CHARACTERIZATION OF *Pm*Spätzle AND SPI*Pm*5 FROM BLACK TIGER SHRIMP *Penaeus monodon*

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ลักษณะสมบัติของ *Pm*Spätzle และ SPI*Pm*5 จากกุ้งกุลาดำ *Penaeus monodon*

นายสิทธิชัย บุญรอด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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สิทธิชัย บุญรอด : ลักษณะสมบัติของ *Pm*Spätzle และ SPI*Pm*5 จากกุ้งกุลาดำ *Penaeus monodon*.(CHARACTERIZATION OF *Pm*Spätzle AND SPI*Pm*5 FROM BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาหลัก : ศ. ดร.วิเชียร ริมพณิชยกิจ

โปรตีนจากระบบภูมิคุ้มกันในน้ำเลือดของกุ้ง ได้แก่ เพปไทด์ต้านจุลชีพ และตัวยับยั้งซีรีนโพรทีเนส พบว่ามีส่วนเกี่ยวข้องในการต้านทานต่อการเข้าทำลายของเชื้อโรค หลังจากการเข้าทำลายของเชื้อโรคในกุ้ง โปรตีน Spätzle ก็ถูกสังเคราะห์ขึ้น และกระตุ้นสัญญาณการตอบสนองต่อการเข้าทำลายของเชื้อผ่านวิถี Toll ด้วยโปรตีน active Spätzle เพื่อที่จะสังเคราะห์เพปไทด์ต้านจุลชีพ ในงานวิจัยนี้ยีน *Pm*Spätzle ทั้ง 3 ไอโซฟอร์ม ถูกพบในกุ้งกุลาดำ *P. monodon* ได้แก่ ยีน *PmSpz1, 2* และ *3* โดยเลือกยีน *PmSpz1* เพื่อศึกษาหน้าที่ โดยจากการทดลองพบการแสดงออกของยีน *PmSpz1* ในทุกเนื้อเยื่อทดสอบ และมีการตอบสนองต่อการเข้าทำลายของเชื้อไวรัสตัวแดงดวงขาว (WSSV) การวิเคราะห์โปรตีน *Pm*Spz1 ในน้ำเลือดด้วยเทคนิค Western blot analysis พบว่าโปรตีน *Pm*Spz1 ส่วนใหญ่จะอยู่ในรูป active *Pm*Spz1 ซึ่งรอที่จะกระตุ้นผ่านวิถี Toll การฉีดรีคอมบิแนนท์โปรตีน *Pm*Spz1 ทำให้กุ้งไม่อ่อนแอต่อการเข้าทำลายของไวรัสตัวแดงดวงขาว สำหรับการฉีดด้วยรีคอมบิแนท์โปรตีน active *Pm*Spz1 ได้กระตุ้นการแสดงออกของยีน *crustinPm1, crustinPm7, ALFPm3, penaeidin3* แต่ไม่พบการแสดงออกยีน *penaeidin5* เพิ่มขึ้น จากงานวิจัยนี้แสดงให้เห็นว่า การแสดงออกที่เพิ่มขึ้นของยีนเพปไทด์ต้านจุลชีพดังกล่าว ยกเว้นยีน *penaeidin5* อยู่ภายใต้การควบคุมของวิถี Toll สำหรับโปรตีน SPI*Pm*5 ได้มีการศึกษาโปรตีนยับยั้งซีรีนโพรทีเนสชนิดคาซาลจากกุ้งกุลาดำ *P. monodon* โดยตรวจพบการแสดงออกของยีน *SPIPm5* ในทุกเนื้อเยื่อทดสอบ และมีการแสดงออกของยีน *SPIPm5* ที่เพิ่มขึ้นภายใต้ภาวะความเครียดจากความร้อน การเข้าทำลายของไวรัสตัวแดงดวงขาว (WSSV) และไวรัสหัวเหลือง (YHV) จากนั้นทำการฉีดรีคอมบิแนนท์โปรตีน SPI*Pm*5 เข้าไปในกุ้ง ซึ่งเป็นการเลียนแบบ การแสดงออกของยีน *SPIPm5* ที่เพิ่มขึ้น เช่นเดียวกับกุ้งที่อยู่ในภาวะความเครียดจากความร้อน พบว่ามีการแสดงออกของยีนทั้งที่เพิ่มขึ้น สำหรับยีน *crustinPm1* และ *penaeidin5* นอกจากนี้ยังพบว่าไม่มีการเปลี่ยนแปลงต่อการแสดงออกของยีน *penaeidin3, hsp70, SPIPm2* และ *SPIPm5* สำหรับรีคอมบิแนนท์โปรตีน SPI*Pm*5 ในน้ำเลือดของกุ้ง สามารถยับยั้งระบบโพรฟีนอลออกซิเดส ในการทดลองเพื่อศึกษาอัตราการรอดของกุ้ง พบว่ารีคอมบิแนนท์โปรตีน SPI*Pm*5 สามารถทำให้กุ้งมีอัตราการรอดที่เพิ่มขึ้นจากการเข้าทำลายของเชื้อไวรัสตัวแดงดวงขาว, ไวรัสหัวเหลือง, แบคทีเรีย *Vibrio harveyi* และ *Vibrio parahaemolyticus* การต้านทานที่เพิ่มขึ้นต่อการเข้าทำลายของเชื้อโรคนั้นสัณนิษฐานว่าเกิดจากการถูกกระตุ้นด้วยรีคอมบิแนนท์โปรตีน SPI*Pm*5 มีผลต่อการลดจำนวนทั้งในไวรัสตัวแดงดวงขาว, ไวรัสหัวเหลือง และการเจริญของเชื้อแบคทีเรีย *V. harveyi* และ *V. parahaemolyticus* แต่ไม่มีผลต่อการแสดงออกของยีนเพปไทด์ต้านจุลชีพ ซึ่งผลของการเข้าทำลายเชื้อโรคอาจถูกต้านทานด้วยรีคอมบิแนนท์โปรตีน SPI*Pm*5 ดังนั้นโปรตีน SPI*Pm*5 จึงมีบทบาทสำคัญต่อระบบภูมิคุ้มกันในกุ้งในการต้านทานต่อการเข้าทำลายของไวรัส และแบคทีเรียด้วย นอกเหนือจากทำหน้าที่เป็นตัวยับยั้งซีรีนโพรทีเนส

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Sittichai Boonrawd : CHARACTERIZATION OF *Pm*Spätzle AND SPI*Pm*5 FROM BLACK TIGER SHRIMP *Penaeus monodon*. Advisor: Prof. Vichien Rimphanitchayakit, Ph.D.

Humoral response proteins in shrimp, antimicrobial proteins (AMPs) and proteinase inhibitors, are implicated in host defenses against the pathogen infection because their expression is changed in response to microbial infection. After the pathogen infection in shrimp, the Spätzle protein is synthesized and activated the pathogenic signaling via Toll pathway by its active protein for the synthesis of AMPs. In this study, three *Pm*Spätzle isoforms, *PmSpz1*, *2* and *3 genes*, were identified in *Penaeus monodon*. The *PmSpz1* gene was chosen for studied in details. Its gene was expressed in all shrimp tissues tested and in response to WSSV infection. Western blot analysis of hemolymph showed that the *Pm*Spz1 mostly existed as an active form awaiting to activate Toll pathway. Injection of a recombinant *Pm*Spz1 rendered the shrimp less susceptible to the WSSV challenge. The recombinant of active Spätzle activated the synthesis of AMP genes: *crustinPm1*, *crustinPm7*, *ALFPm3*, *penaeidin3* but not *penaeidin5*. It is indicating that the expression of all antimicrobial proteins but not *penaeidin5* was expressed under the regulation of Toll pathway. For SPI*Pm*5 protein, the Kazal-type serine proteinase inhibitor from *P. monodon* was also studied. Its transcripts were also expressed in all tissues tested and its gene expression was up-regulated by heat stress, WSSV and YHV. Injection of rSPI*Pm*5 into shrimp to mimic heat stress condition had more or less no effect on the *crustinPm1*, *penaeidin3*, *penaeidin5*, *Hsp70*, *SPIPm2* and *SPIPm5*. The effect of rSPI*Pm*5 protein inhibited the hemolymph proPO activity. In survival experiments, the rSPI*Pm*5 could also prolong the shrimp from pathogen infection, WSSV, YHV, *Vibrio harveyi* and *Vibrio parahaemolyticus* in shrimp. The increased endurance against the microbial infection was due to the inhibitory effects presumably activated by rSPI*Pm*5 on viral replication and bacterial growth but not the expression of AMPs gene. The microbial inhibitory effects may be activated by rSPI*Pm*5. Therefore, the SPI*Pm*5 plays an important role in shrimp innate immunity against the viral and bacterial infection besides its function as a proteinase inhibitor.

Field of Study:	Biotechnology	Student's Signature .....
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bp	base pair
h	hour
kb	kilobase
kDa	kilodalton
M	molar
mg	milligram
ml	milliliter
mM	millimolar
ng	nanogram
nmol	nanomole
nm	nanometer
OD.	optical density (absorbance)
°C	degree Celcius
sec	second
µg	microgram
µl	microliter
µM	micromolar

CHAPTER I

INTRODUCTION

1.1 Shrimp farming development in Thailand

Shrimp farming has been cultured for food supply and the livelihood of coastal people in the past. The shrimp farming has been classified into the several periods. In 1960s, the native shrimp can be harvested from the natural farm or coastal Thailand. The species cultured in this period including banana shrimp *Penaeus merguinensis*, India white shrimp *P. indicus*, school shrimp *Metapenaeus monoceros* and black tiger shrimp *P. monodon*. In 1970-1980, the shrimp farming became popular in a few groups of investors. The shrimp culture developed the main species of *P. merguinensis* and *P. monodon* through the scientific experiment at farm levels and extensive shrimp farming for the exporting to the Japanese market and requirement. In 1980-1986, this period had increased an extensive shrimp farming and changed the farm operation intensity to semi-intensive shrimp culture. It was smaller than the natural farm but provided the significantly higher yields. This period also showed the signs of shrimp massive loss in Taiwan. In 1986-1992, the *P. monodon* rapidly developed the shrimp culture but the banana shrimp declined. Then, Taiwan also transfers the shrimp farming technology to Thailand. Unfortunately, the growth rate of shrimp declined because of the water quality problems. Until in 1992-1996, the shrimp farmers found the falling shrimp production due to the outbreak of pathogen from the viral and bacterial infection. Then, in 1996 – present, the shrimp farming had developed the new shrimp culture techniques, new species, public participation, policy of the government, strategies for the good management farming and extend the international network ((CORIN), 2000; Szuster, 2006)

The increasing mortality of shrimp production remains never-ending due to the microbial infections. The shrimp aquaculture in China and Thailand is significantly declined in 2013 because the shrimp is infected by *Vibrio parahaemolyticus*. It causes acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS). The shrimp aquaculture in China is slightly recovered in 2014 but not in Thailand. Both China and Thailand expected to make improvement the shrimp by 2018 (**Fig 1.1**).

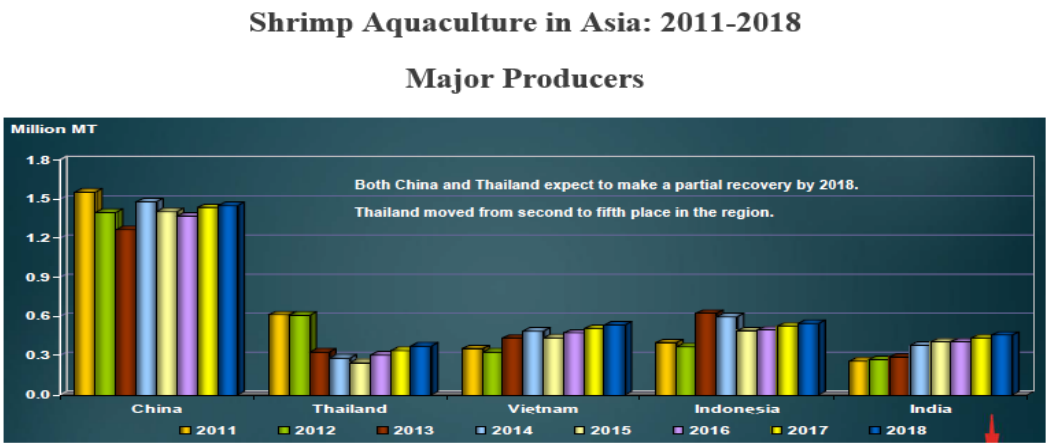

Figure 1.1. Shrimp aquaculture by major producing region during 2011–2018 **(Sources:** FAO (2016) for 2011; FAO (2016) and GOAL (2014) for 2012–2014; GOAL (2016) for 2014–2018**)**

The understanding of shrimp antimicrobial activity is important for the protective infection, help the life of shrimp, good management of shrimp farming and control the diseases in shrimp aquaculture.

1.2 The microbial infection

The major causes of shrimp diseases are mainly by viral and bacterial infection and subsequently cause the high mortality rate in shrimp farming (**Fig 1.2**). Nowadays, there are no drugs available for treatment the infected-shrimp better than the management of pond and the good quality of water. From previous studies, the viral infection has been identified in shrimp such as white spot syndrome disease, yellow head disease, infectious hypodermal and hematopoietic necrosis disease, taura syndrome, hepatopancreatic parvovirus disease and monodon baculovirus disease that cause the vast mortality in farming. In addition, the bacterial infection, *V. harveyi* and *V. parahaemolyticus* (VP_{AHPND}), have been reported that cause slow growth, highmortality and the vast loss production in shrimp aquaculture like the viral infection.

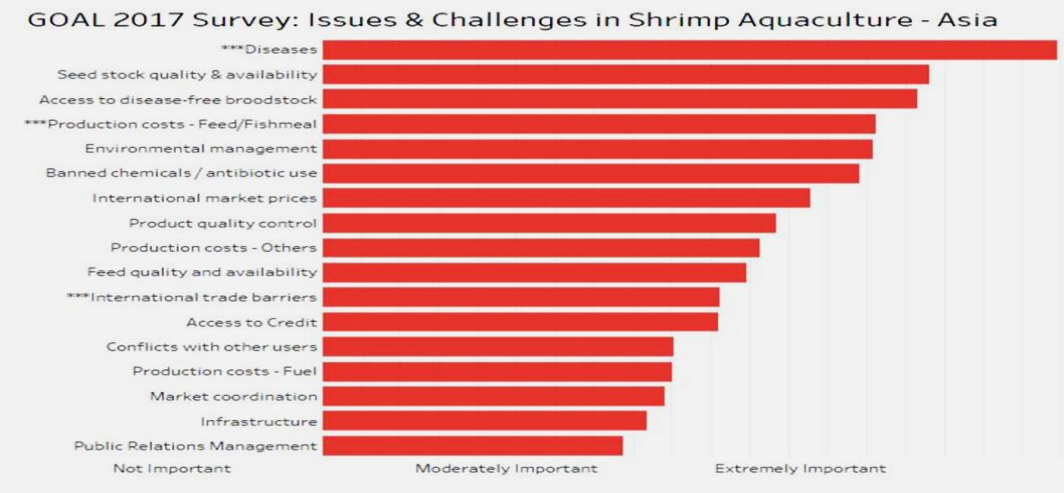

Figure 1.2. Factors affecting shrimp aquaculture in all countries (Source: GOAL 2017)

1.2.1 WSSV

White spot disease (WSD) causes by WSSV infection. WSD is one of high virulent of viral disease in crustacean. WSSV particle consists of the large circular dsDNA virus and rod-shaped containing the viral genome about 300 kb. The virion is a large 80-120 × 250-380 nm symmetrical, particle structure quite similar to baculovirus shape. It is classified in a viral family name as the *Nimaviridae*, genus *Whispovirus* (Liu *et al.*, 2009). The WSSV was first observed in China in 1991 and severe outbreak into the other countries in South-East Asia, the United State, India, west of India, East of the Mediterranean Sea and Europe. The sign of WSD builds the viral white spots on the carapace as a result from abnormalities of calcium accumulation. WSSV can transmitted through the consumption, cannibalism or predation and expose to the water containing with WSSV infection (Verbruggen *et al.*, 2016)

The WSSV isolates was identified to four types including WSSV-CN from China, WSSV-TH from Thailand, WSSV-TW from Taiwan and WSSV-KR from Korea. The five structural proteins of white spot disease virus have been identified. The VP28 and VP19 protein are characterized as envelope protein, VP24 as nucleocapsid protein, VP26 as tegument and VP15 as nucleocapsid/core protein (Escobedo-Bonilla *et al.*, 2008; Verbruggen *et al.*, 2016). Moreover, the binding protein of *Pm*Rab7 from shrimp is a small GTPase protein binding. It binds directly to VP28 and is required for WSSV replication (Liu *et al.*, 2009).

The life cycle of WSSV was shown in **figure 1.3**. The WSSV particles invades through the recipient cell. Interaction of viral proteins and recipient proteins induces the clathrin-mediated endocytosis and local into early endosome. The VP28 interacts with Rab7 protein in the low pH condition and then nucleocapsid of WSSV exits from the endosome. The mechanism of WSSV reaches to nucleus remains poorly understood. For the viral replication, it activates the essential viral genes for reproducible particles. Since the WSSV

does not forming its transcriptional machinery, it depend on the recipient infected cell like parasite infection. The transcriptional factors of recipient cell can bind to the initiation viral promoter for transcriptional step. For the host cell cycle, WSSV can work through the S-phase of cell cycle and depends on the recipient cell for replication processes. The viruses can regulate the recipient cell cycle pass into interaction along with the retinoblastoma (Rb) proteins which are the main regulator of cell cycle processes. In addition, the miRNA synthesis process was related to the apoptosis in infected-cell that inhibit the caspase activity. For endoplasmic reticulum stress (ER stress) responses, the high viral protein production can induce to the ER stress process. The ER stress response protein, unfolded protein response (UPR) is found after viral infection and it can also activate the viral gene expression. Finally in the iron storage, the ferritin binding to iron is important to the host defense system. It can reduces the WSSV proliferation. WSSV replication requires the essential nutrients including iron for energy and can also interfere with the iron and ferritin binding step for WSSV proliferation.

