defense response in the innate immune system against invading pathogens (Christeller, 2005).

Examples of proteinase inhibitors can be seen widely in arthropods. In *Bombyx mori*, the SPIs function to inhibit the microbial proteases and protect the silkworm pupae against pathogens (Zheng et al., 2007). In haematophagous insects such as *Dipetalogaster maximus* and *Triatoma infestans*, these insects secrete the thrombin inhibitors dipetalogastin and infestin, respectively, to prevent blood clotting during blood meal (Campos et al., 2002; Mende et al., 2004). The extracellular

protease inhibitor is produced by the oomycete *Phytophthora infestans*, the cause of disease in potato and tomato, to counter-defense the plant defensive proteinases (Tian et al., 2004; Tian et al., 2005). A male reproduction-related SPI is identified from *Macrobrachium rosenbergii* with inhibitory activity on sperm gelatinolytic activity (Li et al., 2009).

In invertebrate, six families of serine proteinase inhibitors, e.g., Kazal, BPTI-Kunitz, α-macroglobulin, serpin and pacifastin, are well known (Pham et al., 1996; Kanost, 1999). Most inhibitors are recognized by their cognate proteinases according to a common, substrate-like standard mechanism. They share an exposed, rigid binding loop with a very characteristic 'canonical' conformation (Laskowski et al., 1980; Bode, 1992).

1.5 Structural, biological and physiological functions of KPIs

Kazal-type serine proteinase inhibitors (KPIs) are well-known multi-domain SPIs usually containing more than one Kazal domain. Each domain consists of 50–60 amino acid residues with six well-conserved cysteine residues that form three intra-domain disulphide bridges (van de Locht et al., 1995). Generally, the Kazal motif has an amino acid sequence of C-X_a-C-X_b-PVCG-X_c-Y-X_d-C-X_e-C-X_f-C where the subscripts a, b, c, d, e and f are integral numbers of amino acid residues. Most of them are quite variable both within and among the invertebrate species (Cerenius et al., 2010). The reactive site loop of each domain binds tightly to an active site of cognate proteinase by having several contact positions responsible for the interactions between the Kazal domain and the proteinase (Lu et al., 1997; Bode et al., 2000).

The secondary structure of KPI domain consists of one α-helix and an adjacent three-stranded beta-sheet and can be viewed as containing three loops A, B and C (Figure 1.4). The B loop contains a determining P_1 amino acid and hence called the reactive site loop. The P_1 amino acid residue is the second amino acid residue after the second cysteine residue of the domain and is specificity determinant to inhibitory activity for its side chain has to fit into the specificity pocket of cognate serine proteinase (Laskowski Jr and Kato, 1980; van de Locht A et al., 1995).

Each Kazal domain acts as a substrate analogue that stoichiometrically binds competitively through its reactive site loop to the active site of cognate proteinase forming a relatively stable proteinase–proteinase inhibitor complex though the binding of KPI motif to the proteinase is non-covalent. In invertebrate, the typical inhibition constants (K_is) are in the range of nanomolar compared to the millimolar range of K_m values of the synthetic proteinase substrates (Somprasong et al., 2006; Wang et al., 2009; Visetnan et al., 2009; Li et al., 2009a; Li et al., 2009b; González et al., 2007a; González et al., 2007b; Campos et al., 2002). To study the biological functions of KPIs, the regulation of serine proteinases, the target proteinases and the inhibitory specificities can be used as clues to the actual functions of the KPIs.

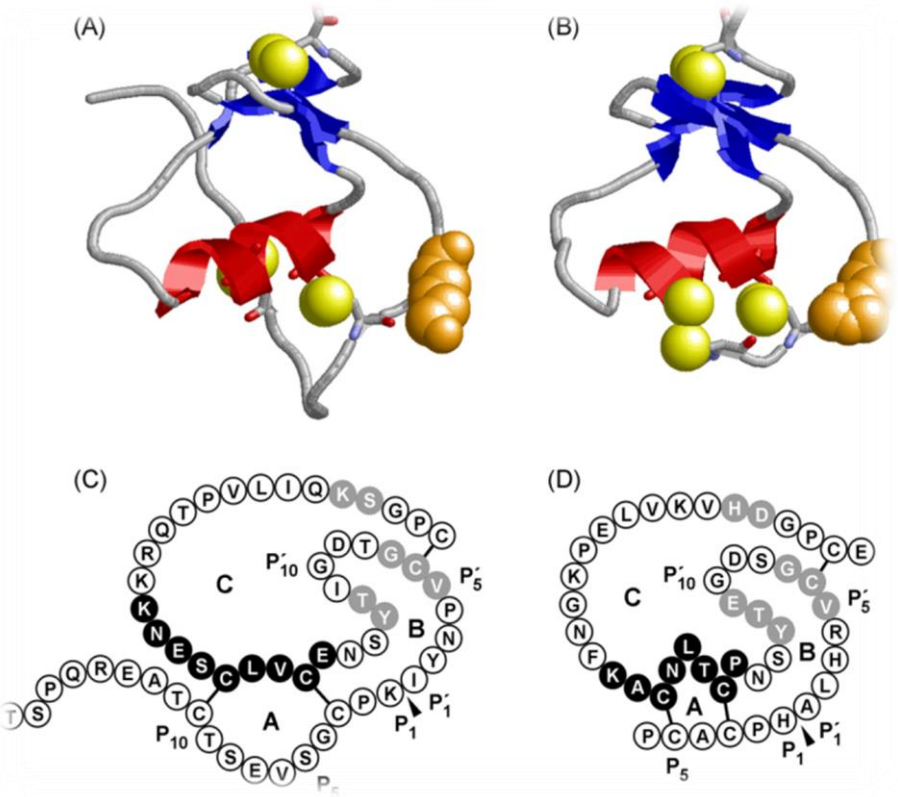

Figure 1.4 The structure of Kazal proteinase inhibitors. The structure of vertebrate KPIs, porcine pancreatic secretory inhibitor derived from the PDB file 1TGS (Bolognesi et al., 1982) (A) and with its covalent primary structure (C) compared with the structure of invertebrate KPIs, rhodniin domain 1 derived from the PDB file 1TBQ (van de Locht et al., 1995) (B) and its covalent primary structure (D). The secondary structure, helices and sheets are shown in red and blue but in black and gray circles in the covalent primary structures, respectively. The sulphur atoms are presented as yellow balls. The space-filled side chains of the P_1 amino acids are in orange color.

1.5.1 Blood feeding

Studying the prevention of the blood clotting in leech *Hirudo medicinalis*, two one-domain "non-classical" Kazal-type proteinase inhibitors, namely the bdellin

B-3 and leech-derived tryptase inhibitor (LDTI) are identified (Sommerhoff et al., 1994; Fink et al., 1986). The P_1 Lys of bdellin B-3 can inhibit trypsin, plasmin and sperm acrosin while the LDTI with the same P_1 Lys inhibits trypsin and chymotrypsin. The inhibitory activity of bdellin B-3 against plasmin prevents the plasmin-mediated platelet activation that leads to platelet clot as a result the bdellin B-3 helps the hirudin to block the blood coagulation (Quinton et al., 2004).

A double-headed Kazal-type proteinase inhibitor, rhodniin, from bug *Rhodnius prolixus* is synthesized to prevent the host blood coagulation and is highly specific for thrombin (Friedrich et al., 1993). Another blood-sucking insect *Dipetalogaster maximus* produces a six-domain Kazal-type thrombin inhibitor but the active inhibitor, dipetalogastin, is double-headed of domains 3 and 4 with the P_1 Arg and Asp, respectively (Mende et al., 1999; 2004). The infestin in the midgut of *T. infestans* plays an important role as anticoagulant during the ingestion of blood meal. The recombinant infestin 4 exhibits very strong anticoagulant activity (Campos et al., 2004b). An eight-domain KPI, brasiliensin, from a related blood-sucking bug *Triatoma brasiliensis* is very similar to infestin (Araujo et al., 2007). The reduction of brasiliensin in the midgut by RNA interference reduces the ingestion of blood stressing the important role of anticoagulant in blood feeding in these *Triatoma* bugs.

1.5.2 Reproduction

In prawn, *Macrobrachium rosenbergii*, a Kazal-type peptidase inhibitor (MRPINK), a double-headed KPI with the P_1 Leu and Pro, has been identified in the male reproductive tract (Cao et al., 2007). The MRPINK also has an inhibitory effect

on the gelatinolytic activity of sperms, catalyzed by a *M. rosenbergii* sperm gelatinase (MSG) (Li et al., 2008).

1.5.3 Prevention of excessive autophagy

Autophagy is a process of cell degradation and recycle of cellular components using lysosome. When the *Kazal1* in *Hydra magnipapillata* is suppressed using the dsRNA feeding, excessive autophagy was observed in gland cells and endodermal digestive cells (Chera et al., 2006). The Kazal1 is a three-domain KPI (P_1 Met, Arg and Arg) expressed from the gland cells. The *Kazal1* knockdown affects homeostatic conditions, for example disorganization and death of gland cells and highly vacuolation of digestive cells. Therefore, the *Kazal1* gene is required for the modulation of autophagy like the pancreatic secretory trypsin inhibitor *SPINK1* and *SPINK3* genes in human and mice, respectively (Masaki et al., 2009).

1.5.4 Protection from microbial destruction

To prevent the microbial invasion, two serine proteinase inhibitors belong to the Kunitz and Kazal families named silk proteinase inhibitors 1 (*Gm*SPI 1) and 2 (*Gm*SPI 2) is identified in the wax moth *Galleria mellonella* (Nirmala et al., 2001a). Moreover, two similar serine proteinase inhibitors, a Kunitz-type *Bm*SPI 1 and Kazal-type *Bm*SPI 2, are also present in the cocoon silk of *Bombyx mori* which inhibit subtilisin and proteinase K (Nirmala et al., 2001b).

1.5.5 Protection against pathogen proteinases and antimicrobial activity

Extracellular microbial proteinases are virulent factors, produced during the development of the infectious diseases. They help the microorganism or pathogens

to penetrate through physical barrier of their hosts (Travis et al., 1995; Christeller, 2005). Therefore, the serine proteinase inhibitors from the host cell have a crucial role to protect themselves. In Chinese white shrimp *Fenneropenaeus chinensis*, one of the four KPIs, *Fc*SPI-1, from the hepatopancreas is identified and shown the inhibitory activity against the microbial proteinases, subtilisin and proteinase K. This KPI may participate in the immune defense response (Wang et al., 2009).

There are several reports about the up-regulation of gene expression of KPI during infection, for instance, the mRNA transcript of mollusk KPI (*AI*SPI), *Argopecten irradians,* encoding a six-domain KPI, is up-regulated after *Vibrio anguillarum* injection (Zhu et al., 2006). In the Zhikong scallop *Chlamys farreri* (*Cf*KZSPI), a twelve-domain KPI gene is highly expressed after *V. anguillarum* challenge (Wang et al., 2008). The expression of a double-headed KPI gene from the hemocyte of white shrimp *Fenneropenaeus chinensis* is up-regulated upon white spot syndrome virus (WSSV) infection (Kong et al., 2009).

1.5.6 Protection from host proteinases

The parasite *Toxoplasma gondii* produces a four-domain KPI, *Tg*PI-1, which strongly inhibits trypsin, chymotrypsin, pancreatic elastase and neutrophil elastase (Pszenny et al., 2000; Morris et al., 2002). The natural *Tg*PI-1 probably functions as protection against digestive enzymes to help the parasite proliferate in the intestine before disseminating to other host tissues. Moreover, another four-domain KPI, *Tg*PI-2, is found to inhibit only trypsin. The actual function of *Tg*PI-2 is not known (Morris and Carruthers, 2003).

1.6 Kazal-type serine proteinase inhibitors in shrimp

A Kazal-type serine proteinase inhibitors are abundant proteins in crustaceans including shrimp (van Hoef et al., 2013). KPI have been reports in many crustaceans like penaeid shrimp, prawn and crayfish, e.g., SPI*Pm* inhibitors from *P. monodon* (Visenan et al., 2009), a *Fc*KPI from *Fenneropenaeus chinensis* (Kong et al., 2009), a *Mr*KPI from *Macrobrachium rosenbergii* (Cao et al., 2007) and *Pl*KPIs from *Pacifastacus leniusculus* (Donpudsa et al., 2010). Recently, a five-domain Kazal serine proteinase inhibitor was identified from the crayfish *Procambarus clarkii* (Zeng and Wang, 2011). Moreover, a one-domain Kazal serine proteinase inhibitor, *Pt*KPI, has been identified in the swimming crab *Portunus trituberculatus* (Wang et al., 2012). In *Penaeus vannamei*, a four-Kazal domain-containing protein has been reported (Vargas-Albores and Villalpando, 2012).

In *Penaeus monodon*, there are at least 9 types of KPIs identified from the EST database (Tassanakajon et al., 2006; Visetnan et al., 2009). A five-domain KPI, SPI*Pm*2, is the most abundant KPI and implicated in host defense against pathogens (Visetnan et al., 2009; Supungul et al., 2002). It also possesses strong inhibitory activities against subtilisin and elastase (Somprasong et al., 2006). Additionally, the SPI*Pm*2 also possesses bacteriostatic activity against *Bacillus subtilis* (Donpudsa et al., 2009).

From the microarray data, the SPI*Pm*2 was found to be up-regulated upon yellow head virus (YHV) and WSSV infection. Several immune-related genes including the SPI*Pm*2 were up-regulated after YHV infection using the suppression subtractive hybridization approach (Prapavorarat et al., 2010). The SPI*Pm*2 is

produced mainly by the hemocytes and secreted into the circulation (Donpudsa et al., 2010). In response to WSSV challenge, the SPI*Pm*2 producing hemocytes in the shrimp are significantly reduced. It seems to be that the hemocytes secrete SPI*Pm*2 into the circulation system. Moreover, the injection of recombinant SPI*Pm*2 (rSPI*Pm*2) into the shrimp before WSSV challenge reduces the WSSV replication and helps prolong the lives of WSSV-infected shrimp (Donpudsa et al., 2010). It is, then, interesting to find out how the WSSV stimulate this secretion of SPI*Pm*2 and fights against the infection.

1.7 Purpose of this thesis research

The potential role of SPI*Pm*2 against the major shrimp pathogen WSSV is examined both in vitro and in vivo. The production and secretion of SPI*Pm*2 in response to WSSV infection is determined. The number of SPI*Pm*2-producing cells is observed using immunocytochemistry. It is also determined whether the virus stimulates the secretion of the SPI*Pm*2.

Injection of the recombinant SPI*Pm*2 (rSPI*Pm*2) into the shrimp before WSSV challenge was examined. To test the antiviral properties of SPI*Pm*2, the shrimp primary cell culture was used to test the antiviral activity of rSPI*Pm*2 to WSSV in term of neutralization and protection. The rSPI*Pm*2 was further investigated for its interaction with WSSV and/or shrimp hemocyte membrane using ELISA technique. Moreover, the localization of SPI*Pm*2 in the normal and WSSV infected hemocytes was observed using confocal laser scanning microscope and transmission electron microscope in conjunction with the use of the immunogold labeling. The yeast two-

hybrid screening technique is used to identify the target proteins of SPI*Pm*2. The study of the target protein may provide insight into the mechanism of action of SPI*Pm*2 on the viral replication process.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

-20^{o}C Freezer (Whirlpool)

-80^{o}C Freezer (ThermoForma)

Amicon Ultra concentrators (Millipore)

Avanti J-30I high performance centrifuge (Beckman Coulter)

Autoclave Model # LS-2D (Rex all industries Co. Ltd.)

Automatic micropipette P10, P20, P100, P200, and P1000 (Gilson Medical Electrical S.A.)