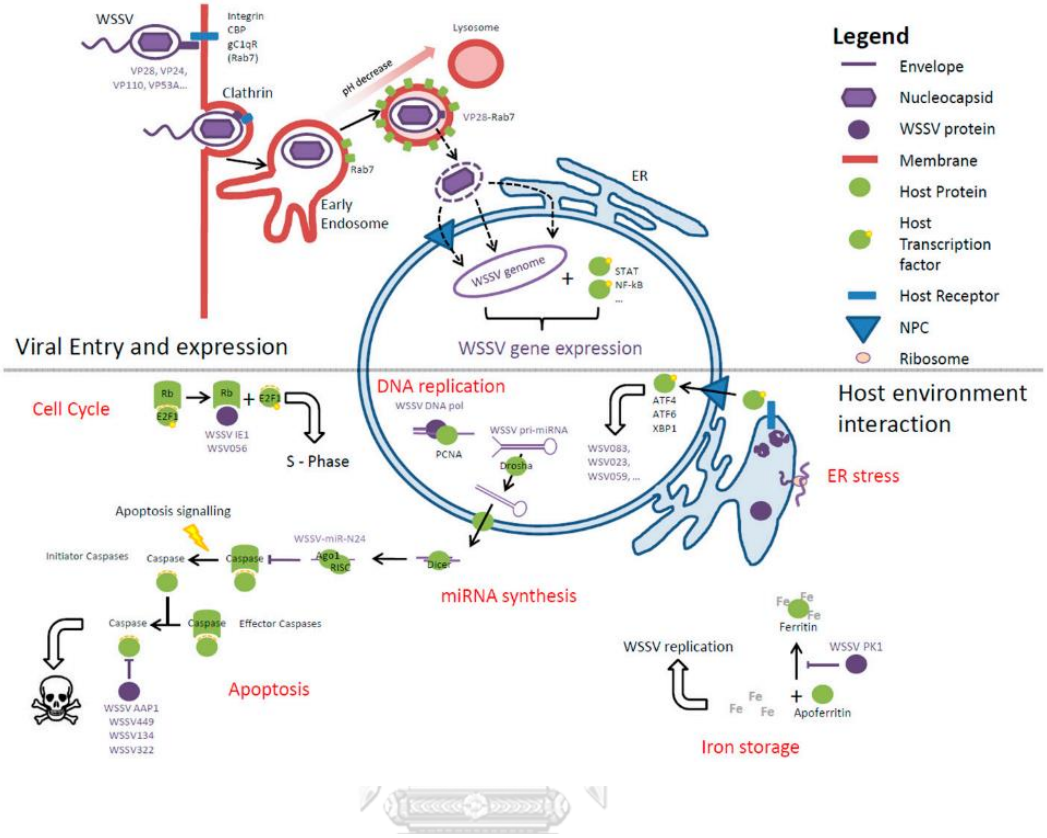

Figure 1.3. The WSSV infection processes in the host cell. The WSSV particle is attached and bound to the host cell membrane. The envelope nucleocapsid is covered in the early endosome. In the pH decrease condition, the nucleocapsid exit from the VP28-Rab7 protein membrane complex and the WSSV genome can pass through the nuclear membrane. WSSV gene expression was processed and the WSSV infection related to the DNA replication, cell cycle, mi-RNA synthesis, inhibition of caspase activity, ER stress and the inhibition of iron and Ferritin in the iron storage process (Verbruggen *et al.*, 2016).

1.2.2 YHV

Yellow head diseases (YHD) that causes the appearance of yellowish swollen cephalothorax, hepatopancreas and white yellow brown gills. The shrimp is also necrosis sign in lymphoid tissue, gills, connective tissues, hemocytes and hematopoietic organs. The lymphoid tissue is a first known target of YHD depend on the histopathology and YHV particle cellular protein studies. The YHD causes by yellow head virus (YHV). The group of 7 YHV types have been identified including YHV-type1 (Thailand), YHV-type2 (GAV, Australia), YHV-type3 (Thailand and Veitnam), YHV-type4 (India), YHV-type5 (Malaysia, Thailand and Philippines), YHV-type6 (Mozambique) and YHV-type7 (Australia) (Thitamadee *et al.*, 2016).

YHV is first reported in Thailand in 1992. There are two types of YHV identified including YHV- type1a and - type1b in *P. monodon* and *L. vannamei*, respectively (Flegel, 2012). The YHV-type1 is the most virulent type. Its particle consists of an enveloped protein (40-60 × 150-200 nm) with genome containing ssRNA (approximately 26 kbp) and classified into the genus *Okavirus*, *Roniviridae*, *Nidovirus*. YHV consists of the major structural proteins such as glycoprotein116, glycoprotein64 and nucleocapsid protein p20 (Liu *et al*., 2009).

YHV particles entry into the cells by endocytosis that the YHV envelope is fused with the cell membrane and uncoated in the endosome complex. The YHV genome releases into the cytoplasm by breaking down the endosomal membrane. The RNA viral genome is replicated and synthesized the nucleocapsid. The nucleocapsid genomes binds into rough endoplasmid reticulum (RER) and transfers through RER-Golgi for envelope forming. Then, the completely viral particles are packaged in secretory vesicles and released by exocytosis at the cell membrane (**Fig 1.4**) (Duangsuwan *et al.*, 2011).

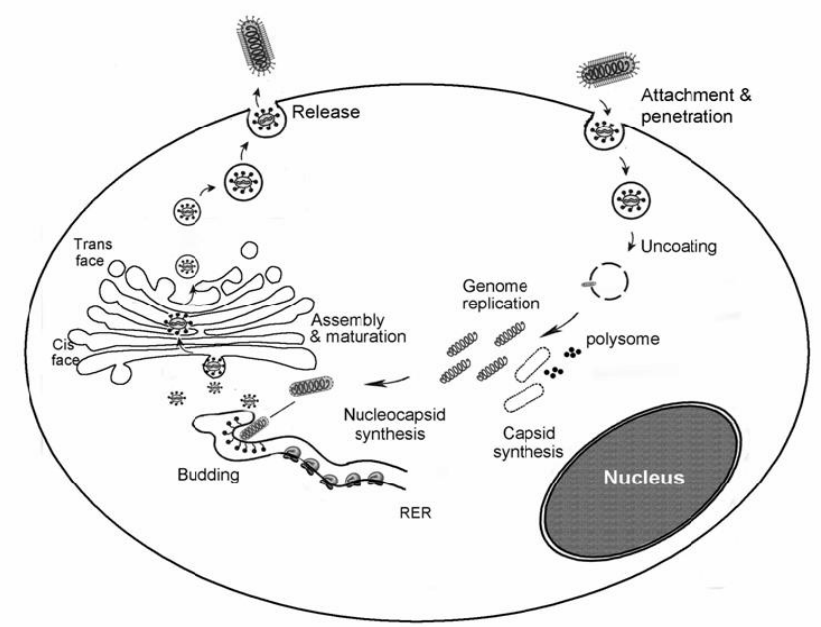

Figure 1.4. The YHV infection in stromal cells and hemocytes in the lymphoid tubule wall. The viral particle entry to the cells by attachment and penetration. After passing through the cell membrane by endocytosis, the virus is uncoated into cytoplasm together with the YHV genome replicated and its nucleocapsid was synthesized. The genome and nucleocapsid bound into rough endoplasmid reticulum (RER) and transfer pass the RER-Golgi. The YHV was packaged in the secretory vesicles and released the viral particles at the cell membrane by exocytosis (Duangsuwan *et al.,* 2011).

1.2.3 IHHNV

Infectious Hyperdomal and Haematopoietic Necrosis disease causes by IHHNV. It is the smallest of infectious virus. It is a non-enveloped icosahedron virus and the diameter within 20–22 nm. The genome of IHHNV contains linear ssDNA with an estimated size of 3.9 kbp. Several types of IHHNV were identified including Type 1, 2, 3A and 3B (Tang *et al.,* 2007). IHHNV was found in the life cycle of shrimp *L. vannamei*. For the IHHN-like virus was found in Australia. Its viral DNA sequences can insert into the shrimp genome. (Tang *et al.,* 2007).

1.2.4 TSV

Taura syndrome virus (TSV) is classified in family of *Picornaviridae* and first reported in 1992. Its particle is a non-enveloped icosahedral virus and symmetry diameter about 31-32 nm. Its genome contains line positive-ssRNA an estimated size of 10 kbp. There are three major and one minor proteins of TSV about 55, 40, 24 kDa and the last one 58 kDa. The TSV can be replicated into cytoplasm of the host cell (Liu *et al.*, 2009).

1.2.5 HPV

HPV causes the hepatopancreatic parvovirus disease. Its particle is the diameter within 22 nm and icosahedral symmetry. The genome of HPV contains negative-ssDNA an estimate size 5-6 kbp. There are the 3 large open reading frames including encoding protein 428 (ORF1), 579 (ORF2) and 818 (ORF3) amino acid and estimated size 50, 68 and 92 kDa, respectively. HPV has been infected into the early and postlarval stages. Moreover, the stunted growth is found in the early juvenile stages (Safeena *et al.*, 2012).

1.2.6 MBV

Monodon-type baculovirus (MBV) is first identified from Taiwan shrimp farming. MBV is well-known in another name *Penaeus monodon* singly enveloped nuclear polyhedrosis virus (*Pm*SNPV) and classified in genus *Nucleopolyhedrovirus*. It is rod-shaped, enveloped and the particle is the diameter within 42-77 nm. The genome of MBV contains dsDNA ranging 80-160 kbp. Its ORF of MBV encodes polyhedrin polypeptide protein about 53 kDa and two minor proteins about 47 and 49 kDa. The gross sign of MBV infection shows the spherical inclusion bodies invasion into the infected-hepatopancreas and midgut tissues (Rajendran *et al.*, 2012).

1.2.7 Vibrio-infection

For bacterial diseases, *Vibrio* species are one of the primary diseases pathogenic agent in penaeid shrimp. *Vibrio* species are gram-negative

facultative anaerobic bacteria of the *Vibrionaceae* family. The *Vibrio* bacteria infection leads the shrimp to slow growth and high mortality. The *Vibrio harveyi* is one of gram-negative and luminous marine bacteria (Dash *et al.*, 2017). The luminescence symptom shows the signs easily visible at night in the infected-shrimp. The shrimp is also found the reduced growth, lethargy, patches on the body and muscle opacity are the vibriosis. The *Vibrio* bacteria has the flagella for motility and also release the exotoxins which causes 100% mortality in *P. monodon* hatcheries (Torky *et al.*, 2016).

Acute hepatopancreatic necrosis syndrome (AHPNS)/ Early mortality syndrome (EMS) causes by *V. parahaemolyticus*. EMS is first reported in China in 2009. It is transmitted by oral, colonize in the gastrointestinal tract of shrimp and produce the toxins. The toxins causes the tissues destruction and dysfunction of the shrimp digestive organ in hepatopancreas. The signs of EMS/AHPNS is an empty stomach and midgut. In early 2013, AHPND caused by VP_{AHPND} are identified and isolated from the AHPND outbreak pond. The VP_{AHPND} produces the sloughing of tubule epithelial cells. From the previous studies, the pVA1 plasmid causes the AHPND. It consists of the conjugation related genes, two plasmid immobilizing and associated with a post-segregational killing system (PSK) genes. The sequence analysis of pVA1 plasmid contains the homolog of the insecticidal *Photorhodus* insect-related (Pir) PirA and PirB toxins (Dangtip *et al.*, 2015; Thitamadee *et al.*, 2016). From previous studies, the first crystal structure $PirA^{VP}/PirB^{VP}$ toxins (**Fig. 1.5B**) of *V. parahaemolyticus* were similar to Cry toxins from *Bacillus thuringiensis*. The structural protein of Bt consists of the domain I protein in pore-formation domain I, the domain II of binding receptor protein and the domain III of sugar binding protein (**Fig. 1.5A**). The Cry insecticidal toxin is an important using in agriculture.

The jelly-roll structure of $PirA^{VP}$ is folded into the eight-stranded antiparallel β-barrel whereas two $PirB^{VP}$ toxin domains is also called the N-

terminal ($PirB^{VP}N$) and the C-terminal ($PirB^{VP}C$), consisting of a seven-α-helices bundle and an eight-stranded antiparallel beta-sheets, respectively. The function of $PirA^{VP}$/$PirB^{VP}$ and Cry toxins was compared. The similarity role of $PirA^{VP}$ and Cry damain III is the specific binding to cell membrane receptor. Three loop of $PirA^{VP}$ is formed to the sugar-binding pocket. For the $PirB^{VP}N$ function, it is similar to Cry toxin domain I that forming a pore protein on the cell membrane whereas $PirB^{VP}C$ structure is similar role with Cry toxin domain II that it can interact with the insect receptor. The $PirB^{VP}C$ is also a receptor binding domain (**Fig. 1.5**) (Lin *et al*., 2017).

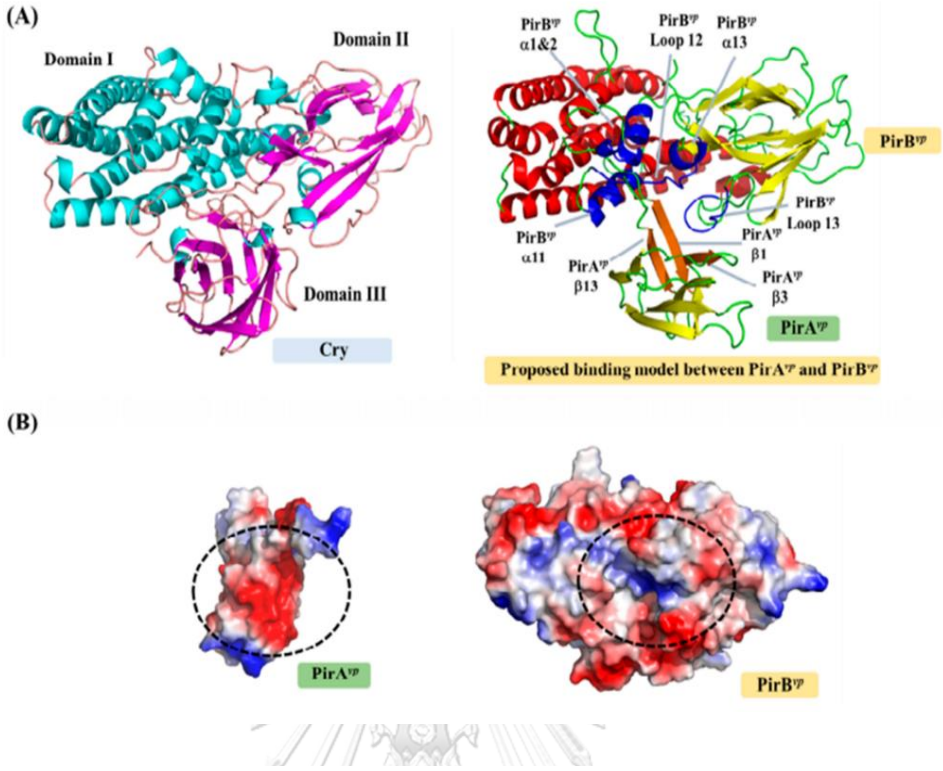

Figure 1.5. The possible binding interface of PirA^{VP}/PirB^{VP} toxins and Cry toxins domain. **(A)** Three domain of Cry toxin, domain I, II and III, respectively. The binding region of PirAB^{VP} was analyzed by refer to Cry toxin domains. **(B)** The PirA^{VP} and PirB^{VP} complex showed the possible binding regions at the surface charges (Lin *et al.*, 2017).

1.3 Crustacean innate immunity

Crustacean relies on the innate immunity but lack the adaptive immunity. The innate immunity is less specific to the pathogen but it is rapidly and efficiently against the invader. The innate immune defense consists of the cellular and humoral immune responses. There are three cellular responses consist of phagocytosis, encapsulation and nodulation whereas the humoral responses involves the infection signaling and secrete the several immune components after pathogen infection such as antimicrobial proteins (AMPs), family of proteinase inhibitors, prophenoloxidase and cytokine-like factors, etc.

1.3.1 Cellular-mediated defense system

The hemocyte is a major defense immune cells in shrimp. The types of hemocytes have been classified including granular (GC), semigranular (SGC) and hyaline cells (HC) (Xu *et al.*, 2014). The several immunity response molecules are induced, activated, accumulated in the granules of hemocytes and secreted into the hemolymph for against the pathogen. For the defense response of cells, it involves the hemocytes such as phagocytosis, encapsulation and nodulation (Jiravanichpaisal *et al.*, 2006).

Phagocytosis is a main cellular-mediated defense system in shrimp and its function is specifically processed by hyaline and semi-granulocyte cells. The hemocytes is directly response to the microbial infection by the chemotaxis, adherence, ingestion, pathogen destruction and exocytosis, respectively (Aguirre-Guzmán *et al.*, 2009).

Encapsulation is another process of hemocytes for elimination the pathogen. It can remove the big antigen by granulocyte and semi-granulocyte which are recognition of the invading agent, surrounding by multiple layers of hemocytes for prevent the pathogen spreading (Aguirre-Guzmán *et al.*, 2009).

For another cell-defense mechanism, the nodulation entraps the pathogen into the center of hemolytical nodules, then, the pathogens are destroyed by the secretory defense molecules from the hemocytes. The nodules formation is processed undergo the activation of proPO system, melanization and destruction of pathogens (Aguirre-Guzmán *et al.*, 2009).

1.3.2 Humoral defense system

1.3.2.1 Pattern recognition proteins (PRPs)

The pattern recognition is the first line in the innate immune system. The immunity system is response to the pathogen by pattern recognition proteins (PRPs). The PRPs specifically recognize to the membrane components of the pathogen including lipopolysaccharide endotoxin (LPS), peptidoglycans (PGN) lipoteichoic acid (LTA) and Beta-1,3-glucan from Gram-negative, Gram-positive bacteria and fungi, respectively (Iwanaga & Lee, 2005). All of microbial cell membrane components is called pathogen-associated molecular patterns (PAMPs). There are the complex of the polysaccharides interact with glycoproteins on the surface of microorganism. For PRPs, lipopolysaccharide (LGBP) or Beta-1,3-glucan binding protein (βGBP) function were characterized (Amparyup *et al*., 2012; Jiravanichpaisal *et al*., 2006). Interaction of PRPs and microbial components enhances the innate immune system and lead to destroy the microorganism infection (Iwanaga & Lee, 2005). The PRPs of black tiger shrimp (*Pm*LGBP) from hemocytes is up-regulated after *V. harveyi* challenge. The binding of *Pm*LGBP and LPS or Beta-1,3-glucan also involve to the prophenoloxidase system (Amparyup *et al*., 2012). In the current, the pattern recognition receptor proteins (PRRs) were characterized their function in shrimp including Beta-1,3 glucanase related proteins (BGRP), Beta-1,3 glucan binding protein (BGBP), c-type lectins (CTL), scavenger receptor (SRs), fibrinogen related proteins (FREPs), thioester containing proteins (TEPs), down syndrome cell adhesion molecule (DSCAM), galactoside-binding lectins (galectins), Serine protease homologs (SPHs),

trans-activation response RNA-binding protein (TRBP) and Toll like receptors (TLRs). All of family PRR proteins are also different specific binding and immune functions (Wang *et al.*, 2013)

1.3.2.2 Apoptosis

Another the defense mechanism of cell, the programmed cell death or apoptosis process is induced after viral infection in crustacean (Leu *et al.*, 2013). The genetic damage, unhealthy and uncontrolled cellular proliferation of cells are removed by the apoptotic processes and also change the physiological and biological of the viral infected-cells for elimination. From previous studies, the apoptosis has protective activity in host cells against WSSV infection (Wang *et al.*, 2013) and also limit the viral replication processes (Xu *et al.*, 2014). The regulator genes of apoptosis have been reported including *PmCasp* (activation of effector caspase), *MjCaspase* (activation of initiator caspase), volt age dependent anion channel (VDAC), tumor suppressor like protein (TSL) and gC1qR (**Fig 1.6**) (Xu *et al.*, 2014). The caspases are the key gene in the apoptosis pathway and also link to the inflammation and melanization (Jearaphunt *et al.*, 2014).

Like apoptosis, WSSV can produce several the viral suppressor proteins or miRNAs for against the host apoptosis processes including AAP-1 (WSV449), WSV222, VP38, WSSV134, WSSV322, WSSV-miR-N24 and hijacking anti-apoptosis of host protein including calreticulin (CRT), inhibitor of apoptosis protein (*Pm*IAP) and fortilin. It is indicating that WSSV can process its replication cycle without the inhibition of host apoptosis process (**Fig 1.6**) (Xu *et al.*, 2014).

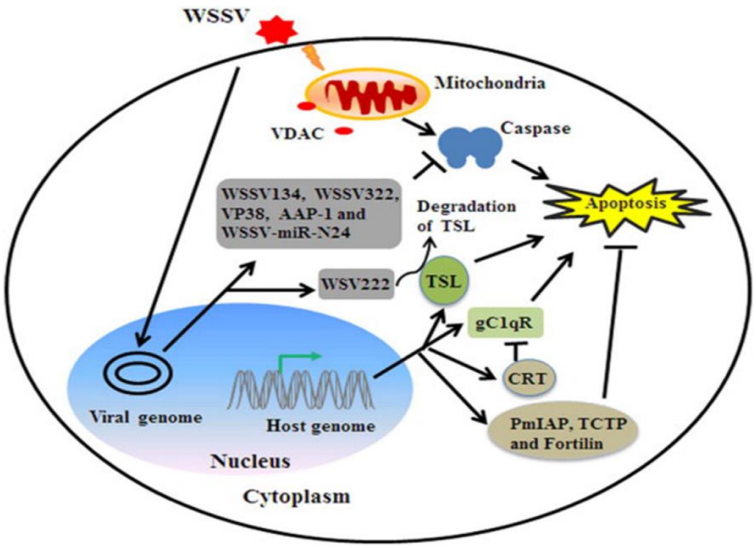

Figure 1.6. The apoptosis model of WSSV-infected shrimp. After the viral infection, the sensor of the host cell activates the apoptosis related-gene components for against WSSV infection. The apoptosis related-genes are induced including caspases, VDAC, TSL and gC1qR. All genes expression inhibits the viral replication processes. In addition, WSSV also produce the anti-apoptosis proteins and miRNAs for prevention its replication cycle such as WSSV134, WSV222, WSSV322, VP38, AAP-1 and WSSV-miR-N24. For anti-apoptosis process, the anti-proteins also activate: CRT, *Pm*IAP, TCTP and fortilin (Xu *et al.*, 2014).

1.3.2.3 Blood coagulation

The blood clotting is the first line of the humoral immune response of the host cells. It is important in the prevention of blood loss, pathogen infection and wound healing from injuries (Maningas et al., 2013). The activation of coagulation process is found in the hyaline cells. The process induces and releases the clotting component proteins for polymerization (Yeh *et al.*, 1998). The clotting process is activated by calcium-dependent transglutaminase (Ca^{2+}-TGase) for linking with the formation of clottable protein (CP) (**Fig 1.7**). The clot backbone consists of branch chain between ε-amino group of a peptide-bound lysine and γ-carboxamide group of a peptide-bound glutamine (Chen *et al.*, 2005). In shrimp, two transglutaminase proteins (STG) including STGI and STGII were characterized. Only the STGII involved the coagulation system (Yeh *et al.*, 2006). In shrimp, the effect of silencing TGase gene experiment

showed significantly decresing to the gene expression of *lysozyme* and *crustin* (Fagutao *et al*., 2012). It means that the TGase can control some innate immune genes in shrimp.

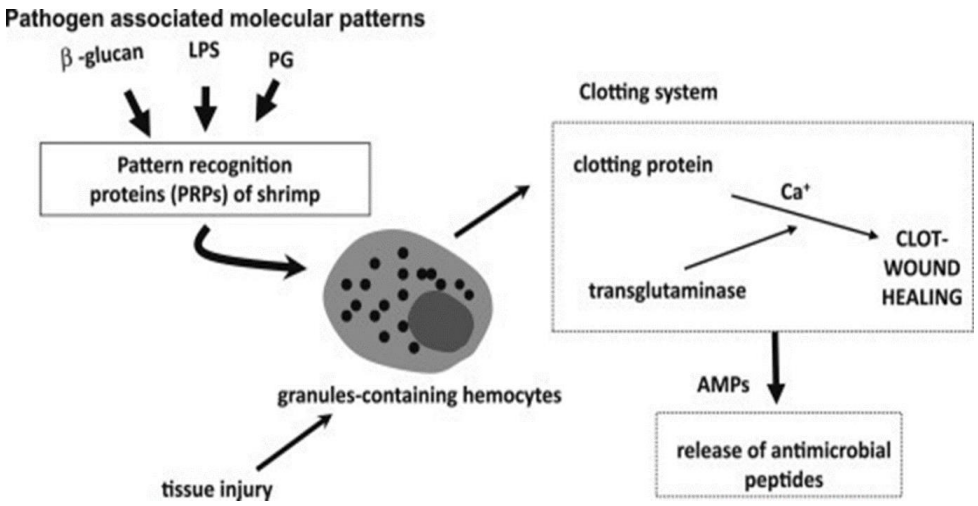

Figure 1.7. Blood clotting cascade in shrimp. The clotting system is activated by pathogenic infection or tissues damage. Transglutaminase (TGase) and clottable protein (CP) are induced from hemocytes and the clotting processes is linked to the expression of AMPs gene. The clotting processes prevent microorganism to enter to the host (Maningas *et al.*, 2013).

1.3.2.4 Melanization

Melanization is an immune defense system of humoral immune response in crustacean. It is an essential defense system for prevention the host from the microbial infection and also injuries. After microbial infection, the melanization is initiated by the activation prophenoloxidase system via the signaling of the cell membrane components to the PRPs (Amparyup *et al.*, 2013). Then, final proteinases are activated by serine proteinase cascade and changed the proPPAE to PPAE. Then, the inactive form of proPOs cleaves to active POs and induce the production by converting the phenol to quinone

(Tassanakajon *et al*., 2018) (**Fig 1.8**). Finally, the killing process produces at the injuries site and around the pathogen infection.

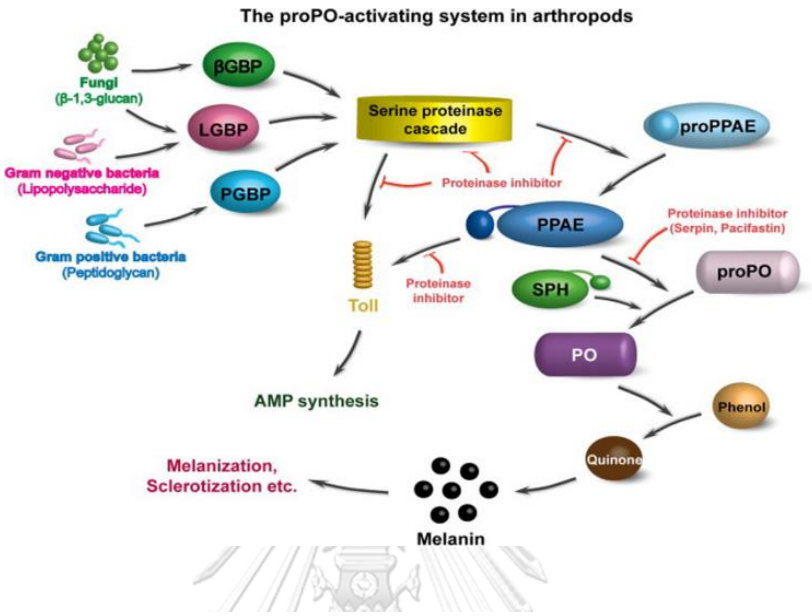

Figure 1.8. The activated-prophenoloxidase system outline in insect. After infection, PAMPs (LPS, PGN and β-1,3-glucan) are recognized by PRPs of host. The serine proteinase activates the proPPAE to PPAE. Then the proPO is converted to PO. The precussor phenol changes to quinone, melanin against around to the pathogen for the melanization and sclerotization. For the AMPs synthesis is induced via the Toll pathway (Amparyup *et al.*, 2013).

1.4 Spätzle

Spätzle is an extracellular protein in shrimp immune system. After microbial infection, Spätzle is secreted into the circulating system as a pro-domain form. Spätzle processing enzyme (SPE) is activated and Spätzle pro-protein domain is cleaved to its active domain. Then, it binds to Toll protein receptor, relay the infection signaling, transmit via complex of transcription factor proteins into nucleus and induce the AMPs synthesis against the microbial infection. In *Drosophila*, the upstream signaling protease cascade is activated the easter zymogen to active zymogen. The active protein binds to

Spätzle pro-domain and cleave to active form C-106. The binding complex of C-106 and Toll receptor activates the signaling across into nucleus and induced the downstream signal of *Drosomycin* gene synthesis (**Fig 1.9**) (Weber *et al*., 2007). Several Spätzle proteins were reported in invertebrate, for examples, *Drosophila, D. melanogaster* (*Dm*Spz1–6) (Parker *et al*., 2001), mosquito, *Aedes aegypti* (Spätzle 1A, 1B and 1C) (Shin *et al*., 2006), White shrimp, *Litopenaeus vannamei* (*Lv*Spz1, 2, and 3) (Wang *et al*., 2012) , silkworm larvae, *Bombyx mori* (*Bm*Spz1) (Y. Wang *et al*., 2007), oak silkworm, *Antheraea pernyi* (*Ap*Spz) (Sun *et al*., 2016), insect, *Manduca sexta*, (*M*sSpz1) (An *et al*., 2010) and Chinese shrimp, *F. chinensis* (*Fc*Spz) (Shi *et al*., 2009).

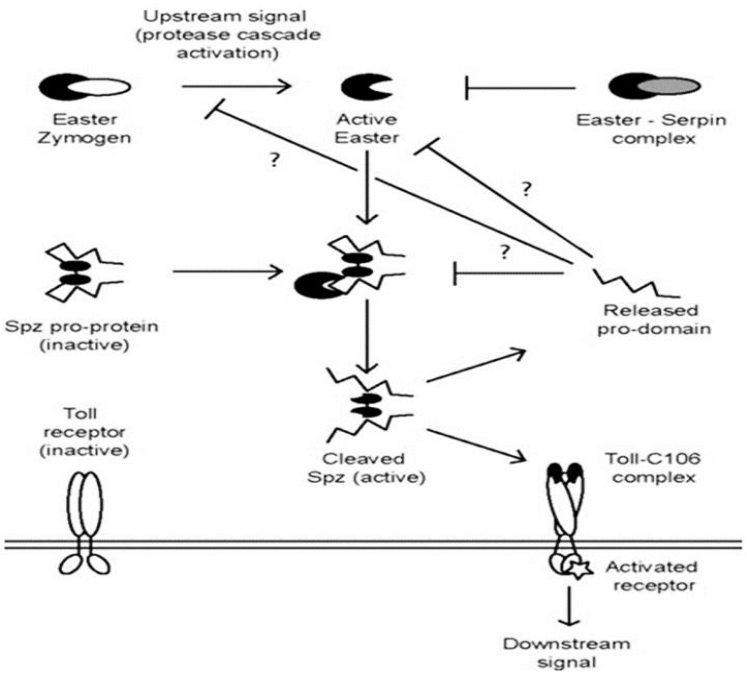

Figure 1.9. Spätzle activation model of *Drosophila*. The easter zymogen is cleaved to active easter zymogen by the activated proteolytic cascade. The active easter zymogen cleaves the pro- Spätzle to active Spätzle form. The Toll receptor is bind to active Spätzle and relay the signaling via Toll pathway into nucleus. Toll-C106 complex is activated the signaling pathway and induced the appropriate AMPs against the pathogen infection (Weber *et al.*, 2007).

AMPs protein plays an important role in innate immune system. Its function is a defense line for against the microbial infection. The infection signaling is detected by cell surface receptor of host cell or PRPs. Then, the signaling is relayed via the Toll and Imd activated protein. The AMPs synthesis in the blood circulating system were induced. Toll signaling pathway is activated by Lys-type Yd88glucan from fungi and some viruses whereas the Imd pathway is triggered by *meso*-diaminopimelic acid (DAP)-type peptidoglycans from Gram-negative, some Gram-positive *baciili* bacteria and some RNA viruses. After activation of Toll receptor, the signaling is sent through the cytoplasmic proteins, MyD88, Tube, Pelle and TRAF6 proteins. Then, the Toll signal transduction activates the Dorsal-Cactus complex protein. The activated Dorsal protein migrates into the nucleus for binding to κB proteins. Finally, the signaling is up-regulated the expression of AMPs gene. For Imd signaling pathway, the IMD proteins and related-proteins activates the Relish protein complex. The first signaling is unknown at the present but another one, the interaction of TAK1 to TAB2 was induced by IMD protein. It affects to the IκB kinase (IKK) complex. The C-terminal half, ankyrin and a death domain are cleaved by a caspase enzyme for activation the Relish complex. The appropriate AMPs protein is synthesized via Kappa B responsible element. Several types of AMPs including anti-lipopolysaccharide factors (ALFs), *crustins*, *penaeidins*, *lysozyme*, *stylicin* and some other-related immune proteins have been reported. They play a key role in the defense immune system for killing or controlling the microbial infection. For another roles, it can be controlled the other immune responses (**Fig 1.10**). The AMPs protein was cleaved from larger proteins and released to the active protein (Tassanakajon *et al*., 2018).

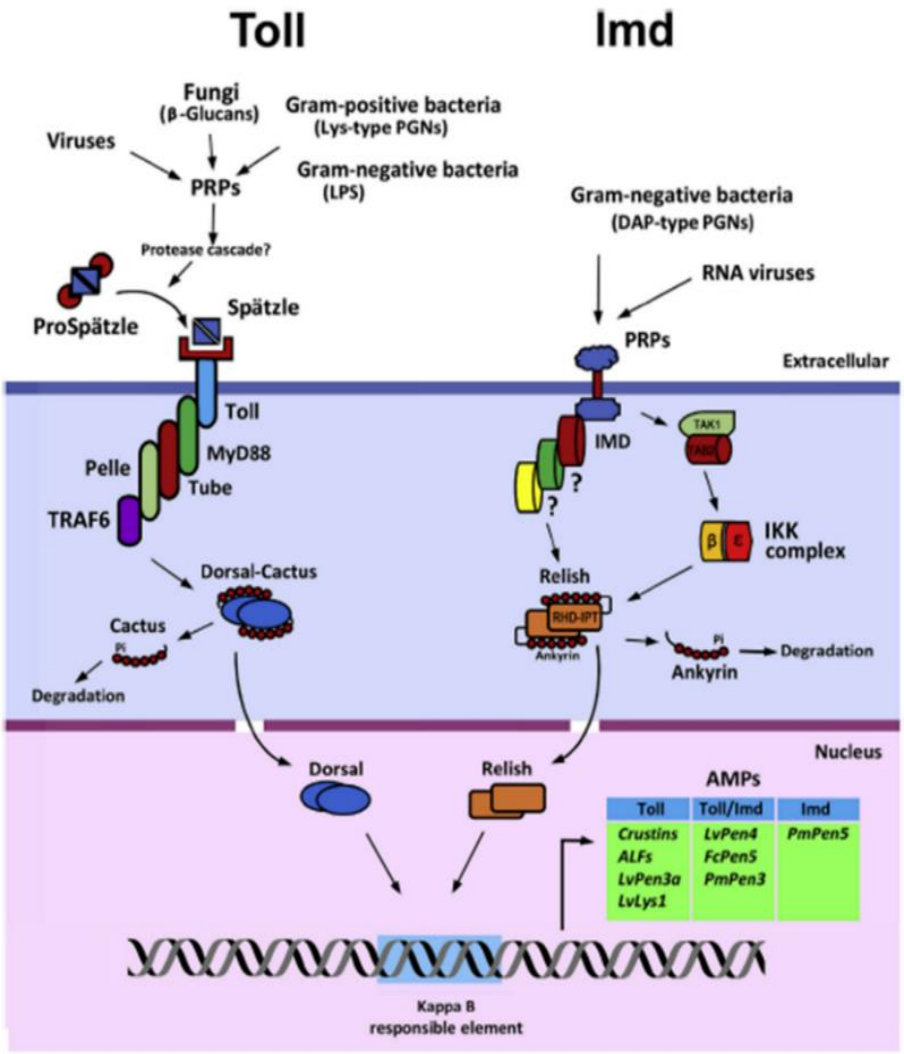

Figure 1.10. Toll and Imd signaling pathways of penaeid shrimp. For Toll pathway, microbacterial infection is activated by Gram-positive and some Gram-negative bacteria, fungi and some viruses whereas Imd pathway also activated by Gram-negative bacteria and RNA viruses. The infection recognizes by the PRPs and relayed the signaling through nucleus. The specific AMPs gene expression is induced against the pathogen infection

1.4.1 Penaeidins

Penaeidins are the first identified antimicrobial protein in shrimp. It is expressed and stored into the blood circulating cells. The penaeidins are cationic peptides and the estimated size 4.7-7.2 kDa and pI 9.0 (Destoumieux *et al.*, 2016). The penaeidins consists of the proline-rich-region (PRR), the cysteine-rich-region (CRR) contain three disulfide bridges of six cysteine residues. There are classified to two penaeidin subfamily. For subfamily I, Pen1 and 5 contain the PRP and CRR domains but subfamily II contains an N-terminal-serine-rich region (SRR), PRR and CRR domains. The defense activity of all domains can be activated against to microbial infection. For examples, the subfamily I inhibits to Gram-positive bacteria and fungi whereas subfamily II also inhibits to Gram-positive and Gram-negative bacteria (Tassanakajon *et al.*, 2018). The penaeidins are stored in granulocytes and semi-granulocytes. In addition, they release in response to microbial infection. Beside the antimicrobial activities, the penaeidins can prolong the shrimp life by the wound healing and moulting (Destoumieux *et al.*, 2016). The penaeidin3 from *P. monodon* shows anti-fungal and -bacterial activities whereas penaeidin5 reduces the growth of bacteria (Hu *et al.*, 2006) and antiviral activity against the WSSV infection (Destoumieux *et al.*, 2016).