Confocal Laser Scannin Microscopy (OLYMPUS FV1000)

FlUOstar Optima (BMG Labtech)

Gel documentation system (GeneCam FLEX1, Syngene)

Hematocytometer (Cole-Parmer)

Hybridization oven (Hybrid)

iCycler iQ^{TM} Real-Time Detection System (Bio-Rad)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

Laminar flow: Dwyer Mark II Model # 25 (Dwyer instruments)

Microcentrifuge tube 0.5 and 1.5 ml (Axygen)

Nipro disposable syringe (Nissho)

Orbital Shaker (Gallenkamp)

Oven series 8000 (Contherm)

PCR Mastercycler (Eppendorf AG, Germany)

PCR thin wall microcentrifuge tube 0.2 ml (Axygen)

pH meter Model # SA720 (Orion)

Pipette tips 10, 20, 200, and 1000 µl (Axygen)

Polylysine slides (Thermo Scientific)

Power supply: Power PAC 300 (Bio-RAD Laboratories)

Refrigerated centrifuge Model # J2-21 (Beckman)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Stirring hot plate (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

96 and 24 well cell culture cluster, flat bottom with lid (Costar)

Water bath (Memmert)

2.1.2 Chemicals and reagents

0.22 µm millipore membrane filter (Millipore)

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen)

Alexa Fluor 568 goat anti-mouse IgG antibody (Invitrogen)

Alkaline phosphatase-conjugated goat anti-mouse IgG (Jeckson ImmunoResearch)

Alkaline phosphatase-conjugated goat anti-rabbit IgG (Jeckson ImmunoResearch)

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

Ampicillin (BioBasic)

Anti-His tag antibody (GE Healthcare)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

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

Boric acid, BH_3O_3 (Merck)

Bromophenol blue (BDH)

Calcium sulfate (Ajax)

Chloroform, $CHCl_3$ (Merck)

Coomassie brilliant blue R-250, $C_{45}H_{44}N_3O_7S_2Na$ (Sigma)

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

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

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

Ethidium bromide (Sigma)

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

Formaldehyde, CH_2O (BDH)

GeneRuler™ 100bp DNA Ladder (Fermentas)

Glacial acetic acid (J.T. Baker)

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine NH_2CH_2COOH (Scharlau)

Horseradish peroxidase-conjugated goat anti-mouse IgG (Jeckson ImmunoResearch)

Hydrochloric acid, HCl (Merck)

Imidazole (Fluka)

Isopropanol, C_3H_7OH (Merck)

Magnesium sulfate (Ajax)

Methanol, CH_3OH (Merck)

2-mercaptoethanol, C_2H_6OS (Fluka)

Nickel grids (Polysciences)

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

Nitroblue tetrazolium (NBT) (Fermentas)

NBT (nitroblue tetrazolium)/BCIP (5-bromo-4-chloro-indolyl phosphate) tablet (Sigma)

Paraformaldehyde (Sigma)

Phosphoric acid (Lab scan)

PMSF (phenylmethanesulfonylfluoride) (Sigma)

Potassium sulfate (Ajax)

Prestained protein molecular weight marker (Fermentas)

Prolong Gold Antifade Reagent (Invitrogen)

Protein A CL4B sepharose (Amersham Biosciences)

Skim milk powder (Mission)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

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

Sodium hydrogen carbonate, $NaHCO_3$ (BDH)

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

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

Sodium hydroxide, NaOH (Eka Nobel)

Sulfuric acid (Carlo erba)

N, N, N′, N′-tetramethylethylenediamine (TEMED) (BDH)

TO-PRO-3 iodide (Invitrogen)

TRIreagent (Molecular biology)

Triton X-100 (Merck)

Tween™-20 (Fluka)

Whatman 3 MM™ filter paper (Whatman)

2.1.3 Enzymes and kit

RevertAID™ First strand cDNA synthesis kit (Fermentas)

Matchmaker™ Gold Y2H System (Clontech)

Make Your Own “Mate&Plate™” Library system (Clontech)

Maxima™ SYBR Green qPCR Master Mix (Fermentas)

NucleoSpin Extract II Kits (Macherey-Nagel)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase (Promega)

T7 RiboMAX™ Express Large Scale RNA Production System (Promega)

Taq DNA polymerase (RBC)

Yeastmaker™ Yeast Transformation System2 (Clontech)

2.1.4 Microorganisms

Escherichia coli strain Rosetta (DE3)

Escherichia coli strain Top10

Escherichia coli strain XL-1-Blue

White spot syndrome virus

2.1.5 Software

SPSS statistics 17.0 (Chicago, USA)

2.1.6 Vectors

pBad/Myc/His-A (Novagen)

pGADT7 (Clontech)

pGADT7-T Control Vector (Clontech)

pGBKT7 (Clontech)

pGBKT7-53 Control Vector (Clontech)

2.2 Shrimps

P. monodon Juveniles, approximately 3-5 g of body weight for knockdown experiment and 15-20 g of body weight for the general purposes, were obtained from shrimp farm (Chuntaburi Province), Thailand. The animals were acclimated for 3 days in indoor tanks with water temperature ranged between 26 and 32 °C and salinity at 15 parts per thousand (ppt) before experimentation. Shrimps were fed twice daily with compound shrimp diet based on 5% of body weight

2.3 Production and purification of the recombinant SPI*Pm*2 protein

The recombinant SPI*Pm*2 (rSPI*Pm*2) was successfully expressed in *E. coli* Rosetta(DE3)pLysS using an expression plasmid pSPI*Pm*2-NS2 by Somprasong et al. (2006) and the methods to produce and to purify the rSPI*Pm*2 by Donpudsa et al. (2009). A single colony of *E. coli* Rosetta(DE3)pLysS containing the pSPI*Pm*2-NS2 was cultured in LB medium under vigorous shaking at 37 °C for an overnight as a starter. The starter was then inoculated into fresh LB medium (1:100) and cultured until the optical density at 600 nm reached 0.6–0.8. Consequently, the culture medium was added 1 M IPTG to the final concentration of 1 mM for induction.

Four hours after IPTG induction, the bacterial cells were collected by centrifugation at 8,000× g for 10 min, frozen completely at −80 °C and thawed at room temperature for three times. The cell pellet was resuspended in PBS buffer (50 mM phosphate buffer pH 7.4) and then sonicated for 2 min. The cell pellet was collected by centrifugation at 10,000× g for 15 min. The pellet was washed twice with 0.5 M NaCl, 2% Triton X–100, twice with 0.5 M NaCl and twice with distilled water.

The pellet was solubilized with 50 mM sodium carbonate buffer pH 10 for an overnight at room temperature. The dissolved protein was collected by centrifugation and the pellet was discarded.

Ni-NTA bead was used to purify the rSPI*Pm*2 protein following the manufacturer standard protocol (GE-Amersham). One milliliter of Ni-NTA bead (50% slurry bead) was loaded into a PD10-disposable column and equilibrated with binding buffer (50 mM carbonate buffer pH 10, 0.3 M NaCl and 50 mM immidazole) for 10 volumes. The column was loaded with the crude protein preparation and, then, washed with the binding buffer for 10 volumes. The recombinant protein was eluted with 5 ml of elution buffer (50 mM carbonate buffer pH 10, 0.3 M NaCl and 300 mM immidazole). The purified rSPI*Pm*2 was dialyzed against PBS buffer pH 7.4. The protein concentration of rSPI*Pm*2 was determined using Bradford method (Bradford, 1976)

2.3.1 Total protein determination

The protein concentration of rSPI*Pm*2 was determined by Bradford assay (Bradford, 1976). Appropriate volume of purified protein was mixed with Bradford working buffer, mixed and, then, incubated for 2 min. The solution was observed the optical density at 595 nm and compared to the standard curve using the BSA as standard protein.

2.3.2 Detection of the recombinant protein by SDS-PAGE

The SDS-PAGE system was performed according to the method of Laemmli (1970) (Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.). The slab gel

(10 × 10 × 0.75 cm) system consisted of 0.1% (w/v) SDS in 12% (w/v) separating gel and 5% (w/v) stacking gel. Tris-glycine buffer pH 8.3 containing 0.1% (w/v) SDS was used as electrode buffer. The gel preparation was described in Appendix B. The cell pellet from recombinant protein production was resuspended in sample buffer and boiled for 10 min before loading to the gel. The electrophoresis was performed at constant current of 20 mA per slab at room temperature from cathode towards anode. The gel was stained with coomassie staining solution to visualize the protein bands.

2.3.3 Detection of the recombinant protein by Western blot analysis

To transfer protein to the nitrocellulose membrane, the recombinant protein was separated on 12% SDS-PAGE gels. The gel was removed from slab. The nitrocellulose membrane, gel and blotting paper were incubated for 15 min in the blotting buffer (48 mM Tris pH 9.2, 39 mM glycine and 20% methanol). They were laid on the Trans-Blot® SD (Bio-Rad) machine in a sandwich model avoiding air bubbles between the layers. Protein transfer was carried out at constant electricity of 80 mA for 1 h. After transferring, the membrane was incubated in blocking buffer (5% (w/v) skim milk, 0.05% (v/v) Tween 20 in PBS buffer) for 1 h at room temperature and, then, washed 3 times for 5 min each in washing buffer (0.05% (v/v) Tween 20 in PBS buffer).

To detect the protein, the membrane was incubated with rabbit antibody diluted 1/10,000 or mouse antibody diluted 1/3,000 in 1% (w/v) skim milk in washing buffer at 37 °C for 1 h. The membrane was washed as above and incubated with secondary antibody, anti-rabbit IgG conjugated with alkaline phosphatase, or secondary antibody, anti-mouse IgG conjugated with horseradish peroxidase, diluted

1/10,000 in the same buffer as primary antibody for 1 h at room temperature. After washing, the membrane was observed in the detection solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and stopped by rinsing the membrane twice with water.

2.4 Purification of WSSV

The WSSV stock was diluted in TN buffer (20 mM Tris-HCl pH 7.4 and 400 mM NaCl,). White shrimp (*Litopenaeus vanamei*) were intramuscularly injected with 100 µl WSSV solution and reared to the moribund stage. The moribund shrimp after having survived to day 5 were collected and the gill tissue was removed for the purification of viral particles. The purification method was slightly modified as described by Xie et al. (2005). Gill tissue was homogenized in TNE buffer (50 mM Tris-HCl pH 8.5, 400 mM NaCl and 5 mM EDTA) and, then, centrifuged at 3,500× g for 15 min at 4 °C. The supernatant was filtrated using 0.22 µM millex-GS syringe-driven filter (Merck Milipore) and further centrifuged at 30,000× g for 30 min at 4 °C to pellet the virions. The pellet was rinsed with TM buffer (50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$) and centrifuged at 3,500× g for 10 min. Subsequently, the pellet was suspended in TM buffer and, then, supernatant was subjected to centrifugation at 30,000× g for 30 min at 4 °C. The pellet was then suspended in TM buffer and divided into aliquots and stored at −80 °C until use.

2.5 Purification of primary antibody

Protein A Sepharose CL-4B (GE Healthcare) was used to purify the IgG antibody form anti-serum according to the manufacturer standard protocol. The bead was applied into the column and washed with 10 volumes of PBS. Anti-sera was loaded into the column and washed with PBS to remove non-specific proteins. After washing, IgG antibody was eluted with elution buffer (100 mM glycine pH 2.5) and the eluted antibody was immediately added with 1 M Tris pH 9.5 to neutralize the solution.

2.6 Immunocytochemistry of SPI*Pm*2 in hemocyte of WSSV infected shrimp

The healthy shrimp were injected with purified WSSV and collected the hemolymph using a modified Alsever solution (MAS: 27 mM sodium citrate, 336 mM NaCl, 115 mM glucose and 9 mM EDTA, pH 7.0) at 0, 6, 24 and 48 h after WSSV injection. The hemolymph was separated the hemocytes from the plasma by centrifugation at 800× g at 4 °C for 10 min and, then, fixed in 4% (w/v) paraformaldehyde. The hemocytes of 2×10^5 cells were spun down onto the poly-L-lysine coated slide at 1,000× g for 5 min. Two sets of slides of hemocytes at different time points were made. They were blocked for non-specific binding with a blocking buffer (PBS containing 1% (v/v) normal goat serum, 1% (w/v) BSA and 0.1% (v/v) Triton X-100) for 1 h at room temperature.

One set of slide was incubated with a primary antibody, rabbit polyclonal antibody specific to SPI*Pm*2. The other set of slide was incubated with normal rabbit anti-serum as control. After 2 h post primary antibody incubation, the slides were

washed in PBS/0.05% Tween-20 (PBS-T) buffer and incubated with a secondary antibody, alkaline phosphatase conjugated goat anti-rabbit IgG (1:2,000 dilution in the blocking buffer) (Jackson Immunoresearch) for 1 h at room temperature. The slides were, then, washed three times in PBS-T buffer, incubated with a detection solution (375 mg/ml NBT, 188 mg/ml BCIP and 1 mM levamisole in 0.1 M Tris–HCl pH 9.5, 50 mM $MgCl_2$ and 0.9% (w/v) NaCl) and visualized under a light microscope (Olympus). The hemocytes of 300 were counted per slides to observe the SPI*Pm*2-positive cells and shown as percentages. The data were obtained from 3 individual shrimp at each time point and shown as mean ± standard deviation. The statistical significance of the data was evaluated using one-way ANOVA followed by post hoc test (Duncan's new multiple range test). Significant differences were accepted at $P < 0.05$.

2.7 Effect of rSPI*Pm*2 on the survival of WSSV-infected shrimp

Shrimp were divided into five groups of ten shrimp. The group number 1-5 was injected with saline, saline, 0.5 nmol rSPI*Pm*2, rabbit serum (1:10 dilution) and anti-SPI*Pm*2 (1:10 dilution), respectively. After 24 h, groups 2–5 were injected with WSSV having the LD_{50} of 3 days and observed the mortality for ten days. The experiment was repeated three times.

2.8 Immunolocalization of SPI*Pm*2 protein and viral proteins, VP28 or WSV477 in hemocytes of WSSV-infected shrimp

Three individuals of normal and WSSV injected shrimp at 6, 24, 48 h, and moribund stage were collected the hemolymph using 4% (w/v) paraformaldehyde (1:1) for fixation. After 10 min, the fixed hemolymph was centrifuged at 800× g at 4 °C for 10 min to separate the hemocytes. The fixed hemocytes were washed twice with and resuspended in phosphate buffered saline (PBS). The hemocytes were counted using hematocytometer. The hemocytes of 10,000 cells per one slide were spun down at 1,000× g for 10 min onto the poly-L-lysine coated slide. To block the non-specific binding, the slides were incubated with blocking buffer (10% fetal bovine serum in PBS) at room temperature for 1 h and then probed with purified rabbit antibody specific to rSPI*Pm*2 and purified mouse antibody specific to VP28 or WSV477 for 1 h at 37 °C. The slides were washed 3 times with washing buffer (0.05% Tween-20 in PBS) and incubated with the secondary antibody conjugated with fluorescence dye, Alexa 488 (goat anti-rabbit antibodies) (Invitrogen) and Alexa 568 (goat anti-mouse antibodies) (Invitrogen), at room temperature for 1 h. To remove the non-specific binding, the slides were washed 3 times with washing buffer and then incubated with TO-PRO-3 iodide (Invitrogen) to stain the nuclear DNA. After washing once with PBS, the slides were applied with mounting medium ProLong Gold antifade (Invitrogen) and visualized the fluorescent signal under a confocal laser scanning microscopy (Nikon).

2.9 Shrimp hemocyte primary cell culture

Three healthy shrimp were collected the hemolymph using a modified Alsever solution (MAS: 27 mM sodium citrate, 336 mM NaCl, 115 mM glucose and 9 mM EDTA, pH 7.0) and immediately centrifuged to separate the hemocytes at 800× g at 4 °C for 10 min. The hemocytes were resuspended in L15 medium with 20% fetal bovine serum (GIBCO) (Jiang et al., 2006). The cell suspension was counted the number of hemocyte using the hematocytometer. The hemocytes were adjusted the cell concentration to 2×10^5 cell per ml with L15 fresh medium and 100 µl and 500 µl of these cells were applied into each well of the 96-well plate and 24-well plate for studying the secretion of SPI*Pm*2 protein and antiviral activity, respectively. The hemocytes were incubated at 28 °C for 24 h for proper cell attachment to the bottom of plates. The culture medium was replaced with 100 ml or 500 ml of fresh medium every day. Under this condition, the cell culture could be maintained more than 5 days with less than 5% cell death in 3 days as determined by trypan blue staining and cell number counting using hematocytometer (Strober, 2001).

2.10 Secretion of SPI*Pm*2 from the hemocytes post WSSV challenge

The hemocyte primary cell culture in 96-well plates was challenged with WSSV by adding the purified WSSV of 1.75×10^5 copies/well into the culture medium. The cells treated with PBS were used for control experiment. The multiplicity of infection was about 10:1. The hemocytes and culture medium were collected at 0, 1, 3 and 6 h after the viral challenge. Western-blot analysis was used to detect the protein expression of *VP28*, *SPIPm2* and *β-actin*. The total protein of

hemocytes and culture medium at different time point were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The protein expression was observed using purified rabbit polyclonal anti-SPI*Pm*2, purified mouse monoclonal anti-VP28 (kindly provided by Professor Paisarn Sithigorngul) and anti-actin (Millipore), respectively. The colors were developed using secondary antibodies conjugated with alkaline phosphatase (purple color) or horseradish peroxidase (brown color).

2.11 Effect of rSPI*Pm*2 against WSSV in hemocyte primary cell culture

To study the antiviral activity of rSPI*Pm*2 against WSSV, the hemocyte primary cell culture in 24-well plates was used to test in term of the neutralization and protection.

2.11.1 Neutralization of rSPI*Pm*2 and purified WSSV

The purified rSPI*Pm*2 and purified WSSV were produced to test the antiviral properties in hemocyte primary cell culture. The purified WSSV (1.75×10^5 copies) was incubated with or without 2 mM rSPI*Pm*2 in PBS for 30 min and the mixture was added into the cell culture. The multiplicity of infection was about 2:1 and the final concentration of SPI*Pm*2 was 0.32 mM per well. After 6 and 24 h post WSSV challenge, the hemocytes were collected and detected the mRNA expression of *VP28* for the analysis of viral propagation by RT-PCR and qRT-PCR.

2.11.2 Protective effect of rSPI*Pm*2 against WSSV

The final concentration of 0.32 mM rSPI*Pm*2 or equivalent amount of PBS as a control were pre-incubated with hemocyte cell cultures for 30 min. To remove

the rSPI*Pm*2, the culture medium was discarded and washed once with fresh culture medium and added with fresh culture medium. The purified WSSV was diluted in PBS (1.75×10^5 copies/well) and then added into the cell culture. These primary cell cultures were incubated at 28 ℃ and collected at 6 and 24 h post WSSV challenge. The hemocytes were detected the mRNA expression of *VP28* and *β-actin* using RT-PCR and qRT-PCR to determine the viral propagation.

2.11.3 Dose-dependent of SPI*Pm*2 against WSSV

The various concentrations of rSPI*Pm*2 (0.02, 0.08, 0.32 and 0.64 mM final concentrations) and WSSV (1.75×10^5 copies/well) were used to test the effect of rSPI*Pm*2. The hemocyte cell cultures were incubated with various concentrations of SPI*Pm*2 or PBS and purified WSSV for 24 h. The hemocytes were collected and detected the mRNA expression of *VP28* and *β-actin* using RT-PCR and qRT-PCR to determine the viral propagation.

2.12 RT-PCR and quantitative real time RT-PCR

Primary hemocyte cell culture or hemocytes were extracted the total RNA using the TRI Reagent (Molecular Research Center) according to the manufacturer's instruction. Hemocytes were homogenized in 200 µl TRI Reagent and, then, the volume of TRI Reagent was adjusted to 1 ml. The homogenate was added 100 µl of chloroform (Merck) and immediately vortexed. The mixture was incubated at RT for 5 min and centrifuged at 12,000× g for 15 min to separate the aqueous solution. To precipitate the total RNA, one volume of isopropanal was added. The precipitated RNA was separated by centrifugation at 12,000× g for 15 min and then washed with

70% ethanol. The RNA pellet was air-dried for 5 min at room temperature and dissolved in the DEPC-treated water. Total RNA concentration was determined using spectophotometer (Beckman).

One µg of total RNA was treated with 1 µl of RQ1 RNase-free DNase (Promega) at 37 °C for 15 min. One µg of DNase-treated total RNA was used to synthesize the first stand cDNA according to the manufacturer's instruction (Fermentas). The gene specific primers for *VP28* gene for WSSV-infected culture and the internal control gene *β-actin* (Table 1) were used to amplify the PCR products. The PCR reaction contained 10× PCR buffer, 200 µM dNTP, 0.2 µM of each specific primer, 1 unit Taq DNA polymerase (RBC) and 4 µl of 1:10 dilution of the cDNA template in a 25 µl PCR reaction volume. The reactions were performed by the following conditions: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s and the final extension at 72 °C for 10 min. The PCR products were determined using 1.5% agarose gel electrophoresis and visualized using Gel Documentation System (Syngene)

The expression level of *VP28* and *β-actin* gene was also analyzed by quantitative real time RT-PCR (qRT-PCR). The gene specific primers used for qRT-PCR are shown in Table 1. The reaction volume was 20 µl containing 10 ml of 2× iQ SYBR® Green Supermix (Bio-Rad), 0.1 mM each forward and reverse primers, and 5 µl of 1:10 diluted cDNA template. All runs employed a negative control without target DNA. The qRT-PCR was performed using the iCycler Thermal Cycler (Bio-Rad). The PCR profile was as follows: 95 °C for 8 min, followed by 40 cycles of 95

°C for 15 s, 60 °C for 20 s and 72 °C for 10 s. Each sample was done in triplicate. The threshold cycle (C_t) for each sample was analyzed by a mathematical model described by Pfaffl (Pfaffl, 2001). The data were shown as means ± standard deviations. Statistical analysis was done using the independent samples t-test and one-way ANOVA followed by post hoc test (Duncan's new multiple range test). Data differences were considered significant at $P < 0.05$.

2.13 Binding activity of rSPI*Pm*2 and WSSV or shrimp hemocyte membrane using ELISA technique

The purified rSPI*Pm*2 was used to test binding with purified WSSV and shrimp hemocyte membrane (SHM) prepared according to Duvic and Söderhäll (Duvic and Söderhäll, 1992).

Enzyme-linked immunosorbent assay (ELISA) was used to determine the binding activity. The purified WSSV and SHM were coated into the bottom of a 96-well plate by adding 100 µl of 1 mg/ml protein equivalent of the samples, incubated at 37 °C for 2 h and washed twice with 200 µl washing buffer (0.05% Tween-20 in PBS). The control wells were incubated with PBS only. To block the non-specific binding, the coated plates were incubated with 5% BSA in PBS for an overnight and then incubated with various amounts of rSPI*Pm*2 (0, 1.2, 2.4, 6 and 12 nM final concentrations in 100 µl PBS) for 1 h at room temperature. The wells were washed once with 200 µl washing buffer.

To detect the rSPI*Pm*2 binding, the wells were incubated with 100 µl mouse monoclonal anti-His-tag (1:3000) in 0.1% BSA in PBS for 1 h and then washed twice

with 200 µl washing buffer. The plate was then incubated with 100 µl alkaline phosphatase-conjugated secondary anti-mouse IgG (1:5000) in 0.1% BSA in PBS for 1 h, washed twice and developed color with *p*-nitrophenylphosphate (Bio-Rad) according to Engvall and Perlmann (Engvall and Perlmann, 1971). To determine the ELISA score by FLUOstar OPTIMA microtiter plate reader (BMG LABTECH) at 405 nm optical density, The reaction was added 100 µl 0.4 N NaOH to stop the reaction. Experiments were performed in triplicate for statistical analysis. The score of A_{405} reading of the WSSV and SHM samples were subtracted from those of PBS controls and, then, from those without rSPI*Pm*2 blank. The results were shown as means ± standard deviations. The statistical significance was evaluated using one-way ANOVA followed by post hoc test (Duncan's new multiple range test). Significant differences were accepted at $P < 0.05$.

2.14 Localization of SPI*Pm*2 in shrimp hemocytes using immunogold labeling

The normal shrimp and WSSV injected hemolymph was directly collected into a fixative solution (4% paraformaldehyde in PBS pH 7.4) and incubated for 1 h at room temperature. Each sample was pooled from three shrimps. To collect the hemocytes, the hemolymph was centrifuged at 800× g and the cells were washed twice with PBS. Three percent agarose gel was prepared by melting in PBS, incubated in 50 °C water-bath and aliquoted 500 µl into 1.5 ml microcentrifuge tube. The pooled hemocytes at high concentration were added into the bottom of agarose gel and immediately quick spin for 2 min. When the hemocytes were immobilized in the solidified agarose gel, the gel was cut into 2 × 2 × 2 mm size and, then, embedded in

LR White (Polysciences) according to a standard protocol recommended by the manufacturer.

The cells were quickly dehydrated for 10 min each in a series of pre-cooled ethanol solutions (30, 50, 70, 90 and 100%). The ethanol was, then, replaced with a 2:1 mixture of ethanol/resin for 20 min, followed by a 1:2 mixture of ethanol/resin for 20 min, and pure resin for 1 h. To infiltrate the sample, the sample was incubated with pure resin at room temperature for an overnight. The resin was replaced with the fresh resin and the sample was incubated further for 2 h. The sample was polymerized at 60 °C for 72 h. The sample was cut into ultrathin sections of 80 nm thick with a glass knife and mounted on 200-mesh gilded nickel grids (Polysciences). The grids were incubated in 0.1 M glycine in PBS buffer for 20 min to block free aldehydes. Non-specific labeling was blocked by pre-incubation with Aurion blocking solution (Electron Microscopy Sciences) in PBS for 30 min at room temperature, washed in PBS 1 min for 3 times.

The sections were, then, incubated with purified antibody, rabbit anti-SPI*Pm*2, diluted in PBS containing 1% (v/v) cold water fish gelatin for 1 h at room temperature, washed with 1% (v/v) cold water fish gelatin in PBS 3 min for 6 times. These grids were incubated with secondary gold-conjugated antibody having gold particle size of 10 nm (Electron Microscopy Sciences) in PBS containing 1% (w/v) BSA, 0.1% (v/v) Tween 20 and 0.1% (v/v) Triton X-100 for 1 h at room temperature, washed again in PBS 3 min for 6 times and post fixed with 2.5% (v/v) glutaraldehyde in PBS for 5 min at room temperature. The grids were rinsed with double-distilled water 3 min for 3 times at room temperature and air-dried. Finally, sections were

contrasted with a saturated solution of uranyl acetate in water for 4 min and lead citrate for 1 min. The samples were examined with an Hitachi H-700 electron microscope at an accelerating voltage of 200 kV.

2.15 Yeast two-hybrid screening

Yeast two-hybrid screening was performed by the Matchmaker Gold Yeast Two-Hybrid System (Clontech). The SPI*Pm*2, a bait gene, was amplified by PCR from pSPI*Pm*2_NS2 (Somprasong et al., 2006) using SPI*Pm*2-F and SPI*Pm*2-R primers (Table 1). The gene was cloned into the *Nco*I and *Bam*HI sites containing the GAL4 DNA-binding domain sequence in the pGBKT7 DNA-BD vector. The recombinant plasmid of pGBKT7-SPI*Pm*2 was transformed into the Y2H Gold competent cells and tested for auto-activation and toxicity. The yeast two-hybrid library was constructed from the hemocyte cDNA (Somboonwiwat, unpublished) and the open reading frames of WSSV (Sangsuriya et al., submitted).

Sub-adult black tiger shrimp were injected with WSSV having an LD_{50} of 3 days. The shrimp hemocytes were collected at 24 h post WSSV infection for total RNA extraction. The Mate & Plate™ Library (Clontech) was used to construct the cDNA library. The various open reading frames of WSSV were cloned into the pGADT7-AD vector containing the GAL4 activation domain and transformed into the Y187 competent yeast cells using Matchmaker Library Construction and Screening Kits (Clontech).

To mate the yeast cells, the yeast cells containing the bait vector and pray vector were cultured following the manufacturer instruction. After mating, the

positive clones were selected on the synthetic medium SD/-Leu/-Trp supplemented with X-α-Gal and aureobasidin A (AbA) (DDO/X/A). The positive clone were picked-up and culture to rescue the recombinant plasmid, then verified the positive clone using co-transformation the bait and pray vector into the Y2H Gold yeast cell and tested for the interaction on SD/-Leu/-Trp/X-α-Gal (DDO/X) and SD/-Ade/-His/-Leu/-Trp/X-α-Gal/AbA (QDO/X/A) agar. The positive prey vectors were extracted the DNA to sequencing, annotation and analysis using bioinformatics tools.

2.16 Production and purification of prey protein WSV477

The prey protein gene, rWSV477, was amplified using the specific primer rWSV477-F and rWSV477-R (Table 1) to construct the recombinant plasmid. The PCR product of WSV477 was cloned into the *Nco*I and *Eco*RI sites in the pBAD/Myc -His A vector containing *myc* epitope and 6×His-tag at its 3′ end. To confirm the correct recombinant clone, the recombinant plasmid was sent for sequencing. The constructed expression plasmid (pBAD-WSV477) was transformed into an *E. coli* strain TOP10 to express the recombinant WSV477 (rWSV477). A single colony of pBAD-WSV477 was inoculated in LB medium and culture overnight. The working starter was then inoculated into the LB fresh medium (1:100) and the bacteria culture was grown to the optical density of 0.6. To induce the protein expression, the culture was added the arabionose solution to 0.02% final concentration and further grown for 4 h. Finally, the bacterial cells were harvested by centrifugation at 5000× g for 5 min.

The bacterial cells were resuspended in PBS and frozen and thawed three cycles. The cells were sonicated and centrifuged to collect the cell pellet at 12,000× g

for 15 min. To dissolve the recombinant protein, the cell pellet was incubated with 50 mM carbonate buffer pH 10 for an overnight and, then, centrifuged to collect the supernatant. The rWSV477 was purified from the crude preparation using Ni-NTA (GE Healthcare), following the manufacturer standard protocol.

The Ni-NTA Sepharose was packed into a column and equilibrated with washing buffer (50 mM carbonate buffer pH 10, 0.3 M sodium chloride and 50 mM imidazole) for 10 volumes. The crude protein was applied into the column. The Ni-NTA beads were washed with washing buffer for 10 volumes to elute the unbound and non-specific binding proteins. The bound protein was eluted with elution buffer (50 mM carbonate buffer, pH 10, 0.3 M sodium chloride and 300 mM imidazole). The purified WSV477 was dialyzed against PBS pH 7.4.

2.17 Production of anti-rWSV477 polyclonal antibody

Two milligrams of the purified rWSV477 was used to immunize mouse for polyclonal anti-rWSV477 production. It was performed by the Biomedical Technology Research Unit, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand.

2.18 Co-immunoprecipitation

To confirm the protein-protein interaction in vitro, the co-immunoprecipitation was carried out. The rabbit antibody specific to rSPI*Pm*2 (Donpudsa et al., 2009) was immobilized and covalently cross-linked with protein A sepharose CL4B (GE Healthcare) using dimethyl pimelimidate (Sigma) as described by Meyer (Meyer et

al., 1984). Fifty microliters of cross-linked bead was equilibrated with binding buffer (PBS pH 7.4 and 1 M NaCl). Twenty micrograms of rSPI*Pm*2 and/or rWSV477 were added and incubated for 1 h at room temperature. To remove the non-specific binding, the column was washed with binding buffer for 15 times to eliminate the unbound protein. The proteins were eluted with 100 mM glycine pH 2.5 and subjected to SDS-PAGE and western blot analysis. The rSPI*Pm*2 and rWSV477 were detected using anti-His tag antibody as primary antibody and developed as protein bands as described above.

2.19 Gene silencing of WSV477 in WSSV-infected hemocyte cell culture and shrimp

Gene silencing was used to study the important role of WSV477 for viral propagation. In this experiment, gene expression of WSV477 in the WSSV-infected primary cell culture and shrimp was suppressed using dsRNA. The WSV477 dsRNA and GFP dsRNA were synthesized using T7 RiboMAX™ Express Large Scale RNA Production System (Promega) following the manufacturer protocol. The selected sequence of WSV477 and GFP gene were amplified from pBAD-WSV477 and pEGFP-1 (Clontech) using specific primers (Table1).

To prepare the hemocyte primary cell culture, the shrimp hemocytes were collected, separated by centrifugation and resuspended in L15 medium supplemented with 20% fetal bovine serum (GIBCO). The hemocyte cell suspension was aliquot into each well of the 96-well plate (2×10^4 cells/100 µl/well) and incubated at 27 °C overnight. Fresh L15 medium supplemented with 20% fetal bovine serum was

replenished. The dsRNA was pre-incubated with histone H2A (calf thymus, type II-A; Sigma) for 10 min at room temperature (Liu et al., 2006) before used. The hemocyte cell cultures, divided into 3 groups of 3 wells, were incubated with PBS, 5 μg of GFP dsRNA or WSV477 dsRNA for 2 h. The purified WSSV (1×10^5 copies) was, then, added and incubated at 27 °C for 24 h. The cultures were collected for total RNA preparation.

Three groups of 5 shrimp (5 g body weight) were injected with 50 μl of 0.85%NaCl, GFP dsRNA (10 μg/g shrimp) and WSSV dsRNA (10 μg/g shrimp), respectively. After 3 h, the shrimp were injected with 50 μl of 0.85% NaCl, GFP dsRNA (10 μg/g) and WSSV dsRNA (10 μg/g), respectively, along with purified WSSV (1×10^3 copies). The shrimp hemocytes were collected at 24 h post WSSV injection for extraction of the total RNA.