1.4.2 Crustins

Crustins are the cationic peptides consists of an N-terminal a glycine-rich, proline, cysteine, aromatic acid-rich region and a whey acidic protein (WAP) domain at C-terminal domain. In *P. monodon*, many types of crustins are known. The type I crustin, carcinin*Pm*1 and *Pm*2, has Cys-rich region and a WAP domain at C- and N-terminus, type II crustin, crustin*Pm*1-*Pm*10, has Gly-, Cys- and a single WAP region domain, and type III crustin, SWD*Pm*1-3, has arginine-proline-region at N-terminus and a C-terminus WAP domain. For crustin*Pm7*, its inhibition activity strongly active in response to both Gram-positive and negative bacteria challenges. Moreover, the antibacterial activities

of crustin*Pm*1 and 7 show the agglutination to bacterial cells (Tassanakajon *et al*., 2018).

1.4.3 Anti-Lipopolysaccharide factors (ALFs)

ALFs are characterized from crustaceans. It contains the hydrophobic region at N-terminus and a LPS-binding domain (LPS-BD). LPS-BD has two conserved cysteine residues positive and negative charge amino residues for cationic and anionic ALFs, respectively. The LPS-BD has been reported that two domains response to the antibacterial activity with disulfide bond, glycine and arginine residues. For another domain, its function inhibits the viral infection by specificity lysine residues. There are several ALFs in *P. monodon*, *ALFPm2*, *ALFPm3* and *ALFPm6* have been identified. The expression pattern of *ALFs* gene respond to the bacterial and viral infections. For inhibition activities, ALF*Pm*3 inhibits the fungi, Gram-positive, Gram-negative bacteria, *V. harveyi*, VP_{AHPND}, and WSSV infection. For ALF*Pm*6, it also inhibits the YHV infection, *V. harveyi* and WSSV infection ((Prapavorarat *et al.*, 2010); Ponprateep *et al.*, 2011).

1.4.4 Lysozyme

Lysozyme is one of antimicrobial protein in shrimp immune system. It cleaves the N-acetylmuramic acid and N-acetyl-glucosamine of meurin and causes the bacterial cell wall lysis. Three type of lysozyme are characterized including chicken- (c-type), goose- (g-type) and invertebrate-type lysozyme (i-type). However, only the c and i-type were found in *P. monodon*. Besides, the lysozymes show the inhibition activity to *V. alginolyticus*, *V. parahaemolyticus* and WSSV infection (Tassanakajon *et al.*, 2013).

1.4.5 Stylicin

Stylicin is an anionic AMPs family. It contains an proline-rich region and at N-, C-terminus. The stylicin from *L. stylirostris* (Ls-Stylicin1), *L. vannamei, P. monodon* and *M. japonicas* (Mj-sty) have been reported. The Mj-

sty transcripts is also expressed in the gill and hemocytes tissues. The Ls-stylicin1 is secreted in hemocytes granular and hyaline cells. The strong inhibition activity of Ls-stylicin1 showed in fungal infected-shrimp but exhibited the poorly inhibitory activity to *V. penaeicidae* infection (Tassanakajon *et al.*, 2018).

1.5 Proteinases and their inhibitors

Proteinase plays an important roles in the physiological and biological processes. The proteinases relates to several processes in living organism such as metamorphosis, wound healing, innate immunity and pathogenesis of the pathogenic infection. In addition, its function can be preventing the loss of blood and eradication the pathogenic infection. The proteinases is regulated by the homologue proteinase inhibitors.

The proteinase inhibitors can be directly against to the homologous proteinases as well as the enzyme-substrate binding such as the serine proteinase (Trypsin)-ovomucoid inhibitor complex (**Fig 1.11A**), the thrombin-hirudin complex (**Fig 1.11B**) and the cysteine proteinase (papain)-cystatin complex (**Fig 1.11C**). The proteinase inhibitors change their conformation of the homologue proteinases complex and also activated its gene expression after microbial infection or injuries. The proteinase inhibitors can be classified to two categories base on their broad spectrum of activity including non-specific and the class-specific proteinase inhibitors. The non-specific proteinase inhibitors are a large molecule protein and less specific to target proteinases such as alpha-2-macroglobulin (A2M). The active site of the target proteinase binds to a generic bait region on A2M molecule, resulting in a conformational change in A2M. One A2M molecule can bind or remove 1 or 2 of the proteinase. For another group, the class-specific proteinase inhibitors shows higher specificity than A2M at binding sites. It binds the target proteinase at active site region and also binding to 1 specific class of proteinase. The class-

specific group is classified in 4 types such as aspartic- (pepstatin), cysteine-(cystatin), metalloproteinase-(tissue inhibitors of metalloproteinase) and serine proteinase inhibitors (alpha1-proteinase inhibitor, antithrombin III) (Hibbetts *et al.*, 1999).

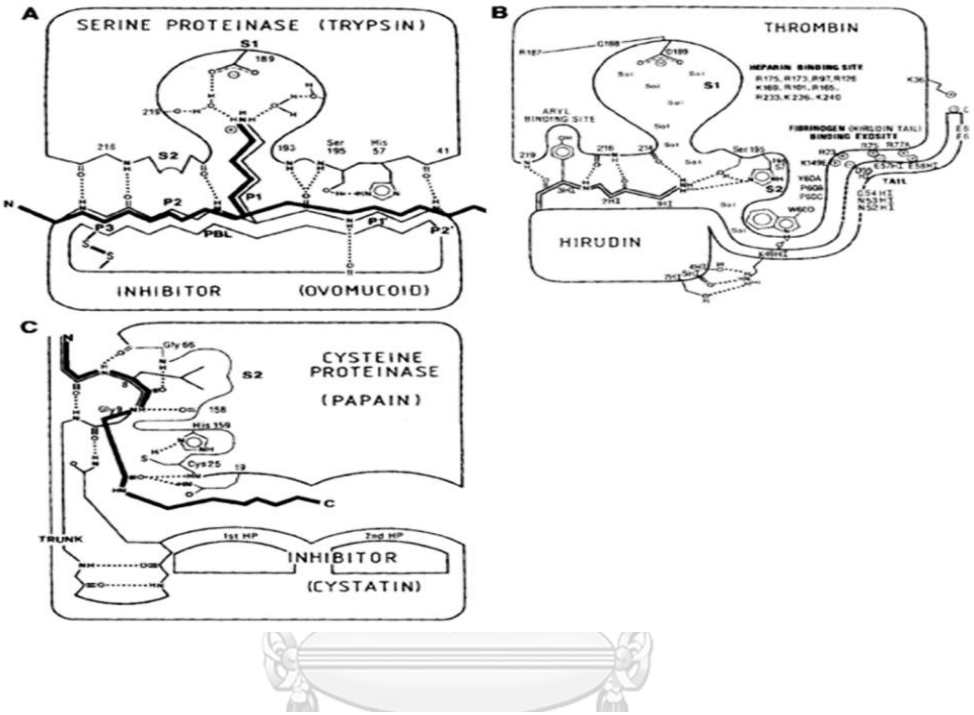

Figure 1.11. Comparison of the canonical substrate-like inhibition mode with that of ovomucoid-serine proteinase (Trypsin) (A) hirudin-thrombin (B) and cystatin and cysteine proteinase (papain) (C) complex (Bode & Huber, 1992).

Several types of serine proteinase inhibitors are found in several invertebrate such as serpins, Kunitz-type inhibitors, Kazal-type inhibitors, Bowman-Birk inhibitors and pacifastin, etc (Kanost, 1999). Reactive site of KPIs domain reacts to the cognate proteinases. The complex protein is called proteinase-proteinase inhibitor complex. The cleavation is influence by P1 amino acid residues and other contact position relate to the complex. The serine proteinase inhibitors has many functions in invertebrate immunity including inhibitory activity to proteinases invader, controlling of endogenous

proteinases in sytem, coagulating hemolymph processes, phenoloxidase activation and proteolytic activation of cytokines (Kanost, 1999). The proteinase inhibitor of vertebrate and invertebrate are shown in **Fig 1.12**.

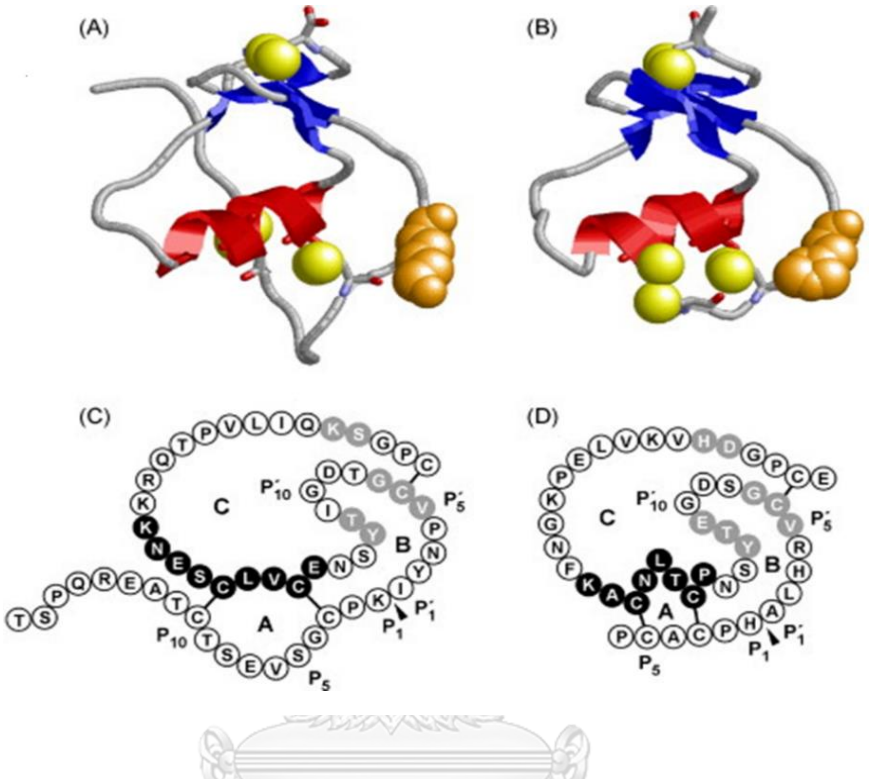

Figure 1.12. Structural of Kazal-type serine proteinase inhibitors from vertebrate and invertebrate. The porcine pancreatic secretory inhibitor of vertebrate KPIs derived from PDB file (A) and the covalent primary structure (C). The rhodniin domain 1 of invertebrate KPIs derived from PDB file (B) and the covalent primary structure (D) (Rimphanitchayakit & Tassanakajon, 2010).

In several crustacean, the KPIs have been studied in shrimp, crab, crayfish and other arthropod species. The gene expression of their KPIs can be up-regulated in infected host. In Chinese shrimp, *Fenneropenaeus chinensis*, *FcSPI* genes are up-regulated in response to bacterial and viral infection. In freshwater crayfish, *Procambarus clarkii*, the gene expression of *hcPcSPI1-4* are involved after microbial infection. The bacteriostatic activity of *hcPcSPI1* and *hcPcSPI2* can be against the *Bacillus subtilis* and *B. thuringiensis*. In

swimming crab, *Portunus trituberculatus*, *PtKPI* transcripts are activated after bacterial and fungal infection.

In black tiger shrimp, there are 9 types of KPIs from the cDNA libraries of hemocyte, hepatopancreas, hematopoietic tissue, ovary and lymphoid organ in the EST database were analyzed (http://pmonodon.biotech.or.th/). SPI*Pm*2, is up-regulated in response to viral challenging (Ponprateep *et al*., 2011). The SPI*Pm*2 stored into the granules and secreted into the cytoplasm after pathogenic infection. The *SPIPm2* transcripts are also increased in response to YHVV and WSSV challenges and also help prolong the mortality rate of viral infected in shrimp. Moreover, its bacteriostatic activity inhibits the *Bacillus subtilis*. The domain 2, 4 and 5 of SPI*Pm*2 can contribute to the bacteriostatic activity (Donpudsa *et al*., 2010; Ponprateep *et al*., 2011). For other KPIs, the two domains of SPI*Pm*4 and 5 also identified. The bacterial inhibitory activity of SPI*Pm*4 interact to subtilisin whereas SPI*Pm*5 strongly interact to elastase and subtilisin but its function lacks the bacteriostatic activity to bacterial infection, *B. megaterium, B. subtilis*, *V. harveyi* 639, *S. aureus* and *E. coli* JM109 (Visetnan *et al*., 2009). In addittion, the gene expression of *SPIPm4* and *5* show increase under the heat stress condition. The SPI*Pm*5 protein is a two-domain Kazal-type serine proteinase inhibitor with the P1 residues Leu and Thr. In cDNA microarray analysis, the *SPIPm5* gene expression was increased after WSSV and YHV challenging (Pongsomboon *et al*., 2011).

1.6 Purpose of Thesis

The humoral response is activated the defense immune system after microbial infection. It depends on the type species of viral, bacterial and fungal infection. The induced-antimicrobial proteins can help and prolong life of infected-shrimp and bind directly to the pathogen for the immobilization and eradication. The function of Spätzle protein in immune system was

investigated. It plays role important for the AMPs synthesis. In order to understanding the Spätzle isoforms, three isoforms were tested the gene expression in hemocyte and gill tissues. The open reading frame of Spätzle were studied from hemocytes. Its transcripts were tested in tissues distribution and WSSV challenge. The recombinant protein expression of pro-Spätzle and active Spätzle were prepared for studying the protective activity of pro-Spätzle and the effect of its active domain on the mortality of WSSV infected shrimp and tested the AMPs transcripts.

For SPI*Pm*5, the possible function and its transcritps in response to the microbial challenging were investigated. The recombinant protein of SPI*Pm*5 was produced for study on the expression of AMPs and some KPIs genes, the transcripts of SPI*Pm*5 gene in tissues test, on prophenoloxidase system in hemolymph, on the protective effect of SPI*Pm*5 in the survival rate of virus and bacteria challenging, on the bacterial clearance assay from hemolymph of bacterial challenging in shrimp, on the effect of the AMPs trancripts from bacterial challenges.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Equipments

Autoclave

Biological safety cabinets

Centrifuge 5804R (Eppendorf)

Centrifuge AvantiTM J-301 (Beckman Coulter)

-20 °C freezer (Whirlpool)

-80 °C freezer (Thermo Electron Corporation)

Gel document (Syngene)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Innova 4000 benchtop incubator shaker (New Brunswick)

Microcentrifuge tubes 0.6 ml and 1.5 ml (Bio-Rad)

Micropipettes P10, P20, P200, P1000 (Gilson Medical Electrical)

Microplate reader (SpectraMAX®M5)

PD-10 column (GE Healthcare)

NanoDrop™ spectrophotometers 2000/2000C (ThermoFisher Scientific)

pH meter model#SA720 (Orion)

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

Real-time thermal cycler (Bio-Rad)

Trans-Blot® SD (Bio-Rad)

Thermal cycler mastercycler gradient (Eppendorf)

Touch mixer model# 232 (Fisher Scientific)

2.1.2. Chemicals and reagents

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

100 bp plus DNA ladder (Fermentas)

2-Mercaptoethanol, C_2H_6OS (Fluka)

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Fermentas)

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

30% (w/v) Acrylamide/Bis solution (Bio-Rad)

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH. (BDH)

Agarose (Vivantis)

Ammonium persulfate, $(NH_4)2S_2O_8$ (USB)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3H_3 (Merck)

Bovine serum albumin (Bio-Rad)

Bromophenol blue (Merck)

Calcium chloride, ($CaCl_2$) (Merck)

Chloroform, $CHCl_3$ (Merck)

Coomassie Brilliant Blue G-250 (Fluka)

Coomassie Brilliant Blue R-250 (Sigma)

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

Ethidium bromide (Sigma)

Ethylenediaminetetraacetic acid (EDTA), disodium salt dehydrate (Fluka)

Formaldehyde (BDH)

Glacial acetic acid (J.T. Baker)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine NH_2CH_2COOH (Scharlau)

Hydrochloric acid, HCl (Merck)

Imidazole (Fluka)

Isopropanol (Merck)

Isopropyl-β-D-thiogalactoside (IPTG) (Sigma)

Methanol, CH_3OH (Merck)

Nitroblue tetrazolium (NBT) (Fermentas)

Nitrocellulose membrane (Bio-Rad)

Potassium chloride, KCl (Ajex)

Potassium dihydrogen phosphate, KH_2PO_4 (Ajex)

Prestained protein molecular weight marker (Fermentas)

RNase A (Sigma)

Sodium acetate, CH_3COONa (Merck)

Sodium bicarbonate, Na_2CO_3 (Ajex)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma)

Sodium dihydrogen orthophosphate, $NaH_2PO_4 \cdot H_2O$ (Carlo Erba)

Disodium hydrogen orthophosphate, $Na_2HPO_4 \cdot H_2O$ (Carlo Erba)

Sodium hydroxide, NaOH (Eka Nobel)

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

Thiosulfate citrate bile salts sucrose (TCBS) (Himedia Laboratories)

TRI Reagent (Genaid)

Tris (hydroxymethyl) aminomethane (Sigma-Aldrich)

Trisma base (Vivantis)

Tryptic soy broth (TSA) (Himedia Laboratories)

Unstained protein molecular weight marker (Fermentas)

2.1.3. Kits

FavorPrep™ gel/PCR purification kit

NucleoSpin®Extract II kit (Macherey-Nagel)

Plasmid miniprep kit (Farvorgen)

RevertAid™ first strand cDNA synthesis kit (Fermentas)

T7 RiboMAX™ express large scale RNA production system (Promega)

2.1.4. Enzymes

Enterokinase (New England Biolabs)

*Nco*I, *Hind*III and *Xho*I (New England Biolabs)

QPCR green master mix LROX (Biotechrabbit)

RBC Taq DNA polymerase (RBC Bioscience)

DNase I, RNase-free (Thermo Scientific)

T4 DNA ligase (Promega)

2.1.5. Substrate

L-3,4-dihydroxyphenylalanine, L-DOPA (Sigma)

2.1.6. Antibiotics

Amplicilin

Kanamycin

2.1.7 Antibodies

Anti-His taq antibody (GE Healthcare)

Anti-*Pm*Spz1 antibody

Anti-SPI*Pm*5 antibody

Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories)

2.1.8. Bacteria and virus strains

Escherichia coli XL-1 Blue

Escherichia coli BL21-CodonPlus (DE3)

Vibrio parahaemolyticus (VP_{AHPND})

Vibrio harveyi

White spot syndrome virus (WSSV)

Yellow head virus (YHV)

2.1.9. Vectors

pGEM-T Easy vector (Promega)

pET-28b(+)

pVR500, a pET-32a(+) derivative

2.1.10. Softwares

Blastp in BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

ExPASy (http://www.expasy.org/proteomics)

GraphPad Prism software (San Diego California, USA)

ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/)

SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/)

SPSS statistic 17.0 (Chicago, USA)

2.2. Methods

2.2.1. Samples preparation

The black tiger shrimp, *Penaeus monodon*, were purchased from local farms and obtained in 17 ppt sea water. The shrimp were reared at an ambient water temperature of 28–30 °C for a few days before experiments.