Hemocytes from cell culture and shrimp were extracted the total RNA using a TRI reagent (Molecular Research Center). The total RNA was treated with RQ1 RNase-free DNase (Promega). One microgram of treated RNA was used as a template to construct the first strand cDNA synthesis using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). The cDNA was detected the expression of WSV477, *VP28*, *SPIPm2*, *ie1* (intermediate early gene 1) and *β-actin* genes by RT-PCR using gene specific primers (Table 1). The *β-actin* gene was an internal control gene. A RT-PCR reaction of 25 μl total volume contained 1× PCR buffer, 200 μM dNTP, 0.2 μM of each specific primer, 1 unit of Taq DNA polymerase (RBC) and 4 μl of 1:10 dilution of the cDNA preparation. The reaction were carried out using the following conditions: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of

denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and the final extension at 72 °C for 10 min. The PCR reactions were analyzed by agarose gel electrophoresis. The intensity of PCR product was detected using GeneCam Flexi, a gel documentation system (SynGene) and further quantified using the GeneTools image analysis software. The expression levels were calculated relative to that of *β-actin* transcripts. The data were statistically analyzed for significant differences between groups using one way analysis of variance (ANOVA) followed by a post hoc test (Duncan's new multiple range test). Significant differences are indicated at $P < 0.05$.

2.20 Proteinase activity assay

Because the rSPI*Pm*2 is a serine proteinase inhibitor, its target protein should be a proteinase. The rWSV477 was characterized for its proteinase activity using zymography (Lantz et al., 1994). The SDS-PAGE supplemented with 0.1% gelatin was prepared. The rWSV477 was loaded into a SDS-PAGE. After electrophoresis, the acrylamide gel was incubated in phosphate buffer pH 7.8 containing 1% Triton X-100 at room temperature for an overnight. The gel was stained with Coomassie Blue Stain. Upon destaining, the gel became dark blue except the lighter area that had proteinase activity.

Table 2.1 Nucleotide sequences of primers for PCR amplification

Primer	Sequence (5′-3′)	Usage
SPI*Pm*2-F	ATCGCCATGGGGAAAATCCGCC	Cloning of *SPIPm2* as bait protein gene in yeast two-hybrid screening
SPI*Pm*2-R	TAGCGGATCCTTAATATCCCTT	Cloning of *SPIPm2* as bait protein gene in yeast two-hybrid screening
rWSV477-F	ATAGCCATGGATATCTTCGTCGAA	Cloning of *WSV477* gene into an expression vector
rWSV477-R	ATAGCCATGGATATCTTCGTCGAA	Cloning of *WSV477* gene into an expression vector
WSV477-F	CATGTGGAATGTCTTTCCTC	dsRNA synthesis
WSV477-R	ACTTTTATTTCTTGAATATT	dsRNA synthesis
GFP-F	ATGGTGAGCAAGGGCGAGGA	dsRNA synthesis
GFP-R	AGAAGGAAGGGCGCTGAC	dsRNA synthesis
WSV477-FRT	CGCGGATCCATGTATATCTTCGTCGA	RT-PCR (Han et al., 2007)
WSV477-RRT	CCGGAATTCTTATAAGAAATGTACAA	RT-PCR (Han et al., 2007)
VP28-FRT	TCACTCTTTCGGTCGTGTCG	RT-PCR of *VR28*
VP28-RRT	CCACACACAAAGGTGCCAAC	RT-PCR of *VR28*
VP28QF	GGGAACATTCAAGGTGTGGA	Realtime RT-PCR
VP28QR	GGTGAAGGAGGAGGTGTTGG	Realtime RT-PCR
SPIPm2-FRT	ATGCAACCACGTCTGTACTG	RT-PCR
SPIPm2-RRT	CTGCAAGGTTCCACATCT	RT-PCR
ie1-FRT	GACTCTACAAATCTCTTTGCCA	RT-PCR
ie1-RRT	CTACCTTTGCACCAATTGCTAG	RT-PCR
β-actin-FRT	GCTTGCTGATCCACATCTGCT	RT-PCR
β-actin-RRT	ATCACCATCGGCAACGAGA	RT-PCR
*β-actin*QF	GAACCTCTCGTTGCCGATGGTG	Realtime RT-PCR
*β-actin*QR	GAAGCTGTGCTACGTGGCTCTG	Realtime RT-PCR

CHAPTER III

RESULTS

3.1 Detection of SPI*Pm*2 protein in various tissues of shrimp

Nine shrimp tissues including hemocyte, intestine, heart, gill, stomach, antennal gland, lymphoid, epipodite and plasma were extracted to detect the expression of SPI*Pm*2 protein. The protein components in shrimp tissues were examined using SDS-PAGE (Fig. 3.1 A) and the expression of SPI*Pm*2 protein was detected using western blot analysis (Fig. 3.1 B). SPI*Pm*2 protein was mainly found in hemocyte while low amount of SPI*Pm*2 was found in lymphoid and epipodite.

3.2 Immunocytochemistry of SPI*Pm*2 on the WSSV-infected hemocyte

Previously, the mRNA expression of SPI*Pm*2 was found to be mainly synthesized in the hemocytes and up-regulated upon WSSV infection. It was, then, interesting to study at the protein level. The immunocytochemistry was used to detect the SPI*Pm*2 protein in the hemocytes. Shrimp were injected with WSSV. The hemocytes from three shrimps were drawn at each time point after infection. The hemocytes were collected at 0, 6, 24, 48 and 72 h after infection (Fig. 3.2 A). The SPI*Pm*2-producing hemocytes were determined using rabbit anti-SPI*Pm*2 (slides a–e) whereas the unimmunized rabbit serum was used as a control (slides f–j). The SPI*Pm*2-producing cells were counted (Fig. 3.2 B).

The results revealed that after the shrimp was infected with WSSV at all time points, the SPI*Pm*2 producing hemocytes were significantly reduced to less than 10%

at 6 h after infection and recovery to about 15%, about half of that observed at time 0, at 24, 48 and 72 h after infection (Fig. 3.2 B).

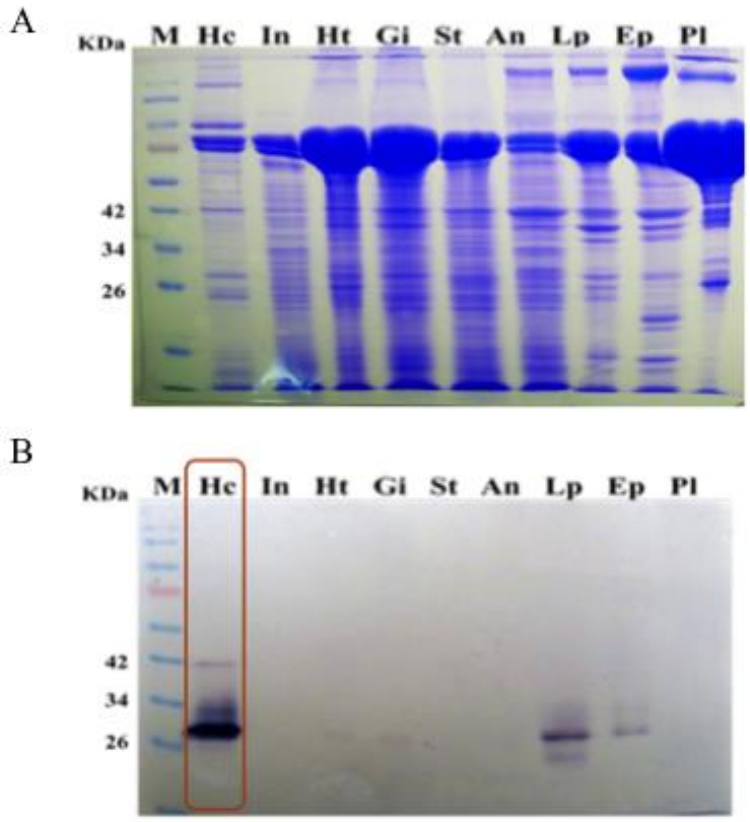

Figure 3.1 Expression of SPI*Pm*2 protein in various tissues of shrimp. Nine shrimp tissues, hemocyte (Hc), intestine (In), heart (Ht), gill (Gi), stomach (St), antennal gland (An), lymphoid organ (Lp), epipodite (Ep) and plasma (Pl) were collected and extracted the protein content. Thirty microgram proteins were subjected to SDS-PAGE and detected the SPI*Pm*2 by western blot analysis using SPI*Pm*2 antibody from rabbit.

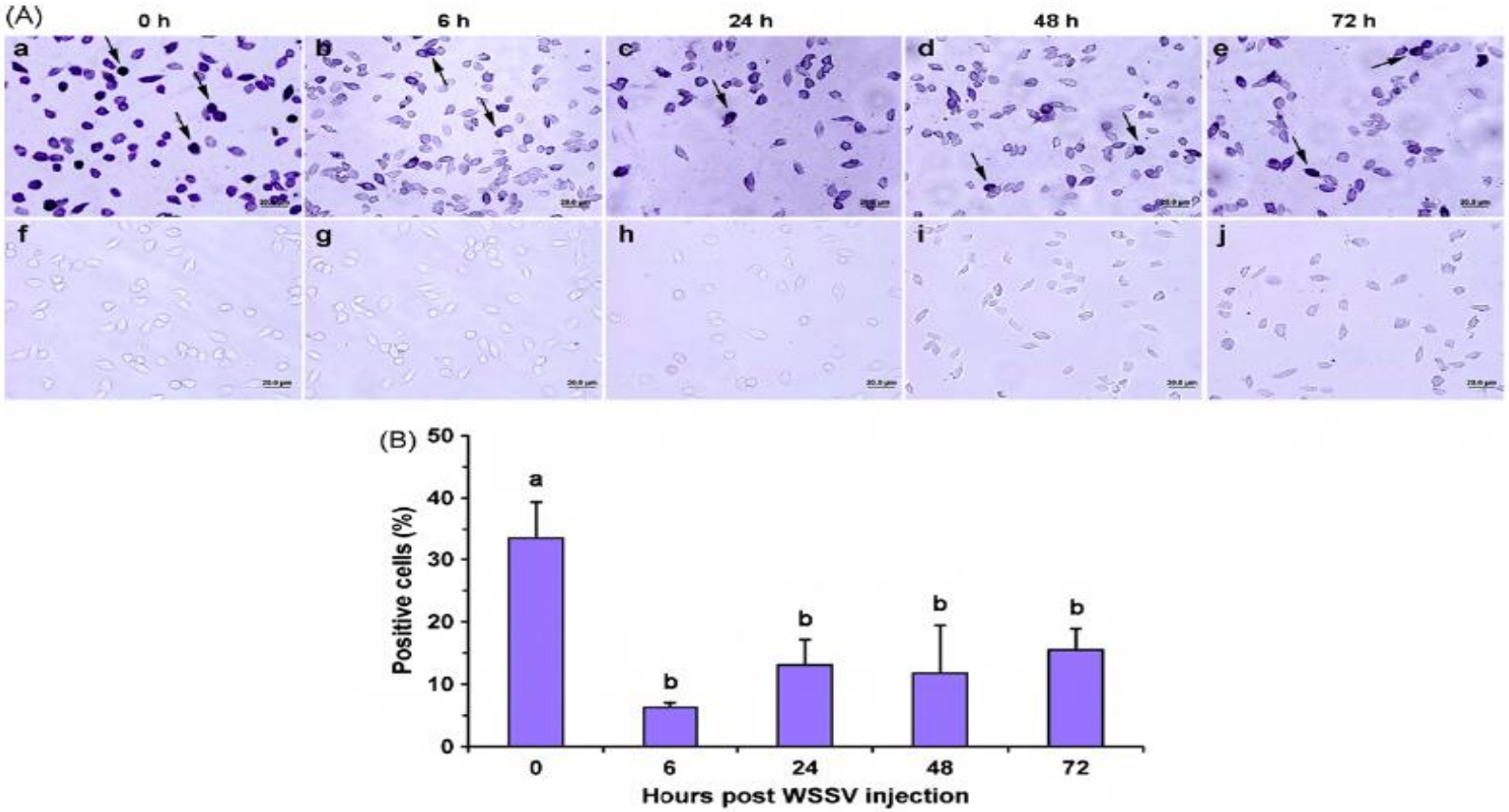
(A)
0 h
6 h
24 h
48 h
72 h
a
b
c
d
e
f
g
h
i
j
(B)
Positive cells (%)
50
40
30
20
10
0
a
b
b
b
b
0
6
24
48
72
Hours post WSSV injection

Figure 3.2 Detection of SPI*Pm*2-producing hemocytes in WSSV-infected shrimp. (A) Hemolymph was collected at 0, 6, 24, 48 and 72 h after WSSV injection. The hemocyte cells were separated and fixed with 4% paraformaldehyde. The hemocytes were attached onto the poly-l-lysine coated slides and subjected to immunocytochemistry detection of SPI*Pm*2. The light microscope was used for determining the SPI*Pm*2-producing hemocytes. Slides a–e are detected using rabbit anti-SPI*Pm*2 whereas slides f–j are rabbit serum controls. The arrows indicate the SPI*Pm*2-positive hemocytes. (B) Three hundred hemocyte cells of each time course were counted and presented by percentages. The bars were shown as means ± standard deviations. The statistical significance of the data was evaluated using one-way ANOVA followed by post hoc test (Duncan's new multiple range test). Significant differences were accepted at $P < 0.05$ as indicated by different letters.

3.3 Production and purification of rSPI*Pm*2

To test the effect of SPI*Pm*2 on the WSSV infected shrimp, the recombinant rSPI*Pm*2 was produced using an *E. coli* system. The crude protein was dissolved from the inclusion bodies with carbonate buffer pH 10 and purified using Ni-NTA column. Figure 3.3 shows the purification of SPI*Pm*2, which was approximately 30 kDa in eluted fraction 2 and 3 (E2 and E3).

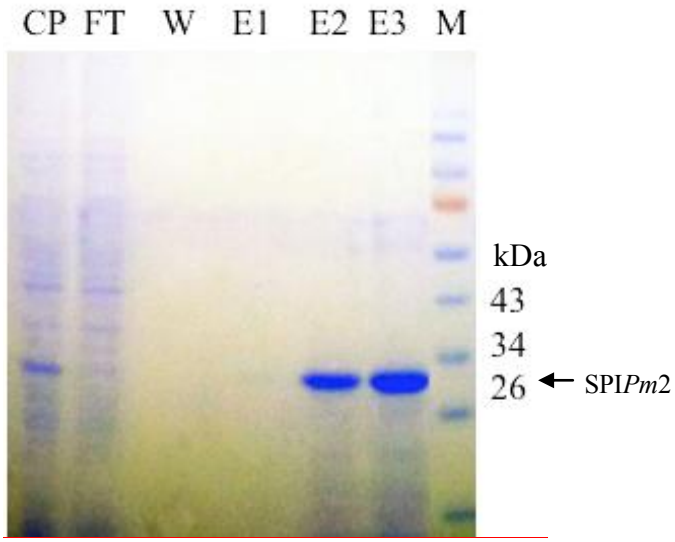

Figure 3.3 Production and purification of the recombinant SPI*Pm*2. The recombinant plasmid, pSPI*Pm*2-NS2, was transformed to expression host, *E.coli* stain rosetta to express the rSPI*Pm*2 protein. The bacteria were grown in LB agar with 50 μM amplicilin and protein expression induced was with 1 M IPTG. After 4 h of induction, the bacterial cells were collected to extract the crude protein. The rSPI*Pm*2 in crude protein was purified by Ni-NTA column. CP = Crude protein, FT = Flow through fraction, W = Wash fraction, E1 = Elution fraction 1, E2 = Elution fraction 2, E3 = Elution fraction 3

3.4 Effect of the recombinant SPI*Pm*2 on the survival of WSSV-infected shrimp

Since the SPI*Pm*2 is one of the viral responsive genes, the increasing in SPI*Pm*2 in plasma may have effect on shrimp immunity against WSSV. To study the function of SPI*Pm*2 in plasma, the recombinant SPI*Pm*2 was produced and purified in *E.coli* system (Donpudsa et al., 2010). To test the effect of SPI*Pm*2 on WSSV infected shrimp, the shrimp were pre-injected with saline (both un-infected and infected controls), SPI*Pm*2, rabbit serum and anti-SPI*Pm*2 for 24 h before WSSV injection. The shrimp mortality was recorded and shown in Fig. 3.4. The cumulative mortality reached 100% at day 4 for the WSSV-infected control and the mortality of the un-infected control was minimal. The shrimp administrated with rabbit serum showed no difference in cumulative mortality from the infected control group whereas the shrimp administrated with anti-SPI*Pm*2 was not significantly different. Interestingly, injection of the recombinant SPI*Pm*2 protein significantly delayed the mortality, 100% cumulative mortality was observed at day 7 as compared to day 4 of the infected control (Fig. 3.4).

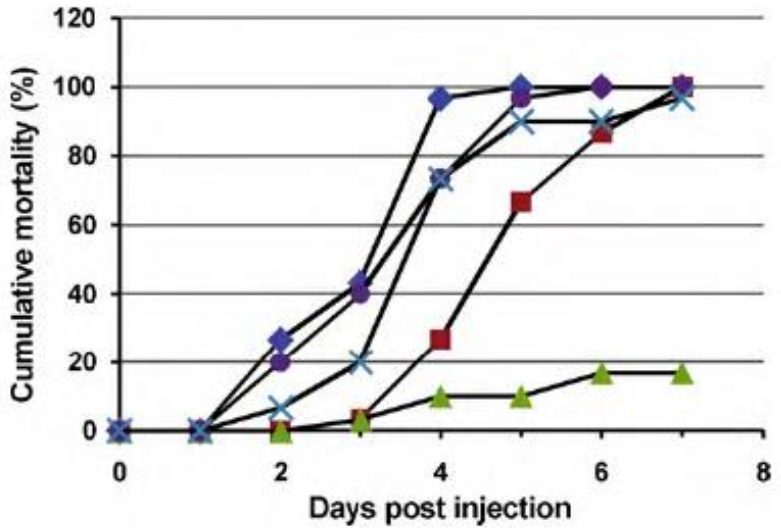

Figure 3.4 Effect of the recombinant SPI*Pm*2 on the survival of WSSV-infected shrimp. The shrimps were divided into five groups of 10 individual shrimp pre-injected with saline (un-infected control, ▲), saline (infected control, ◆), rSPI*Pm*2 (■), rabbit serum (●) and anti-SPI*Pm*2 (✖), respectively, for 24 h before WSSV injection. The shrimp mortality of each experimental group was observed for 10 days. The experiment was repeated three times. The data were the means of three different experiments.

3.5 Localization of SPI*Pm*2 and VP28 in WSSV-infected hemocytes

Because the tissue distribution showed that the mRNA expression of SPI*Pm*2 was mainly in the hemocytes, different hemocyte cell types were analyzed to detect the expression of SPI*Pm*2. The confocal laser scanning microscope was used to visualize the SPI*Pm*2-producing hemocyte using the antibodies specific to the protein. The effect of SPI*Pm*2 upon WSSV infection, the protein expression of SPI*Pm*2 and VP28, viral late protein of WSSV, were detected using different fluorescence-conjugated secondary antibodies specific to different primary antibodies. The green color from Alexa488 and red color form Alexa568 allowed visualization of the

SPI*Pm*2 and VP28, respectively (Fig. 3.5). The nuclei acid was stained with TO-PRO-3 iodide and adjusted the red color to blue to distinguish from the VP28.

Hemocytes were collected from normal shrimp and WSSV-infected shrimp at various time points and moribund shrimp. The bright field image of hemocytes from normal shrimp revealed three types of hemocytes, namely hyaline, semi granular and granular cells. Two cell types, semi-granular and granular, were positively stained for SPI*Pm*2 production but not the hyaline cell. The expression of SPI*Pm*2 protein was located in the cytoplasm of the cell. It is, probably, that the SPI*Pm*2 was produced and stored in secretory granules in the cytoplasm.

The morphology of hemocytes in the early phase and late phase of infection was different. In the early phase, hemocytes looked normal and three different cell types could be found whereas in late phase of infection, hemocytes types were hardly distinguishable as well as those producing SPI*Pm*2. As the infection progressed, the cells became more degranulation.

Upon WSSV infection, the number of the SPI*Pm*2-producing hemocytes was reduced whereas the expression of VP28, viral late protein, was increased. The results showed that the VP28 was not detected at 24 h of infection, but detected late at 48 h and moribund period. In addition, VP28 was located in the nucleus of hemocyte cells appeared to be semigranular or granular cells but depleted of SPI*Pm*2.

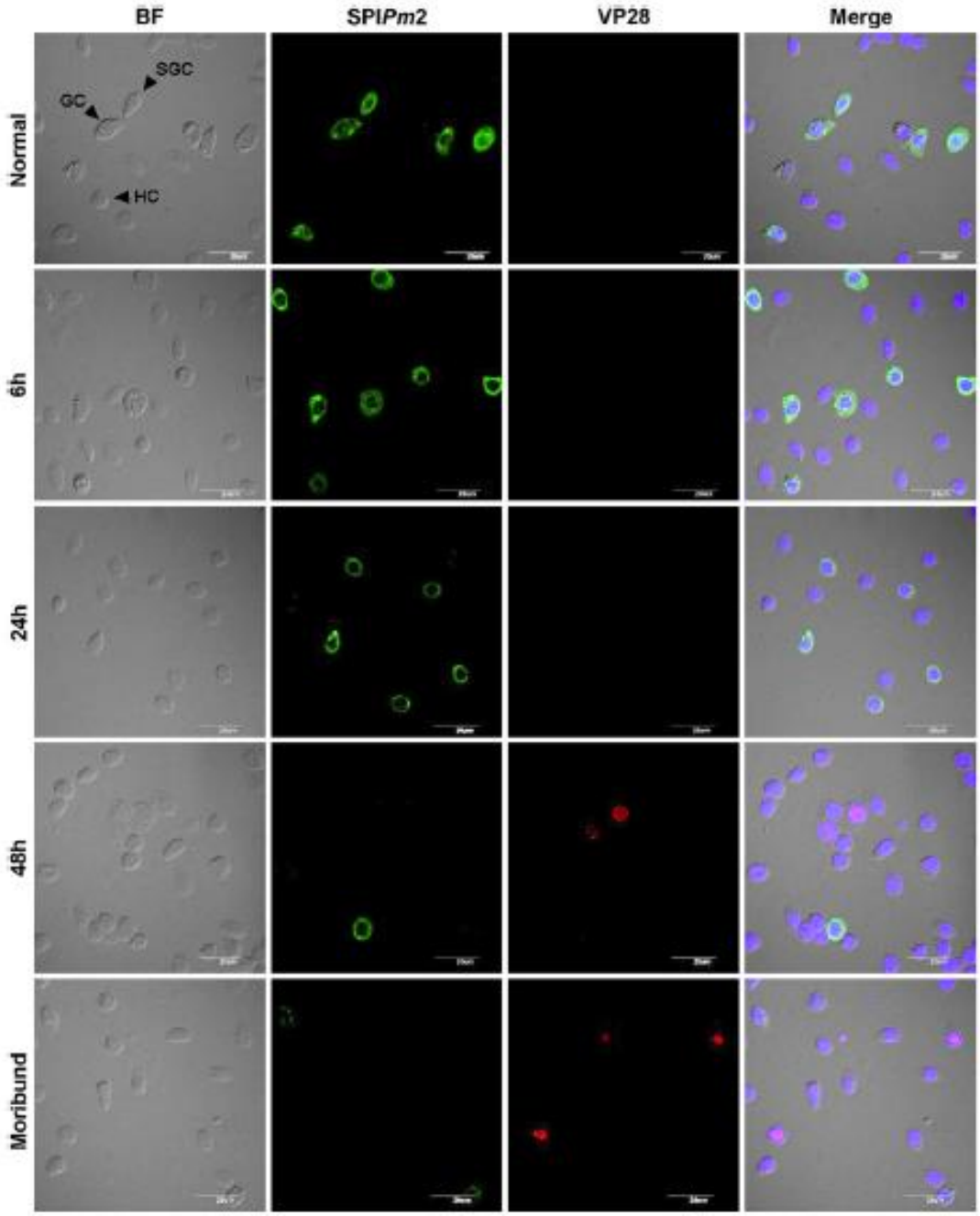

Figure 3.5 Localization of SPI*Pm*2 and VP28 in healthy and WSSV-infected *P. monodon* hemocytes by immunofluorescence confocal laser scanning microscopy. Healthy and WSSV infected hemocytes at 6, 24, 48 h post infection and moribund shrimp were collected and fixed in 4% paraformadehyde. The fixed hemocytes were attached onto poly-L-lysine coated slides. The SPI*Pm*2 and VP28 were detected using purified rabbit polyclonal anti-SPI*Pm*2 and purified mouse monoclonal anti-VP28, respectively.

The hemocytes were then probed with secondary antibodies conjugated with Alexa Flour 488 (green) for SPI*Pm*2 and Alexa Flour 568 (red). Nuclei (adjusted to blue color) were stained with TO-PRO-3 iodide. The HC, SGC and GC are hyaline, semigranular and granular cells, respectively. BFs are bright field images. Bars, 20 mm.

3.6 Primary cell culture of *Penaeus monodon* hemocytes

Shrimp hemocytes were collected and cultured in L15 medium with 20% FBS. Fig. 3.6 shows the hemocyte attached at the bottom of the microtiter plate after incubation overnight. The morphology of the hemocyte has little changed by having a fibroblast-like shape.

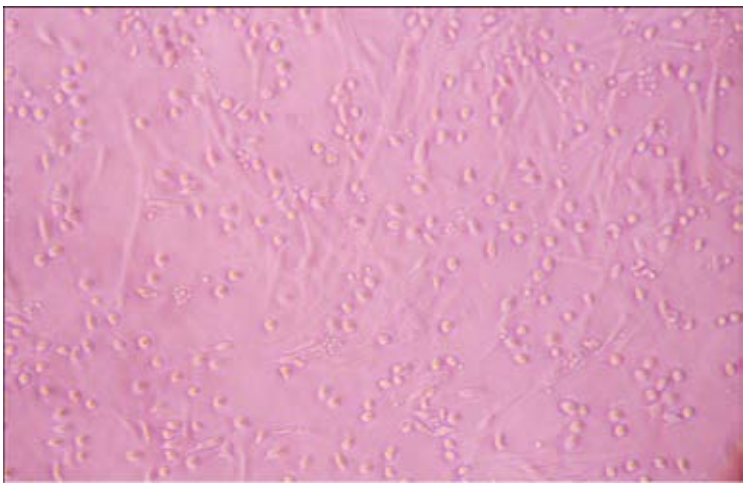

Figure 3.6 Primary cell culture of *Penaeus monodon* hemocytes. The shrimp hemocytes of *Penaeus monodon* were collected and resuspened in the L15 with 20% FBS. The resuspened cell were added into the microtiterplate and then incubated overnight. The picture was visualized under light microscopy (400×).

3.7 Secretion of SPI*Pm*2 from the hemocytes upon WSSV challenge

To show that the SPI*Pm*2 protein from the hemocytes can be directly induced by WSSV infection, a primary cell culture was used because it was void of other shrimp factors in the circulation. Hemocyte primary cell culture of *P. monodon* was cultured and challenged with purified WSSV at a multiplicity of infection of 10:1. The culture medium and hemocytes were collected at different time points after infection. The samples were subjected to SDS-PAGE and western blot analysis using specific antibodies to detect the SPI*Pm*2 and VP28.

Upon WSSV challenge, the SPI*Pm*2 protein was detected in the culture medium indicated that the SPI*Pm*2 was secreted from the hemocytes into the culture medium at 1 h and gradually increased with time (Fig. 3.7 A.). The VP28, a viral late protein, was detected in the hemocytes having the same trend as SPI*Pm*2 (Fig. 3.7 B.) but there was no increase in SPI*Pm*2 in the cells with time (Fig. 3.7 C.).

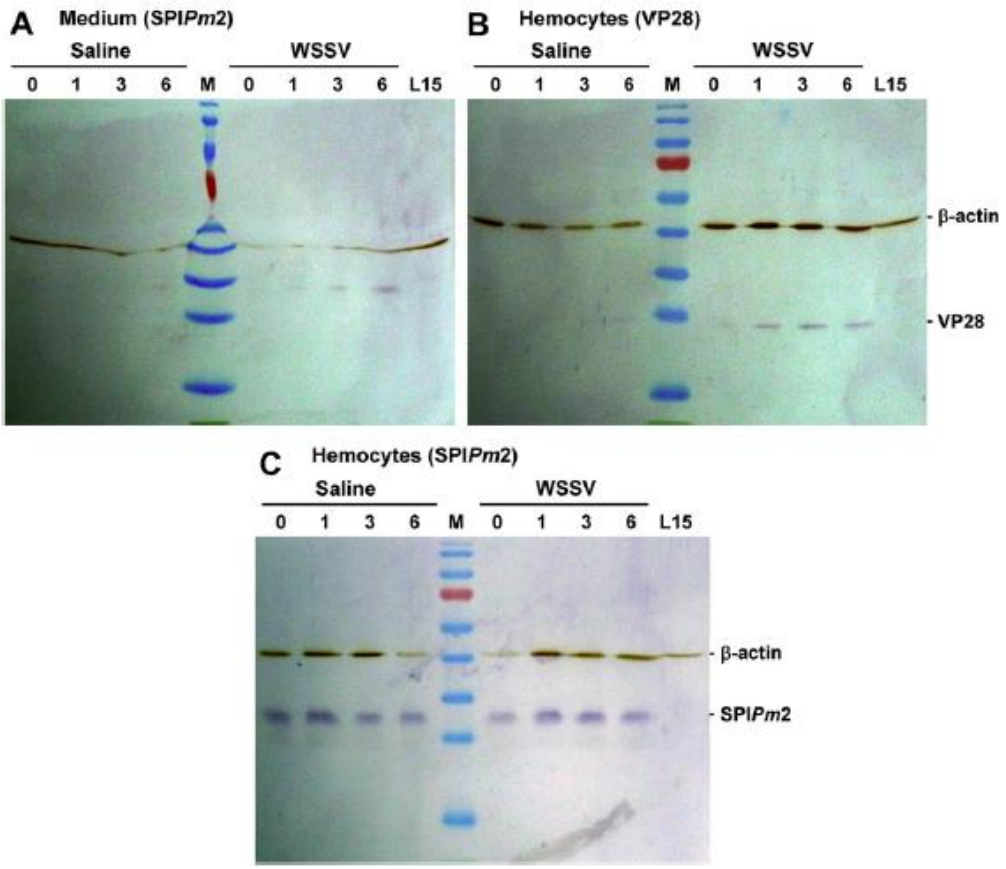

Figure 3.7 Secretion of SPI*Pm*2 from the hemocytes in reponse to WSSV challenge by Western blot analysis. Shrimp hemocytes were collected and cultured in a 96-well microtiter plate. After purified WSSV or PBS was added into the primary cell culture, the hemocytes and culture medium were collected at the time course 0, 1, 3 and 6 h post WSSV challenge. Western blot analysis was used to analyze the protein content in the culture medium (A) and hemocytes (B and C). The SPI*Pm*2 (A and C), VP28 (B) and β-actin were detected using specific antibodies. The detection of SPI*Pm*2 or VP28 and β-actin were developed using secondary antibodies conjugated with alkaline phosphatase (purple color) and horseradish peroxidase (brown color). The aberrant running pattern of proteins in (A) is due to high amount of serum albumin in the culture medium. L15 with FBS is the culture medium. M is pre-stained protein marker.

3.8 Neutralization and protection effect of rSPI*Pm*2 against WSSV

To characterize the function of SPI*Pm*2, the hemocyte primary cell culture was much more convenient for studying the antiviral activity of SPI*Pm*2. Previous result showed that the injection of recombinant SPI*Pm*2 prior to WSSV injection could inhibit WSSV replication and prolong the shrimp life. It was, then, interesting to know how the rSPI*Pm*2 performed its action against WSSV infection.

In this experiment, the neutralization and protective effect were used to test the antiviral activities. In term of neutralization, the rSPI*Pm*2 or PBS was incubated with purified WSSV for 30 min prior to adding into the primary cell culture. In the protective effect experiment, the rSPI*Pm*2 or PBS was pre-incubated with primary cell culture and then removed and washed with fresh medium once before adding the purified WSSV. The multiplicity of infection of about 2:1 was used in this experiment. The primary cell cultures were collected at 6 and 12 h after challenging and then detected the expression of viral late gene, VP28 and β-actin using RT-PCR analysis (Fig. 3.8A and Fig. 3.9A) and qRT-PCR (Fig. 3.8B and Fig. 3.9B). The expression of WSSV late gene VP28 was considered as successful viral replication. The results showed the expression of VP28 was detected at 6 h and 24 h after viral challenge. From RT-PCR and qRT-PCR result, the presence of rSPI*Pm*2 in neutralization and protection experiment at 6 h and 24 h showed the significant reduction of VP28 expression compared with the control experiment, PBS. It seems to be that the rSPI*Pm*2 interferes with the viral multiplication of WSSV.