2.2.2. RNA extraction

The total RNA of hemocytes and other tissues were prepared by TRI Reagent (Genaid). The samples along with TRI Reagent were homogenized, added 200 µl of chloroform and vortexed vigrously. The mixed samples were incubated at RT and separated by centrifugation at 12,000 g for 15 min at 4 °C.

The aqueous solution was collected and precipitated with cool isopropanol at RT for 10 min. The RNA pellet was separated by centrifugation at 12,000 g for 15 min at 4 °C and then washed with 75% ethanol. The RNA pellet was air-dried for 5–10 min and dissolved in DEPC. The total RNA solution was determined the concenration by NanoDrop spectrophotometer.

2.2.3. DNase treatment of total RNA

One µg of the determined RNA was treated along with the 1 U of DNase I, RNase-free (Thermo Scientific). The reaction was incubated at 37 °C for 30 min the contaminated chromosomal DNA by TRI Reagent, added 200 µl of chloroform and vortexed vigorously. The mixture was incubated and separated by centrifugation at 12,000 g for 15 min at 4 °C. The aqueous solution were collected and precipitated with cool isopropanol at RT. The total RNA was also centrifuged, washed with 75% ethanol, re-centrifuged at 12,000 g for 15 min at 4 °C. The RNA pellet was air-dried for 5–10 min and dissolved in DEPC. The total RNA solution was determined the concenration by NanoDrop spectrophotometer.

2.2.4. First-strand cDNA synthesis

The 1st cDNA were prepared by the RevertAid™ first-strand cDNA synthesis kit (Fermentas) from RNA transcripts. The 10 µl of reaction contained 250 ng of total RNA, 1 µl of oligo$(dT)_{18}$, 2 µl of 5×reaction buffer, 1 µl of Ribolock™ RNase inhibitor (20U/ µl), 1 µl of dNTP mix and 1 µl of RevertAid™ M-MuLV reverse transcriptase. The reaction was gently mixed, centrifuge briefly, incubated at 42 °C for 60 min and terminated at 70 °C for 15 min. The reaction were prepared and diluted for amplification by RT-PCR and qRT-PCR or kept at -20 °C.

2.2.5. Semi-quantitative RT-PCR and real-time PCR

The gene expression was investigated in the black tiger shrimp. The genes target were amplified from cDNA template using specific primers (Table

1). The PCR reaction mixture consisted of 1.25 unit *Taq* DNA polymerase (RBC), 10 µM dNTP mix, 10 µM each forward and reverse primers and appropriately 1:10 dilution of cDNA template. The PCR condition started with pre-denaturation at 95 °C for 3 min followed by 30 cycles for gene target or 25 cycles for *EF-1α* of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min, and ended with final extension 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and visualized using Gel documentation system. The expression of *EF-1α* was used as an internal control.

For QPCR method, all reactions were 10 µl containing 2 µl of the 1:10 diluted cDNA template, 0.2 µM of each primer and 1× QPCR green master mix LROX containing DNA polymerase. The qRT-PCR was processed by the real-time thermal cycler (Bio-Rad). The PCR profile started with pre-denaturation at 95 °C for 8 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 30 s. The expression of *EF-1α* was used as an internal control. The experiment was done in triplicate.

The relative expression of gene target was calculated using a comparative method described by Pfaffl (Pfaffl, 2001). The relative expression from PCR band target used GelQuant.NET software provided by biochemlabsolutions. The mean data values were derived from triplicate amplifications and expressed as means ± standard deviations. Statistical analysis was done using the One-way ANOVA followed by Duncan's new multiple range test. Data differences were considered statistically significant at $P<0.05$.

2.2.6. Total protein determination

The purified recombinant protein was determined by Bradford assay (Bradford, 1976). The appropriate of recombinant protein solution was prepared, mixed with Bradford dye reagent and incubated at room temperature

at least 5 min. The solution mixture was measured the absorbance at 595 nm and compared with the standard curve of BSA as standard protein.

2.2.7. SDS-PAGE analysis

The SDS-PAGE system was used the method of Laemmli (1970). The separating and stacking gel solution were set to step follwed in the appendix A. The separating gel solution were mixed and pipetted into the glass plate setting. Then, distilled water was layered over the top of the separation gel. After the polymerized separating gel was completed, the stacking gel was prepared. The protein samples were prepared, mixed vigorously in sample buffer along with Beta-mercaptoethnol and boiled for 10 min before loading into SDS-PAGE. The diluted electrode buffer was prepared. It consists of Tris-glycine buffer, pH 8.3 containing 0.1% (w/v) SDS. The vertical electrophoresis cell ran with constant current of 20 mA per gel at RT. The protein gel was stained with coomassie staining solution, re-stained with destained solution and observed the target protein bands.

2.2.8. Western blot analysis

The recombinant proteins were investigated by Western blot analysis with the specific mouse polyclonal antibody. The proteins were identified on SDS-PAGE gel. The gel, nitrocellulose membrane and blotting paper were incubated for 20 min in blotting buffer (48 mM Tris-pH 9.2, 39 mM glycine and 20% methanol). They were laid on Trans-Blot SD® (Bio-Rad) machine, set on the transfer sandwich layers and avoiding air bubble on membrane. The recombinant protein was transfer to nitrocellulose membrane with a constant electricity of 90 mA for 90 min. After transferring, the membrane was incubated in blocking buffer (5% (w/v) skim milk, 0.05% (v/v) Tween 20 in PBS buffer) for 2 h at room temperature and washed 3 times with washing buffer (0.05% (v/v), Tween 20 in PBS buffer) for 5 min.

To detect the recombinant protein, the membrane was incubated in the primary antibody diluted in 1% (w/v) skim milk in PBST buffer at 37 °C for 2 h. The membrane was washed 3 times and incubated with secondary antibody 1/10,000 in 1% (w/v) skimmilk in PBST buffer for 60 min at 37 °C. The membrane was washed again as above. Finally, the laid membrane was observed in the detection solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) and stopped the reaction with water.

2.3 *Pm*Spätzle

2.3.1. Identification of *PmSpz* isoforms

The *Spätzle* genes of the white shrimp, *Litopenaeus vannamei*, have been identified namely, *LvSpz1*, *LvSpz2* and *LvSpz3* genes (Wang *et al.*, 2007). Like the white shrimp, the black tiger shrimp may have three *Spätzle* genes. In this study, three primer pairs were designed from the nucleotide sequences of *LvSpz1* (accession no. JN180646), *LvSpz2* (JN180647) and *LvSpz3* (JN180648), respectively (Table 1).

The hemocytes and gill tissues of normal shrimp were collected for total RNA extraction and cDNA synthesis. The *Spätzle* genes were detected by semi-quantitative RT-PCR. The samples were run on agarose gel. The major band were excised, eluted, purified and subjected to DNA sequencing by commercial service.

2.3.2. Cloning of *PmSpz1* gene

The *PmSpz1* transcripts were amplified from the hemocytes cDNA of un-infected shrimp. The primers were designed from *LvSpz1* gene sequence from *L. vannamei* (accession no. JN180646) (Table 1) (Wang *et al.*, 2007). The total RNA and cDNA were prepared as described above for the amplification of full-length *PmSpz1* gene. The samples were run on agarose gel electrophoresis, exised, eluted from the gel piece, cloned into pGEM-T Easy vector (Promega) and sequenced by the commercial service.

The *PmSpz1* gene sequence was analyzed for the open reading frame, the deduced amino acid sequence using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) and other bioinformatics information using ExPASy (http://www.expasy.org/proteomics), SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and blastp in BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.3. *PmSpz1* gene expression in tissues

Nine tissues; hemocytes, stomach, hepatopancrease, gill, lymphoid organ, muscle, eyestalk, intestine and heart, were collected from the normal shrimp. The total RNAs were extracted and cDNAs were prepared for the amplification of *PmSpz1* gene by semi-quantitative RT-PCR. The PCR products were analyzed by agarose gel electrophoresis. The expression of *EF-1α* was used as an internal control.

2.3.4. Construction of r*Pm*Spz1 and its active form rac*Pm*Spz1

The expression clones of full-length *PmSpz1* gene and its active domain were constructed for over-production of the recombinant proteins. The primers were designed from the *PmSpz1* gene sequence (Table 1). The forward and reverse primers were added the restriction sites of *Nco*I and *Hind*III, respectively. The full-length gene was amplified, run on agarose gel electrophoresis, purified and ligated into an expression vector pET-28b(+). The pET-28b_PmSpz1 construct was confirmed and sequenced by commercial sequence service for the correct sequence and open reading frame.

The active domain of *PmSpz1* was also cloned for preparation the recombinant protein. To determine the site of cleavage for the active domain, the full-length *PmSpz1* sequence was aligned and compared with the amino acid sequences of *D. melanogaster* (*Dm*_Spz), *M. sexta* (*Ms*_Spz) and *B. mori* (*Bm*_Spz), respectively. The forward primer of active domain was designed from the alignment sequences, after the cleavage site at His109 and added the

restriction site of *Nco*I, CTSPZF (Table 1). The reverse primer, PmSPZR, was used for amplification. The active domain *Spätzle* gene was amplified, run on agarose gel electrophoresis, purified and ligated into an expression vector, pVR500, a derivative of pET-32a(+) (Donpudsa *et al.*, 2009). The pVR500_acPmSpz1 plasmid was produced as fusion protein to the thioredoxin (Trx) tag.

2.3.5. Protein expression of r*Pm*Spz1 and its active form rac*Pm*Spz1

The pET-28b_PmSpz1 and pVR500_acPmSpz1 constructs were transformed into the expression host *E. coli* BL21-CodonPlus (DE3). A single colony of transformant containing the recombinant plasmid was cultured in LB medium under shaking at 37 °C for an overnight. The starter was inoculated into LB broth (1:100) and cultured until an OD_{600} reached 0.6. Consequently, the bacterial culture was induced by IPTG to the final concentration of 1 mM. The culture was continued for 3 h at 37 °C.

After the bacterial culture induction, the bacterial cells were harvested by centrifugation at 2,500 g, 4 °C for 15 min, frozen completely at -80 °C and thawed at room temperature for three times. The bacterial pellet was re-suspended in phosphate buffered saline pH 7.4 (PBS) and sonicated with Sonics Vibracell VCX750 to break the cells. The pellet was collected by centrifugation at 2,500 g, 4 °C for 15 min, washed once with each respective solution: 2% Triton X-100, 0.5 M NaCl in PBS; 0.5 M NaCl in PBS; PBS, and solubilized with 50 mM sodium carbonate buffer pH 10.0 at room temperature. The insoluble matter was removed by centrifugation. The soluble protein was purified using Ni-NTA agarose column.

The Ni-NTA bead was loaded into a PD-10 disposable column and equilibrated with 10 volumes of binding buffer (50 mM carbonate buffer pH 10.0, 0.3 M NaCl and 50 mM immidazole). The column was loaded with the crude protein and washed with the binding buffer. Then, the recombinant protein was eluted with 5 ml of elution buffer (50 mM carbonate buffer pH

10.0, 0.3 M NaCl and 50 mM immidazole). The purified recombinant protein was dialyzed against PBS buffer pH 7.4. The recombinant protein was subject to SDS-PAGE and its concentration was determined by Bradford method (Bradford, 1976). The purified protein of r*Pm*Spz1 was used as antigen for raising mouse polyclonal antiserum at the Institute of Molecular Biosciences, Mahidol University.

For the *Spätzle* active domain, the Trx tag was removed from the recombinant (r) ac*Pm*Spz1 protein by enterokinase digestion. The 0.3 mg of fusion protein was incubated with 0.002 µg of enterokinase (New England Biolabs) at room temperature for 3 days. The rac*Pm*Spz1 protein was re-purified with Ni-NTA column. The completely digestion of rac*Pm*Spz1 protein was subjected to SDS-PAGE. The concentration of rac*Pm*Spz1 protein was determined using the Bradford method (Bradford, 1976).

2.3.6. Expression of *PmSpz1* gene in response to WSSV infection

The expression of *PmSpz1* gene were analyzed by qRT-PCR using primers RTSPZF and RTSPZR (Table 1) and tested its gene expression response to WSSV infection. The normal shrimp were divided into two groups. The challenge group was injected with WSSV, the dilution of WSSV that caused 100% mortality within four days. The control group was injected with normal saline (0.85% NaCl) . The relative gene expression were calculated using a comparative method described by Pfaffl (Pfaffl, 2001).

2.3.7. *Pm*Spz1 in the hemocytes and plasma of WSSV-infected shrimp

After pathogen infection, the Spätzle protein is up-regulated and secreted into the hemolymph. Its function is to relay the infection signal through the Toll receptor into the cells. In this study, the *Pm*Spz1 pro- and active forms were investigated by Western blot analysis after WSSV challenging. The normal shrimp were injected with 1×10^4 copy/ shrimp of purified WSSV. The hemolymph was collected at 0, 12, 24 and 48 hours post

infection (hpi) with 10% sodium citrate at the ratio of 1:3. Then, the hemolymph was centrifuged at 800 g for 15 min to separate the hemocytes from the plasma. The hemocytes were re-suspended with PBS and sonicated to break the cells. The total protein concentration was determined using Bradford assay (Pfaffl, 2001).

To investigate the up-regulation of *Pm*Spz1 protein, the 1000 µg protein of plasma and 200 µg protein of hemocyte lysate at each time point were used. The samples were run on SDS-PAGE gel. The target proteins were transferred to nitrocellulose membrane for the investigation the *Pm*Spz1 protein by Western blot analysis. The β-actin was detected as an internal control protein. The *Pm*Spz1 and β-actin were respectively detected with mouse antibody specific to *Pm*Spz1 and anti-actin (Millipore) and, then, with secondary antibody conjugated with horseradish peroxidase. The protein bands were developed using chemiluminescent substrates and detected by X-ray film.

2.3.8. Effect of rac*Pm*Spz1 on the expression of antimicrobial proteins

The active domain of *Spätzle* protein has been identified the binding with the cell membrane Toll receptor where it activates the up-regulation of AMP genes under the contolling of Toll signaling pathway (Li *et al*., 2013). In this study, the normal shrimp were divided into three groups. The challenge groups were injected with 0.3 and 3 nmol of rac*Pm*Spz1 in 100 µl of normal saline (0.85% NaCl) . The control group was injected with 100 µl of normal saline. The hemolymph was collected at 24 h from three individual shrimp for each concentration of rac*Pm*Spz1. The total RNA and cDNA were prepared for the amplification of the AMP genes by qRT-PCR using specific primers for *ALFPm3, crustinPm1* and *7, penaeidin3* and *5* (**Table 1**) . The expression of *EF-1α* gene was used as an internal control.

The relative expression of AMPs was calculated using a comparative method described by Pfaffl (Pfaffl, 2001). The mean data values were derived from triplicate samples and expressed as means ± standard deviations. Statistical analysis was done using the One-way ANOVA followed by Duncan's new multiple range test. Data differences were considered statistically significant at $P < 0.05$.

2.3.9. Effect of *Pm*Spz1 protein on the survival of WSSV-infected shrimp

After pathogen infection, the Spätzle protein relays the infection signaling through the Toll pathway into the cell and the synthesis of AMP proteins were induced. In this study, the protective effect of Spätzle protein was tested along with the WSSV infection. The normal shrimp were divided into four groups of ten shrimp. The WSSV dilution used had an LD_{50} of 2 days. The shrimp were injected with 66 µl equal volume of suspension of 0.3 nmol of r*Pm*Spz1 protein, 0.3 nmol of r*Pm*Spz1 plus WSSV, WSSV and PBS as control group. The survival experiment was done in triplicate. The cumulative mortality rate were analyzed with the survival program in GraphPad Prism.

2.4. SPI*Pm*5

2.4.1. *SPIPm5* gene expression in tissues

Nine tissues, hemocytes, stomach, hepatopancrease, gill, lymphoid organ, muscle, eyestalk, intestine and heart, were collected from the normal shrimp. The total RNAs were extracted as described above. The cDNAs were synthesized for the amplification of *SPIPm5* gene by semi-quantitative RT-PCR. The PCR products were analyzed by agarose gel electrophoresis. The expression of *EF-1α* was used as an internal control.

2.4.2. SPI*Pm*5 was up-regulated under heat stress

From previous study, the *SPIPm5* gene was up-regulated after the shrimp were exposed to heat shock temperature (Visetnan *et al.*, 2009). In this

study, the shrimp were exposed to heat stress at 33 °C for 1 h and then reared at ambient temperature. The hemolymph was collected from three shrimp each at 24 and 48 h. The control group was also exposed to the ambient temperature. The experiment was done in triplicate. The total RNA and cDNA were prepared for amplification of the *SPIPm5* and *hsp70* gene expression using specific primers (**Table 1**). The gene expression was analyzed by agarose gel electrophoresis. The expression of *EF-1α* was used as an internal control.

2.4.3. Expression of *SPIPm5* gene in response to WSSV and YHV infection

The normal shrimp were divided into three groups of 45 shrimp each. The challenged group was injected with 50 µl of WSSV containing 16,950 copies of WSSV that caused 100% mortality within 4 days. Another challenged group was injected with YHV dilution that killed all shrimp within 5 days. The control group was injected with normal saline. The hemolymph was collected at each time point 3, 6, 12, 24 and 48 h for preparation of the total RNA extraction and cDNA synthesis. The experiment was done in triplicate. The expression of *SPIPm5* gene was analyzed by qRT-PCR using specific primers, RTSPIPm5F and RTSPIPm5R (**Table 1**). The qRT-PCR procedure and the comparative statistic data were done and analyzed as describe above.

2.4.4. Expression of SPI*Pm*5 protein

The recombinant plasmid, pET28b_SPIPm5, was transformed into an *E. coli* BL21-CodonPlus (DE3). A transformant was inoculated in the LB supplemented with kanamycin as a starter. The starter was cultured in LB until the OD 0.6 at 600 nm. The recombinant protein was protuced by IPTG inducing. The culture was continued for 3 h at 37 °C. The bacterial culture was centrifugated at 2500 g at 4 °C for 20 min. The pellet was re-suspended in PBS pH 7.4 and sonicated with Sonics Vibracell VCX750 to break the cells. The rSPI*Pm*5 was expressed as soluble protein, purified with Ni-NTA agarose column, eluted with imidazole and dialyzed against with PBS. The rSPI*Pm*5

protein was run on SDS-PAGE gel. The rSPI*Pm*5 protein concentration was determined by Bradford assay (Bradford, 1976). The rSPI*Pm*5 protein was used as an antigen for raising the mouse polyclonal antiserum at the Faculty of Agriculture, Kasetsart University, Kamphaeng saen Campus.