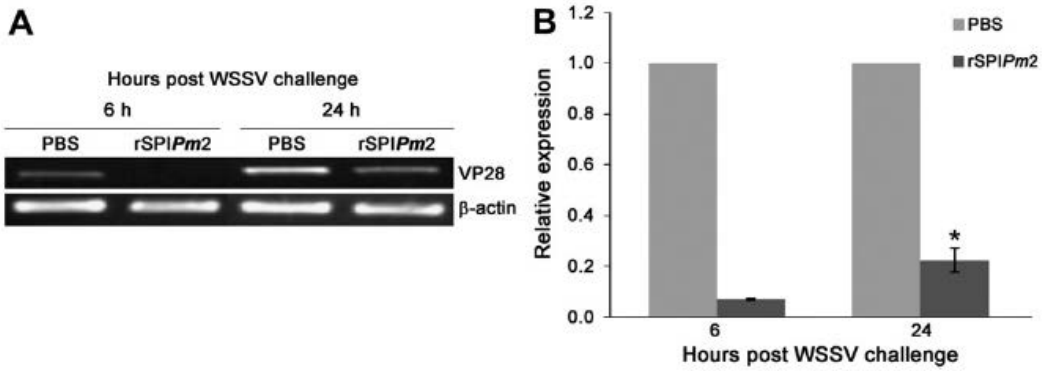

Figure 3.8 Neutralization of rSPI*Pm*2 against WSSV. The purified WSSV was pre-incubated with rSPI*Pm*2 or PBS and added into the primary hemocyte cell cultures. The PBS was used as a control. The cell cultures were collected at 6 and 24 h post WSSV challenge and detected the gene expression of VP28 as an indication of viral replication by RT-PCR (A) and qRT-PCR (B). The β-actin was used as an internal control gene for PCR analysis. The relateive expression of VP28 in WSSV-infected (dark gray) hemocytes was presented by bar graph which have already been normalized against those in the PBS-incubated control (light gray) hemocytes. The results are the average of three independent data with standard deviations. Asterisk indicates statistically significant difference between the results ($P < 0.05$).

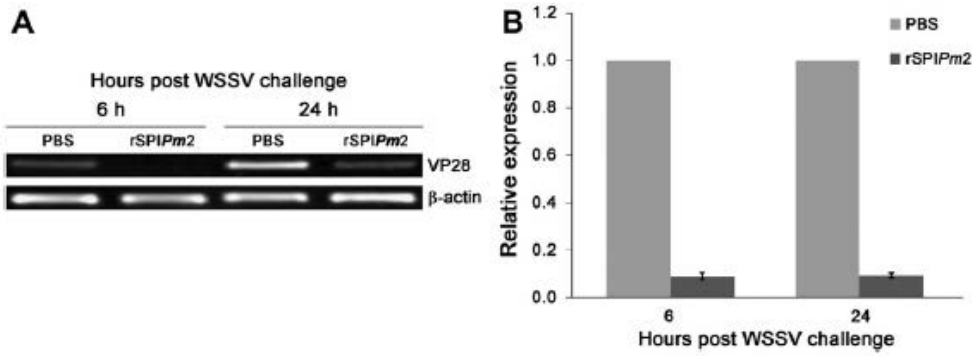

Figure 3.9 Protective effect of rSPI*Pm*2 against WSSV. The primary hemocyte cell cultures were incubated with SPI*Pm*2 before WSSV challenge. PBS was used as a control. The primary cell cultures were collected at 6 and 24 h post WSSV challenge and detected the gene expression of VP28 as an indication of viral replication by RT-PCR (A) and qRT-PCR (B). The β -actin was used as an internal

control gene for PCR analysis. The relateive expression of VP28 in WSSV-infected (dark gray) hemocytes was presented by bar graph which have already been normalized against those in the PBS-incubated control (light gray) hemocytes. The results are the average of three independent data with standard deviations. Asterisk indicates statistically significant difference between the results ($P < 0.05$).

3.9 Dose-dependence of rSPI*Pm*2 against WSSV

Although the antiviral properties of rSPI*Pm*2 were elucidated in term of neutralization and protection, one could question whether the antiviral activity of rSPI*Pm*2 was dose-dependent or not. To solve this, various concentrations of rSPI*Pm*2 were used to test the antiviral activity with purified WSSV. Purified WSSV with the multiplicity of infection of about 2:1 and various concentration of rSPI*Pm*2, 0.02, 0.08, 0.32 and 0.64 μM were added into the cell cultures. PBS was used for control experiment. The primary cell cultures were collected for RT-PCR (Fig. 3.10A) and qRT-PCR analyses (Fig. 3.10B). The results showed that as the concentration of rSPI*Pm*2 was increased, the expression of VP28 was decreased.

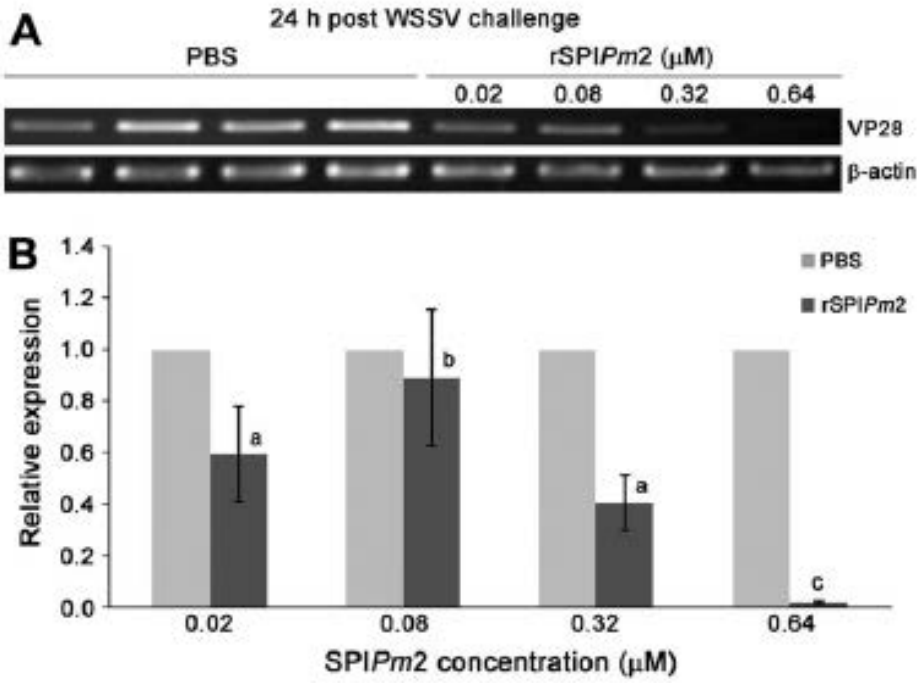

Figure 3.10 Dose-dependence of rSPI*Pm*2 against WSSV. The verious concentrantions of rSPI*Pm*2 and purifed WSSV was incubated with *P. monodon* hemocyte primary cell culture. PBS was used as a control. The hemocyte cell cultures were collected at 24 h post WSSV challenge and detected the gene expression of VP28 using RT-PCR (A) and qRT-PCR (B). The β -actin was used as an internal control gene for PCR analysis. The relateive expression of VP28 in WSSV-infected (dark gray) hemocytes was presented by bar graph which have already been normalized against those in the PBS-incubated control (light gray) hemocytes. The results are the average of three independent data with standard deviations ($P < 0.05$).

3.10 Binding activity of rSPI*Pm*2 to WSSV and shrimp hemocyte membrane

Because the results of neutralization and protection showed that the rSPI*Pm*2 could interfere with WSSV infection in primary cell culture, one would expect that the rSPI*Pm*2 might interact to components of the hemocyte cell and virus. Binding

activity was performed using ELISA technique. The shrimp hemocyte membrane (SHM) extracted from shrimp hemocyte. Purified WSSV were coated into a 96-well microtiter plate and then incubated with various concentrations of rSPI*Pm*2. Anti-His tag antibody was used to detect the bound rSPI*Pm*2 and the chromogenic assay was developed. The result showed the binding activity between rSPI*Pm*2 and purified WSSV or SHM by measuring the absorption at 405 nm. The plot of absorption against the concentration of rSPI*Pm*2 revealed dramatically increase in binding of rSPI*Pm*2 as the concentration of rSPI*Pm*2 is increased and, then, retaining of binding at high concentration of SPI*Pm*2 (Fig. 3.11).

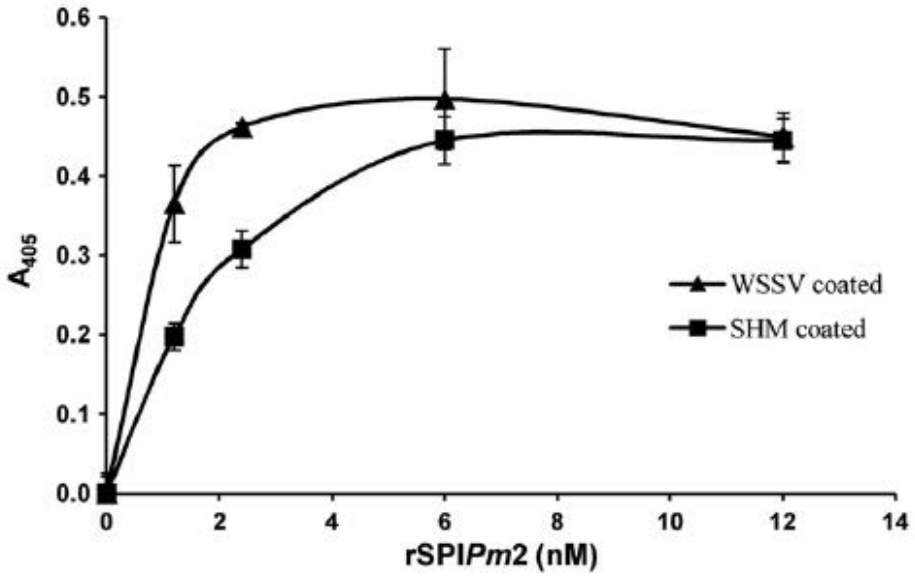

Figure 3.11 Binding activity of rSPI*Pm*2 to WSSV and shirmp hemocyte membrane (SHM). Purified WSSV (▲) and SHM (■) were coated into the bottom of 96-well microtiter plate and incubated with various concentration of rSPI*Pm*2. The binding was detected using anti-His tag antibody and developed color using a secondary antibody. The binding activity of rSPI*Pm*2 to WSSV and SHM was determined by measuring the absorption at 405 nm. The results are means of triplicate data with standard deviations.

3.11 Synthesis and storage of native SPI*Pm*2 in the hemocyte granules

Previously, the confocal micrograph results showed that the native SPI*Pm*2 was found in the cytoplasm of semigranular and granular but not the hyaline cells. Morover, when the hemocytes were infected with WSSV, the SPI*Pm*2 was rapidly secreted in response to viral infection. The localization of SPI*Pm*2 inside the semigranular and granular cells was interesting. To answer this, the immunogold labeling technique was used.

Hemocytes of healthy shrimp and WSSV infected shrimp were subjected to immunogold labeling and then visualized under the transmission electron microscope. Dark spots of gold particles showing the location of SPI*Pm*2 were found in the granules of granular and semi-granular hemocytes in normal shrimp (Fig. 3.12 A and B) but not in the hyaline cell (Fig. 3.12 C). The SPI*Pm*2 molecules were increased drastically in the granular hemocyte collected from the WSSV-infected shrimp (Fig. 3.12 D). The result indicated that the SPI*Pm*2 synthesized was stored in the granules of granular and semi-granular hemocytes awaiting for the release in response to viral infection. Its synthesis was also up-regulated in response to WSSV infection.

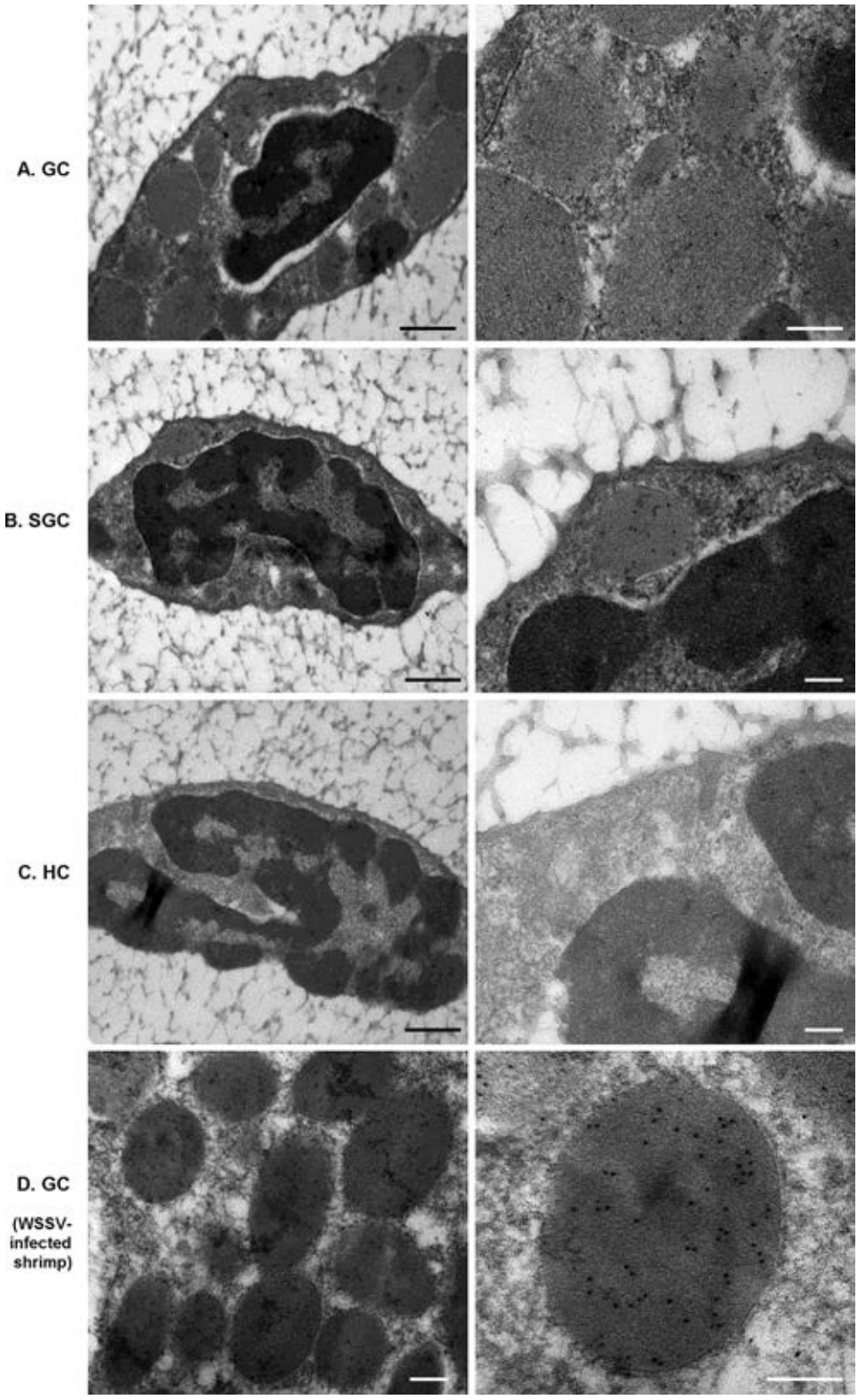

Figure 3.12 Immunogold labeling of SPI*Pm*2 in semigranular, granular and hyaline hemocytes from

Penaeus monodon hemocyte. The normal (A), (B), (C) and WSSV infected hemocytes (D) was collected and embedded with LR white resin. The ultrathin section was developed using immunogold labeling and visualized under transmission electron microscope (TEM). The right micrographs are the magnification of the left micrographs. (A), (B) (C) and (D) are granular cell (GC), semigranular cell (SGC), hyaline cell (HC) and granular cell from WSSV-infected shrimp, respectively. Black bar = 0.5 μ m. and white bar = 0.2 μ m.

3.12 Yeast two-hybrid screening for binding target of SPI*Pm*2

To study the function of SPI*Pm*2, a search for protein targets of SPI*Pm*2 were carried out. Previous results showed the SPI*Pm*2 bound to the SHM and WSSV. It is interesing to identify the SPI*Pm*2 binding proteins. Yeast two-hybrid screening was carried out using the SPI*Pm*2 as a bait protein for screening of two libraries constructed from the hemocyte cDNA and the open reading frames of WSSV genome. Several simple repetitive DNA sequences were obtained from the screening of cDNA library of WSSV-infected hemocytes which could not be used for further sequence analysis (data not shown).

On the contary, five viral proteins: WSV020, WSV399, WSV267, WSV061 and WSV477 as listed in Table 3.1, were identified from the screening of WSSV library. The WSV020, WSV399, WSV267 and WSV061 were unknown proteins. The WSV477 was a known protein previously characterized as an early gene of WSSV. This protein consists of 208 amino acid residues. It was interesting because it was possibly a type of zinc finger regulatory protein with a GTP binding activity (Han et al., 2007).

Table 3.1 Clones detected by yeast two-hybrid from the WSSV open reading frame library.

Clone number	BLAST result	Remark
Clone1/AD and Clone35/AD	WSSV076/WSV020	Unknown protein
Clone2/AD	WSSV458/WSV399	Unknown protein
Clone4/AD	WSSV322/WSV267	Unknown protein
Clone14/AD	WSSV118/WSV061	Unknown protein
Clone46/AD	WSSV004/WSV477	Cys2/Cys2-type zinc finger, ATP/GTP binding motif

3.13 Production and purification of rWSV477

The recombinant WSV477 was produced using an *E.coli* system. To induce the protein expression of rWSV477, arabinose was added and culture for 4 hours. The protein contents of crude protein at 0 and 4 hours after arabinose induction were shown in Fig. 3.13 A staining with Coomassie brilliant blue while the detection of rWSV477 shown in Fig. 3.13 B using western blot analysis. The rWSV477 was appoximately 28 kDa. The rWSV477 from the crude protein extract was purified by nickel-NTA bead (Fig 3.13C). This purified rWSV477 was prepared for antibody production in mouse.

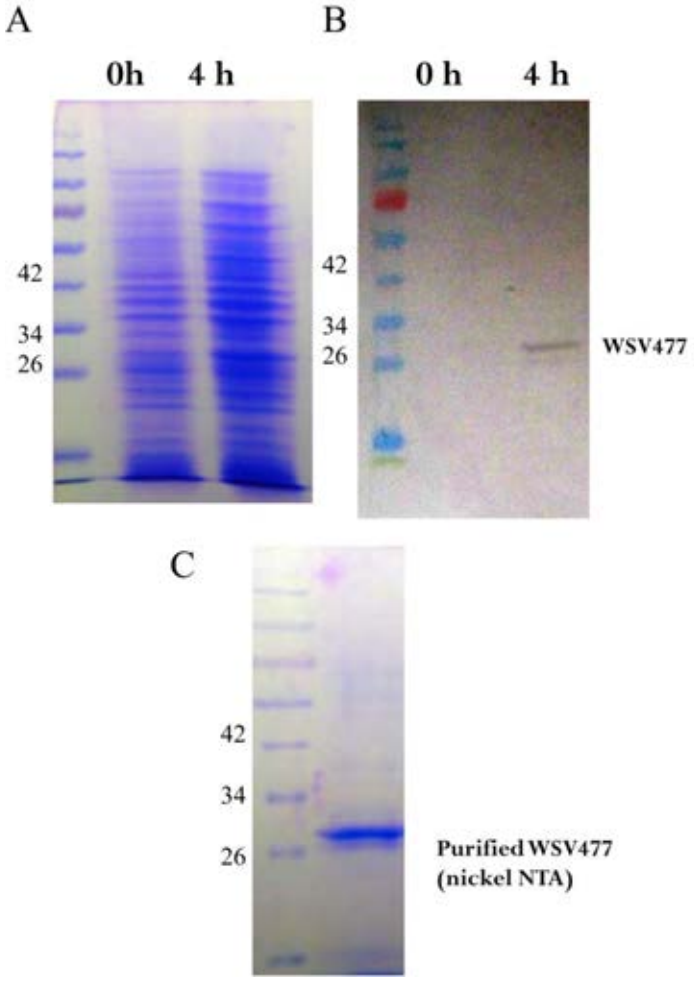

Figure 3.13 Production and purification of rWSV477. The recombinant plasmid, pBad-WSV477, was transformed into an *E. coli* strain top10 and a bacterial cell was, then, cultured in LB broth with ampicillin. To induce the expression of rWSV477, the culture was added the arabinose to the final concentration of 0.02% and cultured for 4 hours. At 0 and 4 hours, the bacterial cells were collected and detected the rWSV477 using SDS-PAGE staining with Coomassie brilliant blue (A) and western blot analysis with His-tag antibody (B). The rWSV477 in crude preparation was purified using Nickel-NTA column (C).

3.14 Confirmation of the interaction between SPI*Pm*2 and WSV477 using co-immunoprecipitation

To confirm the genuine interaction between SPI*Pm*2 and WSV477, the recombinant proteins, rSPI*Pm*2 and rWSV477 were prepared and tested for the interaction using co-immunoprecipitation. The incubated mixture of rSPI*Pm*2 and rWSV477 was applied through a column of protein A agarose beads covalently cross-linked to rabbit anti-rSPI*Pm*2. Either rSPI*Pm*2 or rWSV477 alone was used as controls. If there was no binding, the protein would flow through the column during washing step and would not co-elute.

From the results of the control experiment, the rSPI*Pm*2 was shown to bind with the anti-SPI*Pm*2 crosslinked beads but not rWSSV477 as shown in Fig. 3.14 lanes A and B. The pre-incubation of rSPI*Pm*2 and rWSV477 prior to applying into the column showed that the rSPI*Pm*2 and rWSV477 were co-eluted from the column which indicated the interaction between rSPI*Pm*2 and rWSV477 (Fig 3.14, lane C).

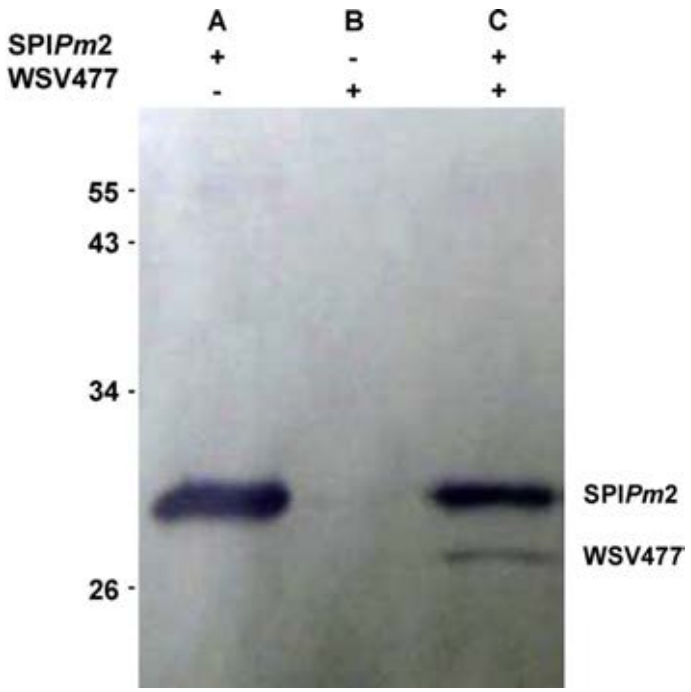

Figure 3.14 Co-immunoprecipitation of the interaction between rSPI*Pm* 2 and rWSV477. The rSPI*Pm*2 and rWSV477 was pre-incubated (C) and loaded into a column of protein A sepharose CL4B cross-linked with rabbit anti-SPI*Pm*2. The proteins were co-eluted with acidic glycine buffer after washing with high salt buffer. Either rSPI*Pm*2 (A) or rWSV477 (B) alone was used as controls. The eluted samples were analyzed by western blot analysis using anti-His antibody.

3.15 Proteinase activity of rWSV477

The SPI*Pm*2 is a proteinase inhibitor and interacts with rWSV477. Usually, any protein that interacts with proteinase inhibitor has a potency to be a proteinase. Therefore, the rWSV477 was tested for its proteinase activity by geletin zymography assay (Lantz and Ciborowski, 1994). The result showed the rWSV477 is not a proteinase (data not shown).

3.16 Co-localization of SPI*Pm*2 and WSV477

The SPI*Pm*2 is an abundant protein in shrimp hemocytes, while WSV477 is an early gene in WSSV infection. The interaction between SPI*Pm*2 and WSV477 was demonstrated in vitro using co-immunoprecipitation. It is, then, interesting to test the interaction of these protein in vivo. The normal and WSSV-infected hemocyte at different time courses: 6, 24 and 48 h were used to observe the co-localization of SPI*Pm*2 and WSV477. The immunofluorescent technique was performed and visualized under the confocal laser scanning microscopy. The two proteins were investigated with antibody specific to SPI*Pm*2 and WSV477. The localization of SPI*Pm*2 and WSV477 was shown as green and red colors, respectively. The confocal laser scanning micrographs shown in Fig. 3.15 revealed that the SPI*Pm*2 was detected in the cytoplasm of granular hemocyte in normal shrimp. As the viral infection progressed, the morhology of hemocytes was difficult to distinguish because of its degranulation. At 6 h post infection, the semigranular cells showed the expression of SPI*Pm*2 (green) and WSV477 (red) in the cytoplasm of the same hemocytes suggesting the co-localization of these proteins. For 24 and 48 h post infection, some hemocytes displayed the co-localization of proteins but it was hard to distinguish the type of hemocytes.

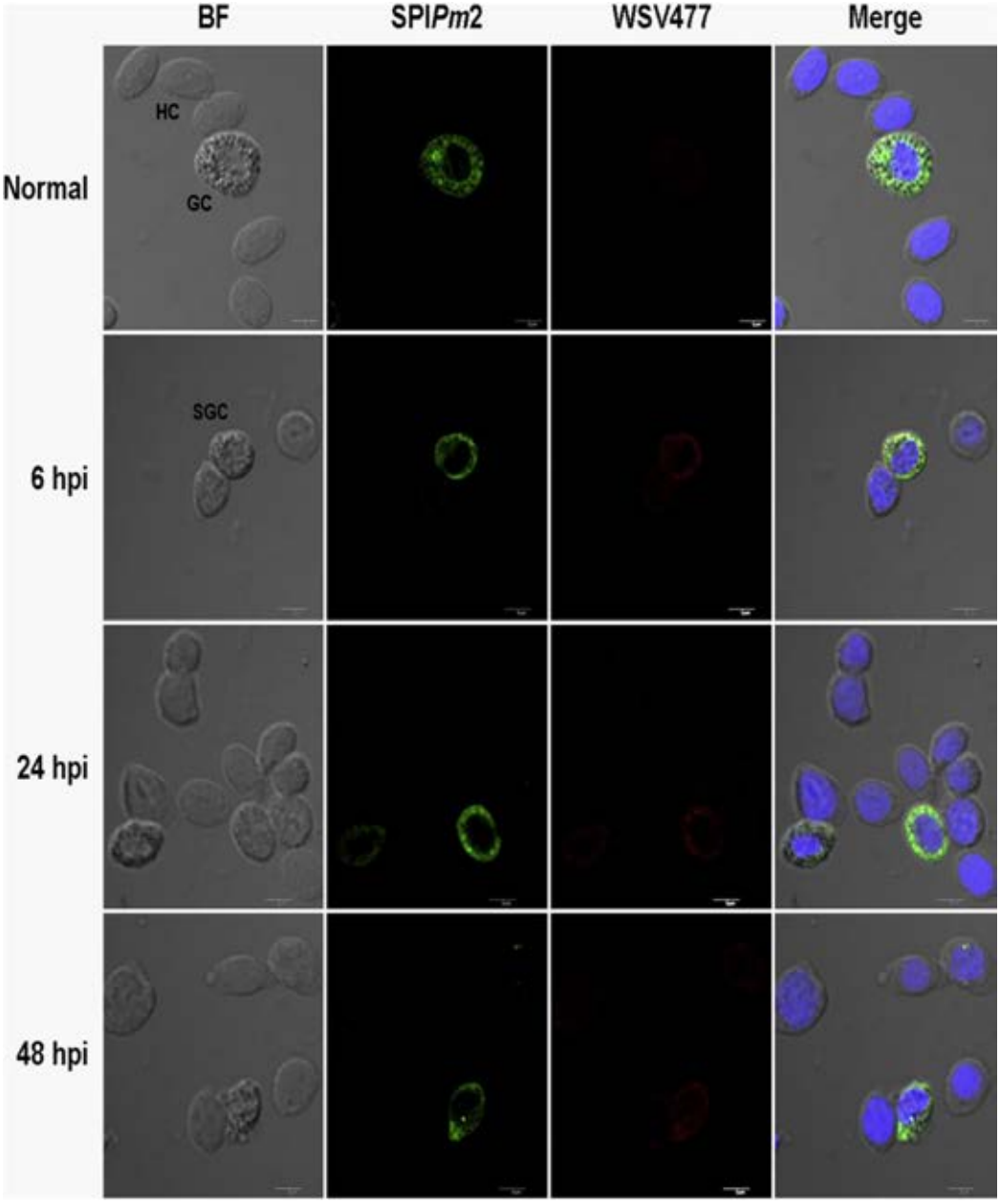

Figure 3.15 Co-localization of SPI*Pm*2 and WSV477 of the normal and WSSV-infected hemocytes under confocal laser scanning microscopy. Normal and WSSV infected hemocytes at 0, 6, 24 and 48 h were collected and fixed by 4% paraformadehyde. The hemocytes were coated to the poly-L-lysine slide and detected the protein expression of SPI*Pm*2 and WSV477 using purified rabbit polyclonal anti-SPI*Pm*2 and mouse anti-WSV477. The positive cells were then probed by secondary antibody conjugated with fluorescent dyes, Alexa Flour 488 (green) for SPI*Pm*2 and Alexa Flour 568 (red) for

WSV477. Nuclei (adjusted to blue color) were stained with TO-PRO-3 iodide. HC, SCG and GC are hyaline, semigranular and granular hemocytes, respectively. BFs are bright field images. Bars represent 5 μm.

3.17 Gene silencing of WSV477 in WSSV infected hemocyte cell culture and shrimp

The SPI*Pm*2 was previously reported to have a capability to inhibit the viral propagation, but the mechanism is not yet known. The interaction between SPI*Pm*2 and WSV477 was indicated by co-immunoprecipitation and co-localization. It was interesting to find out whether the interaction of SPI*Pm*2 to WSV477 could inhibit the viral propagation. The effect of these interaction could not be directly tested on viral propagation. Therefore, the important role of WSV477 was then investigated by RNA interference.

The primary hemocyte culture infected with WSSV was used for gene silencing by WSV477 dsRNA and then detected the expression of *WSV477*, *VP28* (the late viral protein gene) and *SPIPm2* using RT-PCR. GFP dsRNA and normal saline are used in the control experiment. The expression of β-actin gene was the internal control expression. After 24 h post dsRNA transfection, the expression of WSV477 in the cell culture was suppressed (Fig. 3.16 A).

The knockdown of WSV477 in shrimp had no significant effect on the expression of *ie1* but caused substantially reduced expression of *VP28* by 66% as compared to that of *GFP* control (Fig. 3.16 B). The results indicated the important role of WSV477 in WSSV propagation.

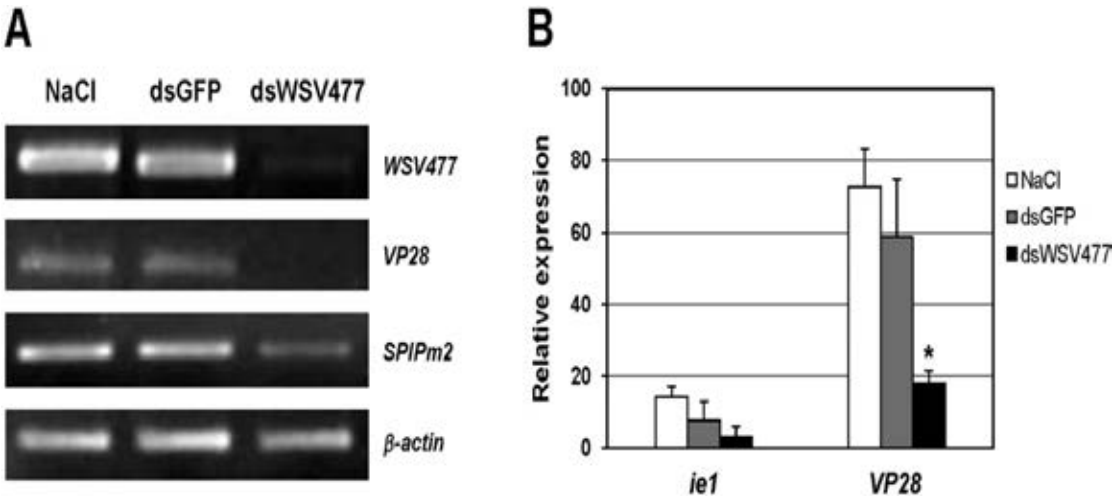

Figure 3.16 Gene silencing of WSV477 on the expression of *VP28*, *SPIPm2* and *ie1* in WSSV-infected shrimp hemocyte culture and shrimp. The shrimp hemocyte cell cultures (A) were transfected with normal saline, GFP dsRNA and WSV477 dsRNA followed by WSSV infection. After 24 h, the hemocyte cultures were harvested for RT-PCR analysis. The expression of *WSV477*, *VP28*, *SPIPm2* and *β-actin* were determined. (B) Three groups of 5 shrimp were doubly injected with normal saline, *GFP* dsRNA and *WSV477* dsRNA. The WSSV was included in the second injection. The shrimp hemocytes were collected for RT-PCR analysis of *WSV477*, *ie1* and *VP28* expression. The data are means of three replicate experiments ± SDs. Asterisk indicates significance at $P < 0.05$.