2.4.5. Effect of rSPI*Pm*5 injection in shrimp

The rSPI*Pm*5 injection may affect the AMPs gene and other immune genes. The normal shrimp were divided into two groups. The shrimp were injected with 0.5 nmol of rSPI*Pm*5 in 30 µl of PBS and 30 µl of PBS, respectively. The experiment was done in triplicate. The hemolymph was collected at 24 h after injection with the rSPI*Pm*5 protein. Total RNA and cDNA were prepared for the detection of the expression of *crustinPm1*, *penaeidin3*, *penaeidin5*, *SPIPm2*, *SPIPm5* and *hsp70* genes by RT-PCR. The experiment was done in triplicate. The PCR products were analyzed by agarose gel electrophoresis. The expression of *EF-1α* gene was used as an internal control.

2.4.6. Gene silencing of *SPIPm5*

Gene silencing was used to study the effect of *SPIPm5* silencing under heat stress condition. The other immune genes was also investigated. The *SPIPm5* dsRNA and *GFP* dsRNA were synthesized using T7 riboMAX™ express large scale RNA production system (Promega) following the manufacturer protocol. The templates of *SPIPm5* (*SPIPm5* gene fragment) and *GFP* [pEGFP-1 (Clontech)] were amplified using specific primers (Table 1). The target band were excised from agarose gel, purified for preparation as templates for *SPIPm5* dsRNA and *GFP* dsRNA synthesis.

The normal shrimp were divided into three groups of 9 shrimp each. The shrimp were injected with 30 µl of *SPIPm5* dsRNA amount 5 µg/g shrimp, *GFP* dsRNA amount 5 µg/g shrimp and normal saline, respectively. After dsRNA injection, the shrimp were exposed to heat stress at 33 °C for 1 h and

reared at ambient temperature for 10 h. The hemolymph was collected for total RNA extraction and cDNA synthesis. The expression of *SPIPm2*, *SPIPm5* and *hsp70* genes was investigated by RT-PCR using specific primers (Table 1). The PCR products were analyzed by gel electrophoresis. The expression of *EF-1α* gene was used as an internal control.

2.4.7. Anti-SPI*Pm*5 antibody injection

Like *SPIPm5* dsRNA injection, the anti-SPI*Pm*5 antibody injection was used to investigate the effect of SPI*Pm*5 depletion on the AMPs transcripts, heat shock protein and KPIs. The normal shrimp were divided into three groups of 9 shrimp each. The shrimp were injected with 30 µl of crude anti-SPI*Pm*5 antibody, 30 µl of anti-SPI*Pm*5 antibody dilution and 30 µl of BSA dilution as control group. The experiment was done in triplicate. After anti-SPI*Pm*5 injection, the hemolymph was collected at 24 h for total RNA extraction and cDNA synthesis. The expression of *SPIPm2*, *SPIPm5*, *crustinPm1*, *penaeidin3*, *penaeidin5* and *hsp70* was detected by RT-PCR using specific primers (Table 1). The PCR products were analyzed by agarose gel electrophoresis. The *EF-1α* gene was used as an internal control.

2.4.8. Effect of SPI*Pm*5 protein on the proPO activating system in shrimp hemolymph

Inhibitory activity of rSPI*Pm*5 on proPO activating system was investigated using a protocol by Ponprateep *et al*. (2017) (Ponprateep *et al*., 2017). The hemolymph was collected with 10% sodium citrate from three normal shrimp. The hemolymph was mixed with PBS at the ratio of 1:3 and vortex vigorously. The protein content in the mixture was determined by using Bradford assay (Bradford, 1976).

The reaction was assayed by pre-incubating the hemolymph mixture of 200 µg along with 0, 0.5, 1 and 2 nmol of rSPI*Pm*5 protein and the 10 µl amount 5 mg/ml of LPS at 30 °C for 15 min. The PBS was used as a control.

The reaction was adjusted to 150 µl with 50 mM Tris-HCl, pH 8.0. Finally, the reaction was added 25 µl of 3 mg/ml the chromogenic substrate, L-3, 4-dihydroxyphenylalanine (L-DOPA), at time zero to started the reaction. The inhibitory activity of rSPI*Pm*5 at various concentrations on proPO activity were measured at A_{490} for every 10 min up to 60 min in a microplate reader. The experiment was done in triplicate.

2.4.9. Effect of SPI*Pm*5 on the survival of pathogen-infected shrimp

2.4.9.1. rSPI*Pm*5 injection and heat stress against WSSV infection

The normal shrimp were divided into five groups of 30 shrimp each. Three groups were injected with 50 µl of 0.5 nmol rSPI*Pm*5, 0.5 nmol rSPI*Pm*5 plus WSSV and WSSV. For two groups were injected with WSSV and PBS before the shrimp were heat-shocked at 33 °C for 1 h and reared in seawater at ambient temperature. The WSSV dilution caused 100% mortality within four days. The experiment was done in triplicate. The cumulative mortality was observed for 14 days. The survival rate of injected shrimp were analyzed with the GraphPad Prism.

2.4.9.2. rSPI*Pm*5 against WSSV and YHV infection

The shrimp were also divided to five groups of 30 shrimp each. The normal shrimp were injected with 50 µl 0.5 nmol of rSPI*Pm*5 plus WSSV, WSSV, 0.5 nmol of rSPI*Pm*5 plus YHV, YHV and PBS. For WSSV and YHV dilution caused 100% mortality within four and five days, respectively. The experiment was done in triplicate. The cumulative mortality was observed for 14 days. The survival rate of injected shrimp were analyzed with the GraphPad Prism.

2.4.9.3. rSPI*Pm*5 against *Vibrio* species

The *Vibrio* bacteria was isolated on Tryptic soy agar plates (Himedia Laboratories) supplemented with 1.5% NaCl. A single colony was picked and cultured on TSB supplemented with 1.5% NaCl. The *Vibrio* bacteria were

grown until OD_{600} reached 1, approximately 3×10^8 CFU/ml for *V. harveyi* injection and VP_{AHPND} immersion experiments. The *Vibrio* bacteria were identified the type species by PCR by specific primers (Dangtip *et al.*, 2015; Maiti *et al.*, 2009).

The normal shrimp were divided into five groups of 30 shrimp each. The shrimp were injected with 50 µl suspension 1×10^6 CFU/ml of *V. harveyi*, 0.5 nmol of rSPI*Pm*5 plus *V. harveyi*. For VP_{AHPND}, the shrimp were injected with 0.5 nmol of rSPI*Pm*5 and immersed in seawater containing 1×10^6 CFU/ml of VP_{AHPND}. The infection control shrimp were also injected with *V. harveyi* and immersed in VP_{AHPND}-seawater. The normal control group was injected with PBS. The two *Vibrio* infection used caused 100% mortality within 6–7 days. The cumulative mortality was observed for 14 days. The survival rates of shrimp was analyzed with the survival program in GraphPad Prism.

2.4.10. Inhibitory effect of SPI*Pm*5 on viral replication

The SPI*Pm*5 may inhibit the viral replication by activated the related immune genes. In this study, the normal shrimp were injected with the various amounts of 0, 0.5, 2, 8 and 16 nmol of rSPI*Pm*5 plus WSSV and YHV dilutions. The PBS injection was done for a control group. The viral dilutions used caused 100% mortality within four and five days, respectively. At 24 h after injection, the hemolymph was collected for total RNA extraction and cDNA synthesis. The expression of *VP28* gene and *YHV* gene [the segment of *ORF1b* and *ORF2* genes of YHV genome (Ochoa-Meza *et al.*, 2013) was analyzed by RT-PCR using specific primers (**Table 1**). The PCR products were analyzed by agarose gel electrophoresis. The expression of *EF-1α* gene was used as an internal control. The experiment was done in triplicate.

2.4.11. Inhibitory effect of SPI*Pm*5 on bacteria in infected shrimp

The inhibitory activity of SPI*Pm*5 against to the *Vibrio* bacterial growth were tested with the various amount of rSPI*Pm*5. The normal shrimp were

injected with 50 µl of 0, 0.5, 1 and 2 nmol of rSPI*Pm*5 plus 3×10^8 CFU/ml *V. harveyi* or plus immersion with 3×10^8 CFU/ml VP_{AHPND} in seawater. The bacterial dilution used caused 100% mortality within four–five days. After *Vibrio* infection, the hemolymph was collected from 3 shrimp for each amount of rSPI*Pm*5 at 5, 15, 30, 60 and 180 min, respectively.

The infected hemolymph was diluted 100-fold with normal saline. The 10 µl of diluted hemolymph were dropped on TCBS agar plates (Himedia Laboratories). For control group, the shrimp were injected with normal saline. Their hemolymph was also collected and dropped on TCBS and incubated at 30 °C for 13–14 h (Xu *et al.*, 2014). The numbers of *Vibrio* colonies were counted and the data shown were means ± standard deviations. The experiment was done in triplicate. Statistical analysis was done using the One-way ANOVA followed by Duncan's 'new multiple rang test. Data differences were considered statistically significant at $P<0.05$.

2.4.12. Effect of SPI*Pm*5 on AMP gene expression in infected shrimp

It is possible that the rSPI*Pm*5 may activate the AMP gene expression against the *Vibrio* infection. In this study, the normal shrimp were injected with 50 µl of 0, 0.5, 1 and 2 nmol of rSPI*Pm*5 plus 3×10^8 CFU/mL *V. harveyi* or immersed with 3×10^8 CFU/mL VP_{AHPND} in seawater. The control groups were injected with normal saline. The hemolymph was collected from three shrimp each for various amounts of rSPI*Pm*5 at 1 h after infection. The expression of AMP genes: *ALFPm3*, *crustinPm1*, *crustinPm7*, *penaeidin3*, *penaeidin5* and *lysozyme*, was investigated by RT-PCR using specific primers (**Table 1**). The PCR products were analysed by agarose gel electrophoresis. The expression of *EF-1α* gene was used as an internal control.

Table 1 Sequences of primers used in this study

Primer	**Sequence (5'→3')**	**Usage**
SPZF	GCGACTCTCGTATCTATCCGC	Full-length
SPZR	TGGCCAGGGAGTCCTAGATCC	
PmSPZF	GCGCCCATGGGCTCCGTCCAACC	Gene specific, Cloning
PmSPZR	GCGCAAGCTTTGCTGGAAGATGG	
RTSPZF	CGCTTGGAGACCTGTCTGTT	RT-PCR, qRT-PCR
RTSPZR	TAGGGATCGCATGGGTCGTA	
CTSPZF	ATATCCATGGCGGGTGGTCCTGA	Gene specific, Cloning
ALFPm3F	ATACTAGAATTCCAAGGGTGGGAGGCT GTGGCA	RT-PCR of *ALFPm3*, *crustinPm1*, *Penaiedin3*, *Penaiedin5*, *lysozyme* genes
ALFPm3R	TATTATGGATCCCTATGAGCTGAGCCAC TGGTTGGCCT	
CrustinPm1F	CTGCTGCGAGTCAAGGTATG	
CrustinPm1R	AGGTACTGGCTGCTCTACTG	
CrustinPm7F	GGCATGGTGGCGTTGTTCCT	
CrustinPm7R	TGTCGGAGCCGAAGCAGTCA	
Pen3F	GGATCCTAATACGACTCACTATAGGAT GCGTCTCGTGGTCTGCCT	
Pen3R	GGATCCTAATACGACTCACTATAGGCC TGTGAATACACGAGGA	
Pen5F	TTGGTCTATGCTTTGCAAGG	
Pen5R	ACAGATAGTTAAAGTGAAAGAC	
LysozymeF	CCGTTACCAAGCCTTACTG	
LysozymeR	TAATCGGTCGCGTAGTCCTC	
LvSPZ1F	TGGGGACTCTCCTTACGA	RT-PCR of *LvSPZ1* gene (Wang *et al.*, 2012)
LvSPZ1R	GGGAACAGACAGGTCTCCA	
LvSPZ2F	ACAGCCCACCTGACTGTACC	RT-PCR of *LvSPZ2* gene (Wang *et al.*, 2012)
LvSPZ2R	ATCGCTACTGACGAGGCAAT	

Table 1 Sequences of primers used in this study (Cont.).

Primer	**Sequence (5'→3')**	**Usage**
RTSPIPm5F	ATTCTCTTCGCTGTGGCTGT	qRT-PCR and RT-PCR of *SPIPm5* gene
RTSPIPm5R	TCACAAGACCCATGATGCAC	
PmHsp70F	CTTCGACAACCGCATGGTGA	RT-PCR of heat shock protein 70 (Junprung *et al.*, 2017)
PmHsp70R	GAAGAGGGAGCCGATCTCCA	
RTSPIPm2F	ATGCAACCACGTCTGTACTG	RT-PCR of *SPIPm2* gene (Ponprateep *et al.*, 2013)
RTSPIPm2R	CTGCAAGGTTCCACATCT	
T7/SPIPm5F	GGATCCTAATACGACTCACTATAGG GGAAAAGGAGGCAAATTCAGACT	Gene silencing of *SPIPm5* gene
T7/SPIPm5R	GGATCCTAATACGACTCACTATAGG ATATCCCTTCTTGATAGGCG	
T7/GFPF	TAATACGACTCACTATAGGATGGTG AGCAAGGGCGAGGA	Gene silencing of *GFP* gene
T7/GFPR	TAATACGACTCACTATAGGTTACTT GTACAGCTCGTCCA	
VP28F	TCACTCTTTCGGTCGTGTCG	RT-PCR of WSSV (Ponprateep *et al.*, 2013)
VP28R	CCACACACAAAGGTGCCAAC	
YHVF	GGTTTTAGGTGACATTAGGGTCT	RT-PCR of YHV gene (Ochoa-Meza *et al.*, 2013)
YHVR	GATGATTGAAAGGGATTGTCG	
EF-1αF	GGTGCTGGACAAGCTGAAGGC	Internal control :RT-PCR, qRT-PCR
EF-1αR	CGTTCCGGTGATCATGTTCTTGATG	

CHAPTER III

RESULTS

3. *Pm*Spätzle

3.1. Identification of *PmSpz* isoforms

The *PmSpz1* clones and their partial sequences were identified from the *P.monodon* EST database (Tassanakajon *et al.*, 2006). Two clones of *Spz3* genes were identified in the GenBank database, one from *P. monodon* (accession no. KM225266) and another from *Fenneropenaeus chinensis* (accession no. EU523114) (unpublished). Moreover, the Pacific white shrimp, *L. vannamei*, has been reported that there are three *Spätzle* genes, *LvSpz1*, *LvSpz2* and *LvSpz3* (Wang *et al.*, 2012). Like *L. vannamei*, the *P. monodon* may have three isoforms equivalent to *LvSpz* genes. Since the protein sequences of *PmSpz1* and *PmSpz3* are highly homologous to *LvSpz 1* and *LvSpz3* sequences, three primer pairs of *PmSpz* genes were designed from three *LvSpz* genes for the amplification the *Spätzle* gene in the black tiger shrimp.

The *PmSpz1* and *3* genes were amplified from the hemocyte cDNAs and the *PmSpz2* gene was amplified from the gill cDNAs. Three *Spätzle* from *P. monodon* were analyzed by RT-PCR and the *Spätzle* gene fragments were observed (**Fig. 3.1.**). The bands were excised, cleaned up and sequenced. The nucleotide sequences were analyzed in BlastX. The results indicated that there were three *PmSpz* genes in *P. monodon* and their sequences were equivalent to the *LvSpz* genes. The three *PmSpz* sequences were deposited into the GenBank with the accession number KY053796, KY053798 and KY053797, respectively. The *PmSpz1* and *3* were expressed fairly common in the hemocytes whereas the *PmSpz2* was detected in gill but hardly detected in hemocytes. The *Spätzle* gene fragments were compared with the reference gene expression of *EF-1α*.

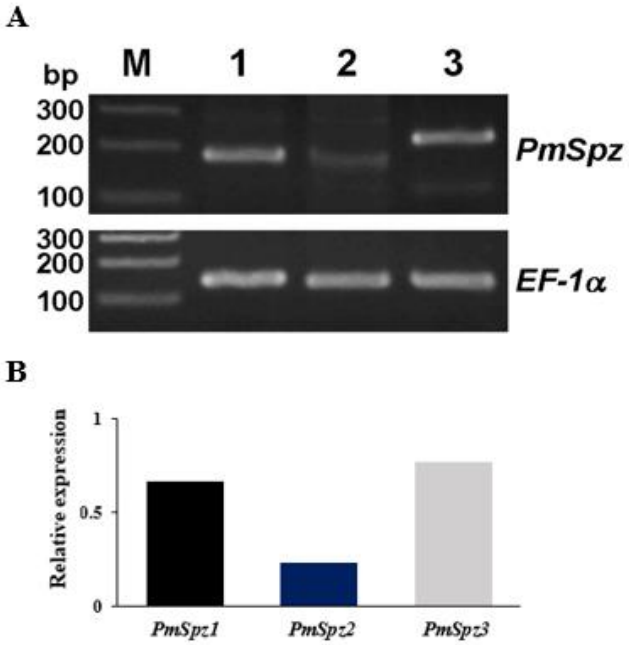

Figure 3.1. The expression of three *PmSpz* genes from the normal shrimp. (A) The *PmSpz* genes were amplified from hemocyte cDNA and the specific primers of *PmSpz1*, *PmSpz2* and *PmSpz3* were designed from *LvSpz 1*, *LvSpz2* and *LvSpz3* gene sequences. Lanes M, 1, 2 and 3 are DNA ladder, PCR products from *PmSpz* isoform 1 (*PmSpz1*, hemocytes), *PmSpz* isoform *2* (*PmSpz2*, gill) and *PmSpz* isoform 3 (*PmSpz3*, hemocytes), respectively and (B) the relative expression analysis of *PmSpz1*, *2* and *3* genes were analyzed. *EF-1α* was used as internal control.

3.2. *PmSpz1* gene and its active domain

From nucleotide sequence analysis, the *PmSpz1* gene from hemocytes is equivalently to *LvSpz1* gene. The *PmSpz1* clone was identified from the *P. monodon* EST database. The primers were designed from the nucleotide sequence of *LvSpz1*. The band was amplified, purified and sequenced. The nucleotide sequences were analysed by using bioinformatic programs. The *PmSpz1* sequences showed that it consisted of an open reading frame of 714 bp encoding a protein of 237 amino acids and the signal peptide of 25 amino acid residues. The mature protein had an estimated molecular mass of 23.7 kDa and

a *pI* of 5.34. The Blastp showed the Spätzle conserved domains at 116-–208 amino acid residues of mature protein (**Fig. 3.2.**).