CHAPTER IV

DISCUSSION

Marine and fresh water animals are constantly and directly exposed to bacteria and virus in their environment. Constant protection is very important for their success to survive. Hemolymph of these animals are filled with many types of immune effectors that can rapidly fight against the microorganism in different ways. Varieties of antimicrobial peptides, as front-line defenses, are identified in *P. monodon* such as anti-lipopolysaccharide factors, crustins and penaeidins (Tassanakajon et al., 2010). Melanization, opsonization and coagulation are the other defensive reactions against the invaders (Jiravanichpaisal et al., 2006). In addition, the proteinases and proteinase inhibitors play an important role as the immune effectors in some processes of the innate immune system (Kanost, 1999; Rimphanitchayakit and Tassanakajon, 2010). Activation and inhibition of the processes are regulated by them.

The hemocytes are effective tissue which has an important role to defense the pathogenic microorganism in shrimp immune system. The defensive molecules in several pathways of shrimp immunity such as phagocytosis, blood clotting, phenoloxidase system and antimicrobial peptides are expressed mainly in the hemocyte. Moreover, the increase in the expression of the various immune genes occurs in the hemocytes upon the pathogenic microorganism invasion.

The SPI*Pm*2, a member of Kazal-serine proteinase inhibitor (KPI) family, is identified from the hemocyte library of *P. monodon*. The SPI*Pm*2 are mainly expressed in the hemocytes. The suppression subtractive hybridization approach

(Prapavorarat et al., 2010) and cDNA microarray (unpublished) reveal that the expression of SPI*Pm*2 is up-regulated upon YHV and WSSV infection. In this research, our expression studies had confirmed that the SPI*Pm*2 was up-regulated; it was expressed more in an early phase after WSSV infection in shrimp and gradually reduced with time. The result indicated that the SPI*Pm*2 might have a role in the shrimp immune response against WSSV infection. Similarly, report on *Pacifastacus leniusculus* also shows the up-regulation of a four-domain Kazal-serine proteinase inhibitor, PAPI 1, upon WSSV infection (Liu et al., 2006). The up-regulation is also observed in a two-domain KPI gene from Chinese shrimp, *F. chinensis*, and two two-domain KPI genes from red swamp crayfish, *P. clarkii* in response to WSSV challenge (Kong et al., 2009; Li et al., 2010).

Since the *SPIPm2* gene expression was up-regulated upon WSSV infection while the SPI*Pm*2 protein was hardly detected in the hemocytes from shrimp infected with WSSV for 24 h. It is possible that the number of hemocytes producing SPI*Pm*2 might be decreased because of the viral infection. Immunocytochemistry of the hemocytes was employed and it showed that during the course of WSSV infection up to 72 h, the percentages of SPI*Pm*2-producing hemocytes were reduced by more than half. These suggested that the SPI*Pm*2-producing hemocytes continuously secreted the SPI*Pm*2 as long as the infection was in progress and led to the reduction of the SPI*Pm*2-producing hemocytes while at the same time, its gene expression was up-regulated.

The immunofluorescent labeling technique was used to observe the location of SPI*Pm*2 and a WSSV late gene product VP28 in different shrimp hemocytes, granular, semigranular and hyaline cells. We later found that the SPI*Pm*2 was located

in the cytoplasm in the secretory granules of semigranular and granular hemocytes (see below). As the infection progressed, the hemocytes that showed the expression of VP28 protein are severely viral-infected but the SPI*Pm*2 cannot detect in these hemocytes. As the hemocytes secreted more and more SPI*Pm*2, it became agranular cells. The infected cells contained hardly any SPI*Pm*2 but the appearance of the cells, though looked degenerate, seemed to be either semigranular or granular hemocytes. This result was more or less in agreement with those of Wang et al. (Wang et al., 2002) and Jiang et al. (Jiang et al., 2006) that the WSSV selectively infected semigranular and granular hemocytes in *Penaeus merguiensis* and *Penaeus chinensis*, respectively.

From the above results, it is suggested that the virus can induce the secretion of SPI*Pm*2 from the SPI*Pm*2-producing hemocytes. To test this notion, we used the hemocyte primary cell culture which was easily to establish and able to avoid in situ influences from other factors in the shrimp circulation, particularly the SPI*Pm*2 itself. The secretion of SPI*Pm*2 was readily detected as early as 1 h after the WSSV challenge and increased as the infection progressed after adding the WSSV into the culture.

Using immunofluorescence technique, it indicates that the SPI*Pm*2 is synthesized, stored in semigranular and granular hemocytes, and secreted into the circulation as a hemolymph protein. To confirm the location of SPI*Pm*2 inside the cells, the immunogold labeling technique was used. We showed that the SPI*Pm*2 protein was stored in the granules of the SGC and GC, while the hyaline cells (HC) lacked the storage granule and, hence, did not accommodate SPI*Pm*2 protein. It has been shown that the very same cell types that produce SPI*Pm*2, namely SGC and GC,

in *Penaeus merguiensis* and *Penaeus chinensis* are also targets of WSSV attack (Wang et al., 2002; Jiang et al., 2006). In shrimp immune system, the different types of hemocyets have been identified the specific functions. Semigranular and granular cell are proposed to function in encapsulation, phagocytosis, prophenoloxidase (ProPO) system and cytotoxicity, while the hyaline cell functions in phagocytosis (Johansson et al., 2000).

To study the effect of SPI*Pm*2 protein on the survival of WSSV-infected shrimp, the recombinant SPI*Pm*2 was administrated by muscular injection along with WSSV. The mortality rate was observed. It was found that the rSPI*Pm*2 significantly delayed the mortality rate of shrimp. However, administration of anti-SPI*Pm*2 did not accelerate the mortality rate as expected because the increase in WSSV replication might not be high enough.

The antiviral activity of rSPI*Pm*2 was further investigated by using shrimp primary cell culture. In the experiments, the rSPI*Pm*2 was tested for its action whether it was neutralization of the virus or protection of the cells from virus. Interestingly, it was found that the replication of WSSV was retarded in both experiments of neutralization and protection. In addition, increasing amount of rSPI*Pm*2 resulted in more strength of antiviral activity.

The finding that the SPI*Pm*2 impeded the WSSV replication implied that the SPI*Pm*2 might interact with the cell or the virus or both. Using an ELISA technique, we showed that the SPI*Pm*2 bound to both the virus and shrimp hemocyte membrane. The bindings were saturable as more and more rSPI*Pm*2 was added. The binding to the cell membrane is intriguing. The WSSV infection requires that the virus recognizes its receptor on the cell surface before it is uptake by some means into the

cell. One way that SPI*Pm*2 can interfere with the infection is to bind to the viral receptor and block the viral entrance mechanism. So far, there are a few cellular proteins shown to interact with WSSV and claim to be WSSV receptor proteins or, at least, involved in WSSV infection. Three proteins of WSSV-binding proteins from the *P. monodon* hemocyte membrane bind to VP28 (Sritunyalucksana et al., 2006). Two of these are WBP25 and WBP30 (SPI*Pm*2). Additionally, WSSV envelope protein VP187 is able to interact with the *Metanephrops japonicus* cell surface protein β-integrin (Li et al., 2007). Gene silencing of *Pm*Rab7 and β-integrin inhibits the viral infection (Li et al., 2007; Ongvarrasopone et al., 2008).

Because the SPI*Pm*2 binds both WSSV and shrimp hemocyte membrane, it would be easy to speculate that, on one side, the SPI*Pm*2 may block the viral entry by binding to the viral protein VP28 and, on the other side, it may block the viral receptor on the cell surface. However, the speculation will be proven satisfactory only if the binding counterparts of SPI*Pm*2 on both sides be identified. We, then, may be able to uncover the mechanism of antiviral activity of SPI*Pm*2. In this study, the yeast two-hybrid screening was used to search for both the cellular and viral targets of SPI*Pm*2. Unfortunately, only five viral candidate proteins were identified. All but one was unknown. The only known protein was the 208-amino acid WSV477. The WSV477 was an early protein in WSSV life cycle, which might play a key role in DNA replication and virus proliferation. It was possibly a zinc finger regulatory protein with GTP-binding activity (Han et al., 2007)

Because the SPI*Pm*2 is a proteinase inhibitor, it is accustomed to think that the interacting counterpart is a proteinase. WSV477 protein was tested for the proteinase activity, but it was not a proteinase as anticipated. To confirm whether the interaction

was genuine, the co-immunoprecipitation assay was employed. The result confirmed the specific interaction between SPI*Pm*2 and WSV477. Therefore, it indicated that the SPI*Pm*2 did not function as a proteinase inhibitor in this instance.

With the finding that the SPI*Pm*2 bound to WSSV as mentioned above, it raised a question whether their binding affected the WSSV replication. That was how importance the WSV477 was to the virus. Moreover, the two proteins should be localized in the same cells. RNA interference was used to show that the expression of VP28 was down-regulated after *wsv477* gene knockdown. This result was exactly the effect of SPI*Pm*2 on WSV477 replication. However, the knocked down of *wsv477* had no significant effect on *ie1* expression since the expression of *ie1* was as early as 2 hpi whereas the expression of WSV477 began at 4 hpi (Han et al., 2007; Li et al., 2009).

Using confocal laser scanning microscope, the two proteins were co-localized in the cytoplasm of the same WSSV-infected hemocytes, both the granular and semigranular hemocytes. The results suggested that the SPI*Pm*2 was involved in WSSV response only during the early phase of infection by binding to the WSV477 in the infected cells. It was expected that the binding of SPI*Pm*2 toWSV477 retarded the function of WSV477 resulting in the decrease in WSSV replication.

Besides response to WSSV infection, we believed that the SPI*Pm*2 might have proteinase inhibitory function as well for it had three out of five active proteinase inhibitory domains.

CHAPTER V

CONCLUSION

A five-domain Kazal-type serine proteinase inhibitor, SPI*Pm*2, from *Penaeus monodon* has been implicated in antiviral responses for its up-regulation upon viral infection. The SPI*Pm*2 was detected in the hemocytes using immunocytochemical study of *P. monodon* hemocytes. The percentage of SPI*Pm*2-producing hemocytes was reduced by about half after WSSV infection. The result of immunofluorescence technique showed that the SPI*Pm*2 was located in the granules in the cytoplasm of semigranular and granular but not the hyaline hemocytes. Immunogold labeling technique further showed that the SPI*Pm*2 protein was stored in the granules of the hemocytes and the SPI*Pm*2 protein was increased upon viral infection. Moreover, the SPI*Pm*2 was secreted readily from the semigranular and granular hemocytes upon WSSV challenge and progression of infection. The WSSV-infected cells were either semigranular or granular hemocytes or both and depleted of SPI*Pm*2.

The injection of rSPI*Pm*2 into the shrimp prolonged the mortality rate of WSSV-infected shrimp. Using RT-PCR and qRT-PCR, the presence of rSPI*Pm*2 in neutralization and protection experiment at 6 h and 24 h significantly reduced the WSSV VP28 expression and the reduction of VP28 expression was dose-dependent on the amount of SPI*Pm*2. The antiviral activity was very likely due to the binding of SPI*Pm*2 to the components of viral particle and shrimp hemocyte cell membrane. The result indicated that SPI*Pm*2 was involved in the innate immunity against WSSV infection in shrimp.

Using yeast two-hybrid screening, five viral proteins: WSV020, WSV399, WSV267, WSV061 and WSV477 that interacted with bait protein SPI*Pm*2, were identified from the screening of WSSV ORF library. All but WSV477 were unknown. The WSV477 was reported to be a Cys2/Cys2-type zinc finger regulatory protein having ATP/GTP-binding activity. In vitro pull down assay confirmed the protein-protein interaction between rSPI*Pm*2 and rWSV477. Confocal laser scanning microscopy demonstrated the SPI*Pm*2 and WSV477 were co-localized in the cytoplasm of the shrimp hemocytes. The silencing of WSV477 gene resulted in down-regulated of viral late gene VP28, the same result obtained when the SPI*Pm*2 is used to neutralize the WSSV. In this instance, the SPI*Pm*2 did not function as proteinase inhibitor but inhibited the regulatory function of WSV477.

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Appendices

Appendix A

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Preparation for polyacrylamide gel electrophoresis

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

acrylamide	29.2	g
bis-acrylamide	0.8	g

Adjust volume to 100 ml with distilled water.

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

Tris (hydroxymethyl)-aminomethen 18.17 g

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

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

Tris (hydroxymethyl)-aminomethen 12.1 g

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

2. SDS-PAGE

- **12% Separating gel**

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

- **5.0% Stacking gel**

H_2O	2.7	ml

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

- **5x Sample buffer**

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

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

3. Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

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

Dissolve in distilled water to 1 litre. Do not adjust pH with acid or base (final pH should be 8.3).

Appendix B

Presentations

1. Oral presentation of "Interaction between a Kazal-type serine proteinase inhibitor and viral protein WSV477 in the black tiger shrimp *Penaeus monodon*" (2012) The 9th Asia-Pacific Marine Biotechnology Conference, Kochi City, Japan.

2. Oral presentation of "Characterization of a Kazal-type serine proteinase inhibitor SPI*Pm*2 and its partners from the black tiger shrimp *Penaeus monodon*" (2012) The Science Forum 2012, Chulalongkorn University, Thailand.

3. Poster presentation of "Antiviral role of a Kazal-type serine proteinase inhibitor SPI*Pm*2 from the black tiger shrimp *Penaeus monodon*" (2011) The 3rd BMB International Conference, Chiang Mai, Thailand.

4. Oral presentation of "Expression of a Kazal-type serine proteinase inhibitor SPI*Pm*2 from the black tiger shrimp *Penaeus monodon*" (2010) 15th Biological Sciences Graduate Congress, University of Malaya, Malaysia.

Publications

1. Donpusa S, **Ponprateep S,** Prapavorarat A, Visetnan S, Tassanakajon A, Rimphanitchayakit V. A Kazal-type serine proteinase inhibitor SPI*Pm*2 from black tiger shrimp *Penaeus monodon* is involved in antiviral responses. Developmental and comparative Immunology 2010; 34:1101-1108.

2. **Ponprateep S,** Tassanakajon A, Rimphanitchayakit V. A Kazal-type serine proteinase inhibitor SPI*Pm*2 from black tiger shrimp *Penaeus monodon* is capable of neutralization and protection of hemocytes from the white spot syndrome virus. Fish and Shellfish Immunology 2011; 31:1179-1185.

3. **Ponprateep S,** Kornsunee Phiwsaiya, Tassanakajon A, Rimphanitchayakit V. Interaction between Kazal-type serine proteinase inhibitor SPI*Pm*2 and viral protein WSV477 reduces the replication of white spot syndrome virus. Fish and Shellfish Immunology 2013; xxx:1-8.

BIOGRAPHY

Miss Sirikwan Ponprateep was born on July 10, 1981 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi in 2003. She graduated with the degree of Master of Science from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2008. She has studied for the degree of Doctor of Science at the Department of Biochemistry, Chulalongkorn University since 2008.

She had published her works in the research journals on the topics of "A Kazal-type serine proteinase inhibitor SPI*Pm*2 from black tiger shrimp *Penaeus monodon* is involved in antiviral responses", "A Kazal-type serine proteinase inhibitor SPI*Pm*2 from black tiger shrimp *Penaeus monodon* is capable of neutralization and protection of hemocytes from the white spot syndrome virus" and "Interaction between Kazal-type serine proteinase inhibitor SPI*Pm*2 and viral protein WSV477 reduces the replication of white spot syndrome virus".