The alignment sequences of *Spätzle* gene, the *DmSpz1* (*D. melanogaster*), *MsSpz1* (*M. sexta*), *BmSpz1* (*B. mori*) and *PmSpz1* (*P. monodon*) showed the six conserved Cys-residues at the carbonyl-terminal domain as cysteine knot domain. For the sequence alignment of *Spätzle* genes, they showed the protease cleavage site of *Spätzle* genes at the C-terminal of His109 of *P. monodon* which was equivalent to His109, Arg195, Arg169 and Arg152 of *L. vannamei*, *D. menalogaster*, *M. sexta* and *B. mori*, respectively (**Fig. 3.3.**). All but *PmSpz1* gene were cleaved by trypsin-like protease. No basic amino acids, Arg and Lys, were in the vicinity of His109 of *Pm*Spz1.

```
  CTCTCATATTGGTAGGTTGGTCATCATAATTCATAAAACAATTTAAATGGTTTTCATCCACCAT  64
ATTGTATTAATGCTATCACTTTGATACAATAACAATAAAAGAAATAATACATAATTGATTTTCTTG 130
TAC ATG TATAATTTTCAGTCGGTCGTTCTGGCTTGTGCCGGAGTCGTTCTGGGCTCCGTCCAACCC 196
   M  Y  N  F  Q  S  V  V  L  A  C  A  G  V  V  L  G  S  V  Q  P

CCAGCATATGGTCATCATCCACAGCCTGCTTACGGTCATCGTGCGCCTGCTTACGGTCATCAGCCT 262
P  A  Y  G  H  H  P  Q  P  A  Y  G  H  R  A  P  A  Y  G  H  Q  P

GCCTACAAGCACGGTTACTGCGATCCAACTGTGGCGCCTTCGTGTGCTACCAACTCAACTCTCTCC 328
A  Y  K  H  G  Y  C  D  P  T  V  A  P  S  C  A  T  N  S  T  L  S

TATTGCTTGGAAGATGCTGAGTATCCCGAGTACGAGATCAAGGGGGCCATCAGTGCTGATCATCTG 394
Y  C  L  E  D  A  E  Y  P  E  Y  E  I  K  G  A  I  S  A  D  H  L

TTCGCCAAGAAGTATGCTGATATCGCCGACCAGTCTGCTGATGATCTGGTAGACATGGTAAGCAAG 460
F  A  K  K  Y  A  D  I  A  D  Q  S  A  D  D  L  V  D  M  V  S  K

GAGCAGGAAGAGGCTTTCGATTACTCTTACTACACAGGGGCATCCACCGGGGACTCTCCTTATGAT 526
E  Q  E  E  A  F  D  Y  S  Y  Y  T  G  A  S  T  G  D  S  P  Y  D

GCCACCCACTGGGGTGGTCCTGAGGGCTACATTTGTCCCTCAGATGTGGTGTATGCCATGCCCAAA 592
A  T  H  W  G  G  P  E  G  Y  I  C  P  S  D  V  V  Y  A  M  P  K

CGTGCCCAAAACGTGGAGGGTAAATGGCGCGTGATTGTCAACGACGTTCACTATTACACCCAGACT 658
R  A  Q  N  V  E  G  K  W  R  V  I  V  N  D  V  H  Y  Y  T  Q  T

GCCCGCTTGGAGACCTGTCTGTTCCCAGAGGCTGCTTGCCGTGCCCTTGCCCCTTGCTATCAGAGT 724
A  R  L  E  T  C  L  F  P  E  A  A  C  R  A  L  A  P  C  Y  Q  S

CACTGTACCCAGAAGTCAGTGTACCACCGCCTCCTGTCGTACGACCCATGCGATCCCTATAAGGGT 790
H  C  T  Q  K  S  V  Y  H  R  L  L  S  Y  D  P  C  D  P  Y  K  G

CTCTTCATCGACATCTACAAGCTGCCTTCTGCCTGCTCCTGCCATCTTCCAGCA TAA TACGCC    853
L  F  I  D  I  Y  K  L  P  S  A  C  S  C  H  L  P  A  *
```

Figure 3.2. The open-reading frame of *PmSpz1* and its deduced amino acid sequence from cDNA *PmSpz1* clone. The signal peptide is underlined. The start and stop codons are shown in boldface. The asterisk indicates stop codon. The Spätzle conserved domain is shaded green.

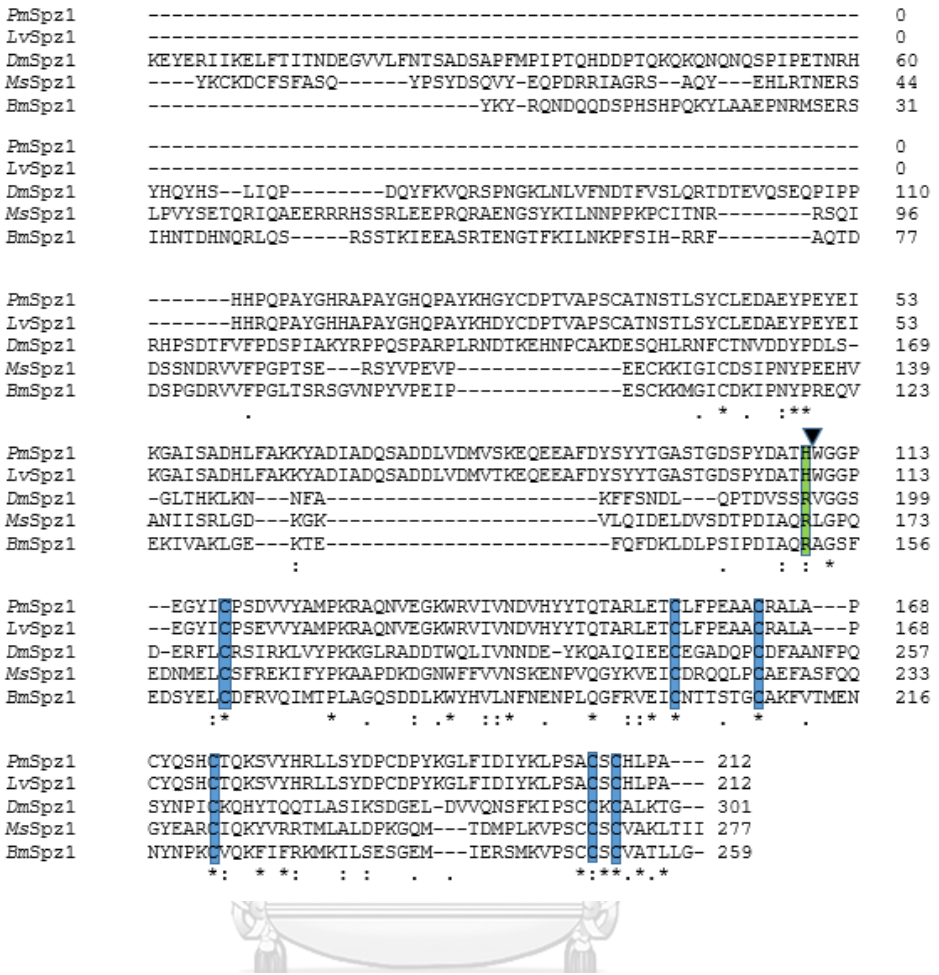

Figure 3.3. Amino acid sequence alignment of *Pm*Spz1 protein with the white shrimp *Lv*Spz1 (JN180646), *D. melanogaster* Spz1A (*Dm_Spz*, NM_079802), *M. sexta* Spz1A (*Ms_Spz*, accession no. GQ249944) and *B. mori* Spz1 (*Bm_Spz*, NM_001114594). The six conserved cysteine residues of the cystine knot domain are shaded blue. The proteolytic cleavage sites that activate Spz proteins are indicated by black triangle after the green-shaded amino acids.

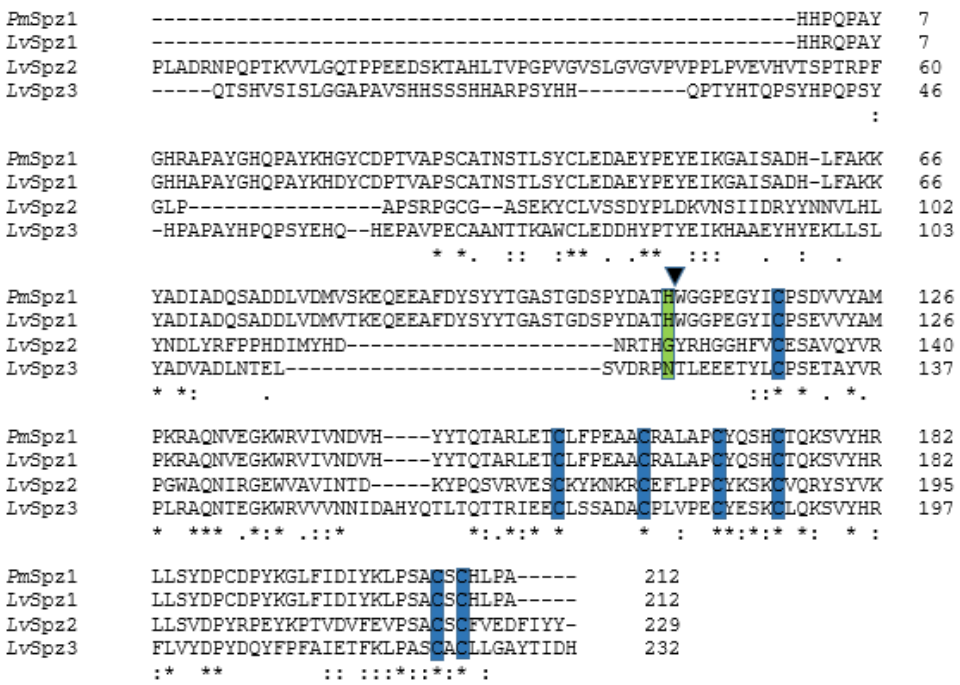

Figure 3.4. Amino acid sequence alignment of *Pm*Spz1 with the white shrimp, *L. vannamei* *LvSpz1* (JN180646), *Lv*Spz2 (JN180647) and *Lv*Spz3 (JN180648).The seven conserved cysteine residues of the cystine knot domain are shaded blue. The proteolytic cleavage sites that activate Spz proteins are indicated by black triangle after the green-shaded amino acids.

The *Pm*Spz1 protein without signal peptide residues showed 97.64%, 29.24% and 48.63 identity with the *Lv*Spz1, *Lv*Spz2 and *Lv*Spz3, respectively. The aligment sequences also showed the seven conserved Cys-residues at the carbonyl-terminal domain as cysteine knot domain and cleavage site of *Spätle* genes (**Fig 3.4**).

3.3. Expression of *PmSpz1* gene in shrimp tissues and in response to WSSV infection

The expression of *PmSpz* gene was tested in nine tissues from the normal shrimp including hemocytes, stomach, hepatopancreas, gill, lymphoid organ, eye, muscle, intestine and heart. The *PmSpz1* gene was investigated by RT-PCR using specific primers. The results suggested that the *PmSpz1* gene were expressed in all tissues but only the muscle showed less expression (**Fig. 3.5A**).

The hemocytes were used for testing the expression of *PmSpz1* gene in response to WSSV infection and the expression of AMP genes. From previous studies, the *PmSpz1* gene has been reported that it is up-regulated in response to the injection with both heat-killed Gram-positive *Staphylococcus aureus* and Gram-negative *V. harveyi* (Arayamethakorn *et al*., 2017). In this study, the *PmSpz1* gene was investigated in response to the WSSV infection from hemocytes. The hemocytes were collected at 3, 6, 12, 24 and 48 h from WSSV-infected shrimp for preparation total RNA and cDNA synthesis. The *PmSpz1* transcripts were analyzed by qPCR. The results concluded that the *PmSpz1* gene was expressed several fold compared with the control group (**Fig. 3.5B**).

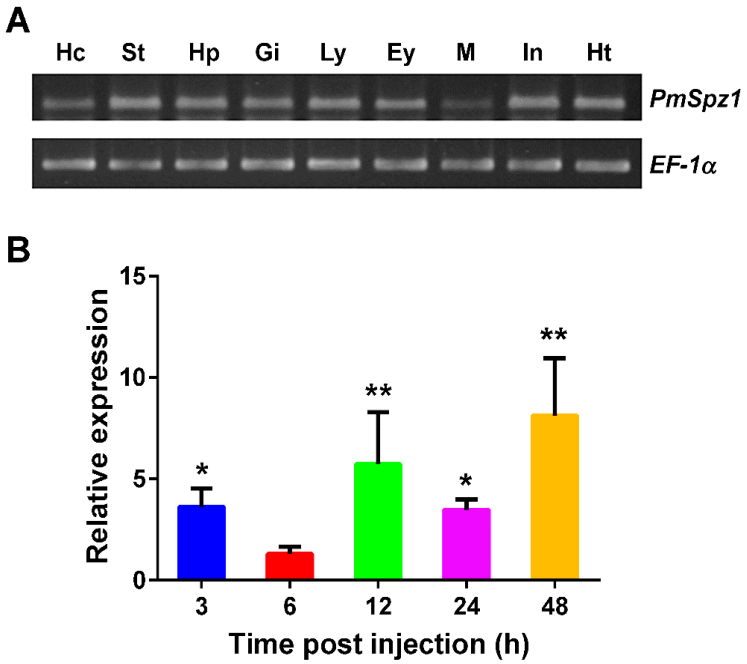

Figure 3.5. Expression of *PmSpz1* gene in shrimp tissues and in response to WSSV infection. (A) Its expression was found in nine tissues tested: hemocytes (Hc), stomach (St), hepatopancreas (Hp), gill (Gi), lymphoid organ (Ly), eye (Ey), muscle (M), intestine (In) and heart (Ht). (B) After WSSV infection, the *PmSpz1* gene expression were investigated from the hemocytes. They were collected at various times and analysed by qRT-PCR. The results are means ± SDs of three independent amplifications. The * and ** indicate significant differences more than 2-fold and 10-fold, respectively, over the normal saline control. For both experiments, the expression of *EF-1α* was used as an internal control.

3.4. Hemolymph *Pm*Spz1 in WSSV-infected shrimp

The Spätzle protein is a secretory protein and secreted into the blood circulation in shrimp. It can be activated, relay the infection signal through the Toll receptor on the cell membrane and induced the synthesis of AMPs against the pathogenic infection (Ferrandon *et al.*, 2004; Shia *et al.*, 2009; Weber *et al.*, 2003) In this study, the Spätzle protein was detected from the hemolypmh of infected shrimp using anti-*Pm*Spz1 antibody by Western blot analysis. The hemolymph were collected at 0, 6, 24 and 48 h and prepared the hemocyte

lysate and plasma. The samples were analysed by SDS-PAGE. The results were interesting that the two forms of *Pm*Spz1 were detected in plasma but the active form was found only in the hemocyte lysate (**Fig. 3.6.**). For the pro-protein, it decreased in plasma as the infection progressed. However, the active form of *Pm*Spz1 was found in large proportion in the hemocyte lysate and plasma. For the other un-infected samples, we also found the *Pm*Spz1 active form (data not shown).

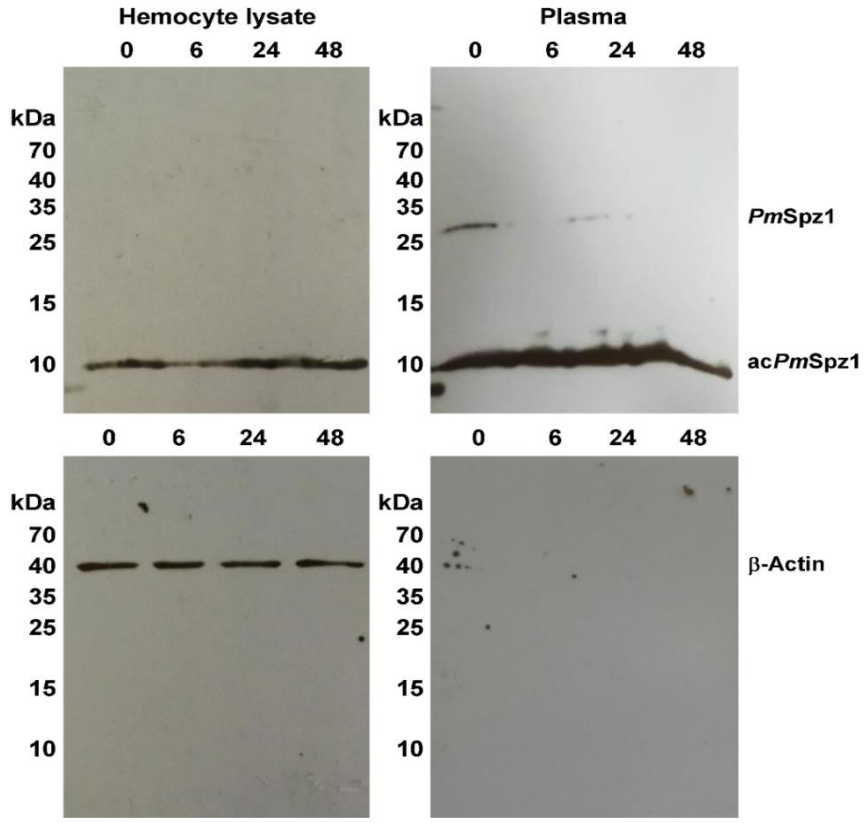

Figure 3.6. Investigation of the *Pm*Spz1 protein in hemocyte lysate and plasma from WSSV-infected shrimp. The hemocyte lysate and plasma were prepared from WSSV-infected shrimp at 0, 6, 24 and 48 hpi. They were subjected to SDS-PAGE and transfer membrane for Western blotting. The *Pm*Spz1 was detected using anti-*Pm*Spz1 as primary antibody and secondary antibody conjugated with horseradish peroxidase. The protein bands were developed using chemiluminescent substrates and detected by X-ray film. The β-actin was detected as an internal control. Nevertheless, the β-actin was not detected in the plasma.

3.5. Protein expression of *Pm*Spz1 protein and its active domain

The *Pm*Spz1 and its active protein were prepared for the study of their activity. The gene fragments of the mature *Pm*Spz1 and its active domain were cloned into an *E. coli* expression vector. Two *Pm*Spz1 proteins were over-produced using an *E. coli* BL21 (DE3) CondonPlus.

The mature *Pm*Spz1 protein was expressed as inclusion bodies. The crude protein was dissolved with carbonate buffer pH 10 and purified using Ni-NTA column. The purified protein was analysed by SDS-PAGE (**Fig. 3.7.**) . The r*Pm*Spz1 protein was approximately 30 kDa although the calculated MW was 26.2 kDa (**Fig. 3.10, lane 1**) . It was also investigated with the anti-His TAG antibody (**data not shown**).

The rac*Pm*Spz1 protein was also over-produced as inclusion bodies. It was a fusion protein to Trx Tag of 26.0 kDa (**Fig. 3.8**) . The fusion protein rac*Pm*Spz1 fusion protein was solubilized and completely cleaved with enterokinase (**Fig. 3.9**) . The rac*Pm*Spz1 was re-purified by Ni-NTA column (**Fig. 3.10, lane 2**) for removing the Trx Tag. The cleaved-rac*Pm*Spz1 was 13.3 kDa in size.

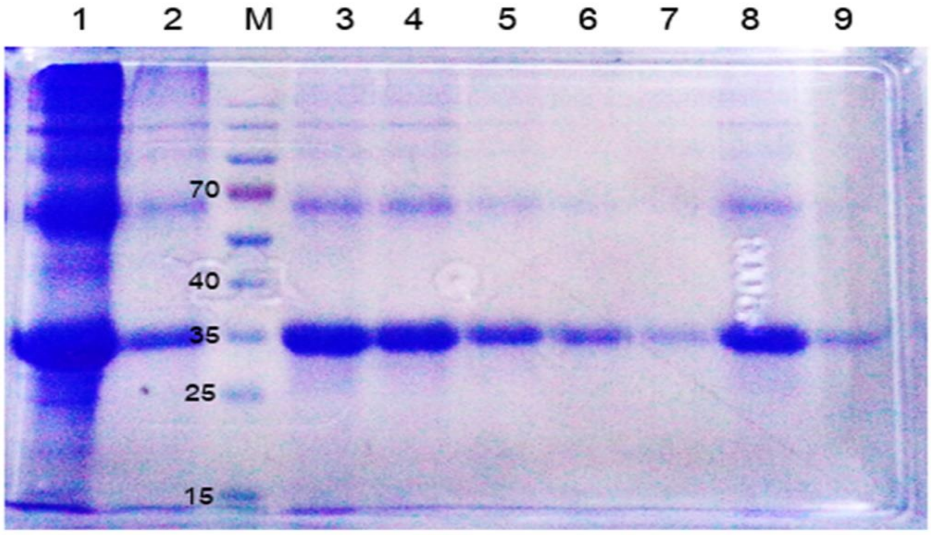

Figure 3.7. The recombinant *Pm*Spz1 proteins was over-produced in *E. coli* BL21 (DE3) CodonPlus. The r*Pm*Spz1 in crude protein was purified by Ni-NTA column. The r*Pm*Spz1 was analysed by SDS-PAGE. Lane 1 = crude protein, Lane 2 = Flow through, Lane 3 = Elution fraction1, Lane 4 = Elution fraction2, Lane 5 = Elution fraction 4, Lane 6 = Elution fraction 5, Lane 7 = Elution fraction 6, Lane 8 = Elution fraction 3, Lane 9 = Wash fraction. Lanes M = protein standard marker.

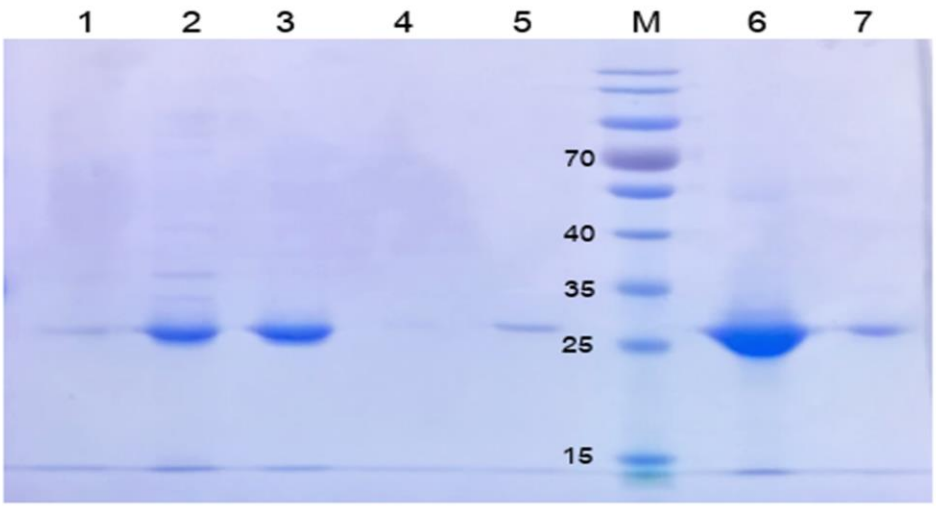

Figure 3.8. The fusion protein, Trx_rac*Pm*Spz1, was also over-produced in *E. coli* BL21 (DE3) CodonPlus. The Trx_rac*Pm*Spz1 in crude protein was purified by Ni-NTA column. The Trx_rac*Pm*Spz1 was analysed by SDS-PAGE. Lane 1 = Wash fraction, Lane 2 = Flow through, Lane 3 = Elution fraction1, Lane 4 = Elution fraction 2, Lane 5 = Elution fraction 3, Lane 6 = Elution fraction 4, Lane 7 = Elution fraction 5, Lanes M = protein standard marker.

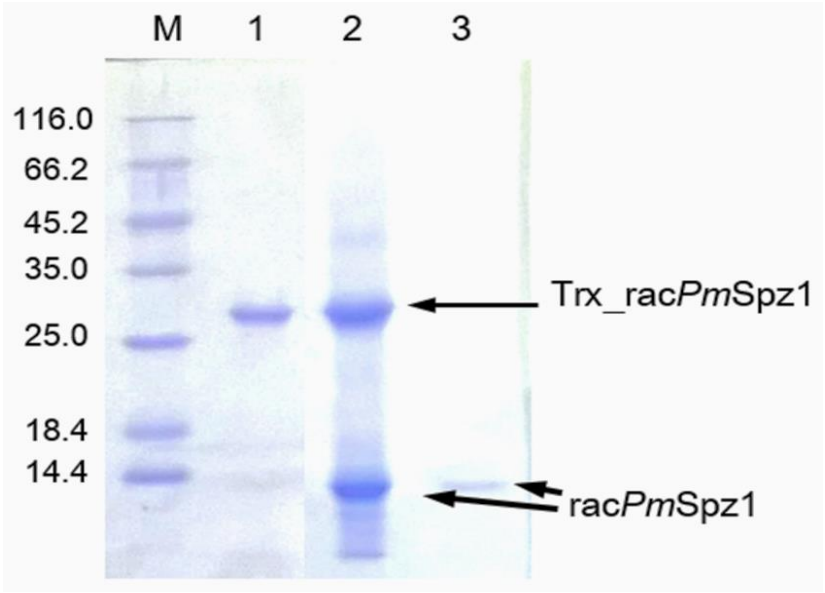

Figure 3.9. The recombinant ac*Pm*Spz1 proteins was prepared from the fusion protein, Trx_rac*Pm*Spz1. The Trx_rac*Pm*Spz1 was cleaved with enterokinase in completion. The racPmSpz1 protein was re-purified through the Ni-NTA column and dialyzed against PBS. The purified racPmSpz1 protein was analyzed by SDS-PAGE. Lane 1 = Trx_rac*Pm*Spz1 fusion protein, Lane 2 = Trx_rac*Pm*Spz1 in crude protein, Lane 3 = rac*Pm*Spz1 in supernatant. Lanes M = protein standard markers.

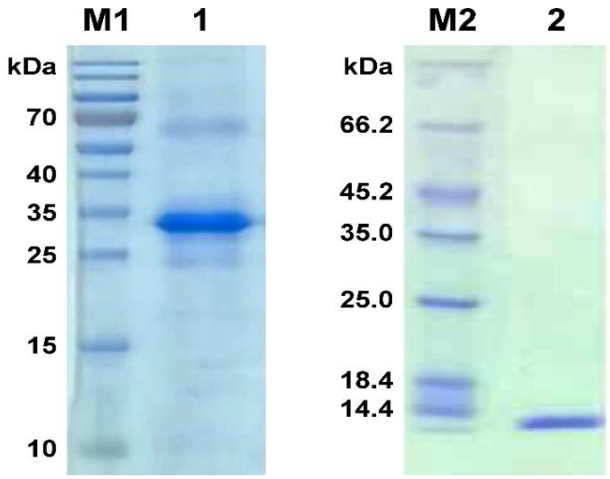

Figure 3.10. The recombinant *Pm*Spz1 and rac*Pm*Spz1 proteins were over-produced in *E. coli* BL21 (DE3) CodonPlus. The purified r*Pm*Spz1 (lane 1) and rac*Pm*Spz1 protein (lane 2) was analyzed by SDS-PAGE. Lanes M1 and M2 are two different protein standard markers.

3.6. Effect of r*Pm*Spz1 protein on the survival of WSSV-infected shrimp

The Spätzle protein is involved in immune response to pathogen infection. The Spätzle protein can induce the antimicrobial process in the shrimp. The normal shrimp were injected with *Pm*Spz1 plus WSSV dilution compared with the WSSV and control groups. The results showed that the r*Pm*Spz1 protein was able to prolong the life of shrimp more than 40% up to ten days compared with the control group (**Fig. 3.11**).

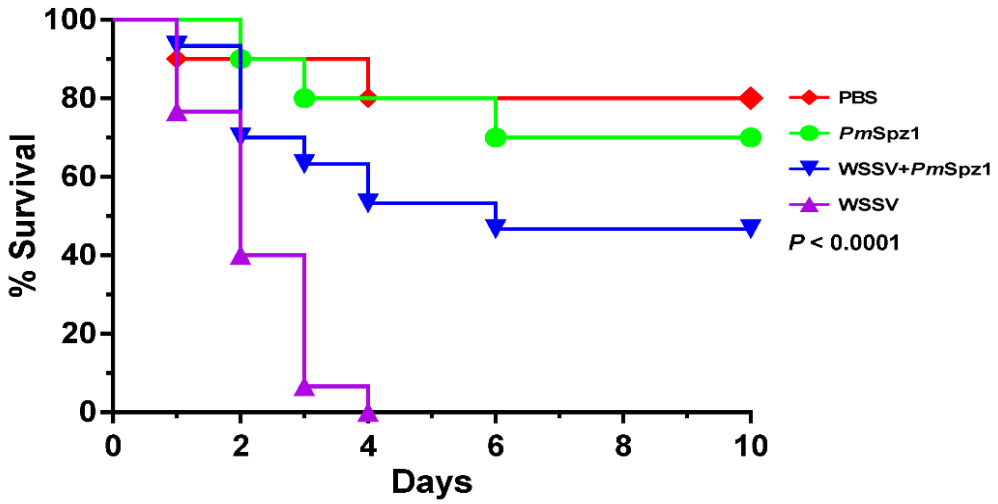

Figure 3.11. The Kaplan-Meier survival plots showing the protective effect of r*Pm*Spz1 protein on the WSSV-infected shrimp. The protective effect of r*Pm*Spz1 were investigated. The shrimp were divided into four groups and injected with 1× PBS (♦), r*Pm*Spz1 (●), WSSV+r*Pm*Spz1 (▼) and WSSV (▲). Some shrimp died in the PBS and *Pm*Spz1 control groups because of moulting and cannibalism. The *P*-value calculated by log-rank test in the GraphPad Sigma Plot indicates significant difference.

3.7. rac*Pm*Spz1 stimulated the expression of some antimicrobial peptide genes

The rac*Pm*Spz1 protein activated the Toll receptor and induced the signaling through Toll pathway for the AMPs synthesis. To investigate the AMP gene expression, the 0.3 and 3 nmol amount of rac*Pm*Spz1 protein were injected into the normal shrimp. The hemolymph was collected at 24 h and tested by qPCR. The results showed that the AMP genes; *ALFPm3*, *crustinPm1*, *crustinPm7*, *penaeidin3* and but not *penaeidin5*, were up-regulated after injection with 3 nmol rac*Pm*Spz1 protein and also the gene expression patterns were different (**Fig. 3.12.**). The results summarized that the rac*Pm*Spz1 protein injection induced the signaling of the synthesis of AMPs. All but not *penaeidin5* were induced under the Toll pathway (Visetnan *et al*., 2015). From previous study, the expression of *ALFPm3* and *crustinPm1* genes was up-regulated under the Toll pathway control. For another genes, *penaeidin3* and *crustinPm7* was partly under the regulation of Toll and Imd pathways whereas that of *penaeidin5* was expressed under the control of Imd pathway.

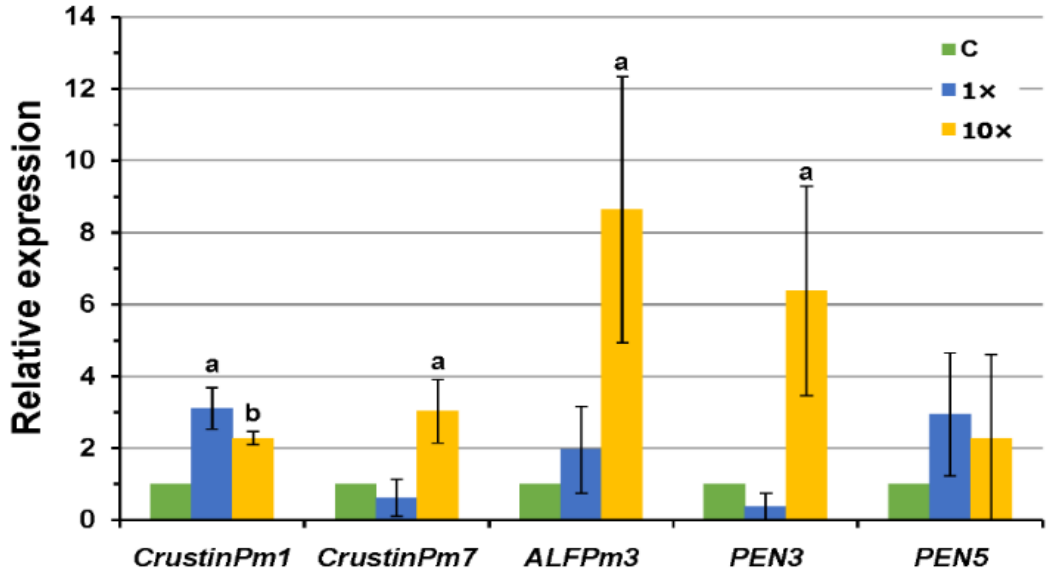

Figure 3.12. Effect of rac*Pm*Spz1 protein injection. The normal shrimp were injected with 0.3 (1×) and 3 (10×) nmol of the active domain rac*Pm*Spz1 protein. The AMP genes, *crustinPm1*, crustin*Pm7*, *ALFPm3*, *penaedin3* and *penaeidin5*, were analyzed by qRT-PCR. The data represent means ± SDs of three independent qRT-PCR amplifications. The expression of *EF-1α* gene was used as an internal control. Letters indicate significant differences above the normal saline control at $P < 0.05$.

4. SPI*Pm*5

SPI*Pm*5 is a two domain Kazal type serine proteinase inhibitor. The P1 residues of SPI*Pm*5, Leu and Thr, can inhibit the subtilisin and elastase (**Fig 3.13A**) . From previous study, the *SPIPm5* gene has been identified from the hemocyte cDNAs. Its gene expression is up-regulated upon heat-stress and YHV infection in shrimp. It is possible that the SPI*Pm*5 may have important function in shrimp immune system involving stresses (Visetnan *et al.*, 2009). In this research, we investigated the effect of SPI*Pm*5 response to stresses and its activities further.

4.1. Expression of *SPIPm5* gene in shrimp tissues

For tissue distribution experiment, the *SPIPm5* transcripts were detected in nine tissues from the normal shrimp. Total RNA and cDNAs were prepared. The *SPIPm5* gene was analyzed by RT-PCR. The *SPIPm5* transcripts were detected in all nine tissues, hemocytes, stomach, gill, lymphoid organ, muscle, intestine and heart but hepatopancreas and eyestalk were expressed less **(Fig. 3.13B).** *EF-1α* was used as an internal control. The hemocytes were used for investigation the effect of *SPIPm5* and other related immune gene expression in infected shrimp.

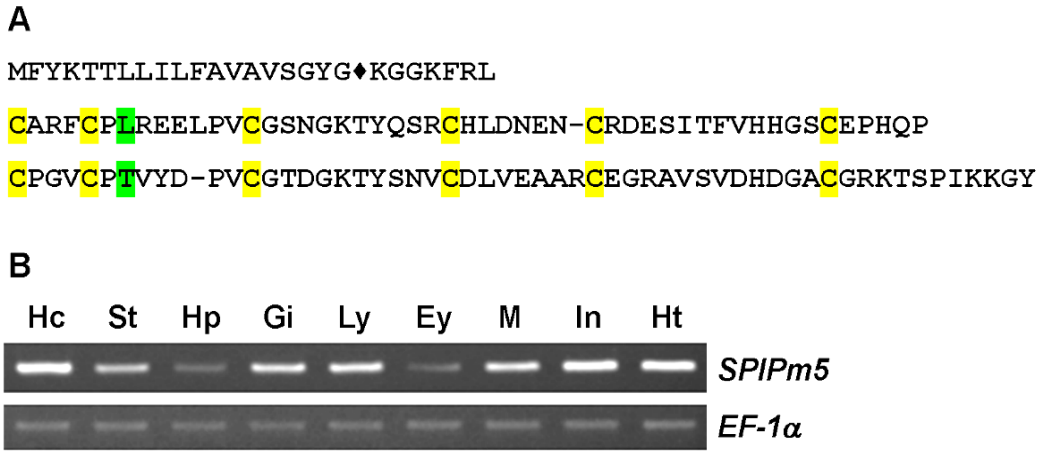

Figure 3.13. The SPI*Pm*5 amino acid sequences and its gene expression in the normal shrimp tissues. (A) The complete and amino acid sequence of SPI*Pm*5 protein. Black diamond indicates the potential signal peptide cleavage site. Conserved cysteine residues and the P1 amino acids are shaded yellow and green, respectively. (B) The expression of *SPIPm5* gene was found in nine tissues: hemocytes (Hc), stomach (St), hepatopancreas (Hp), gill (Gi), lymphoid organ (Ly), eyestalk (Ey), muscle (M), intestine (In) and heart (Ht). *EF-1α* was used as an internal control.

4.2. Up-regulation of *SPIPm5* gene under heat stress

The expression of *SPIPm5* gene was tested in heat stress temperature. From previous study, the normal shrimp were exposed to heat shock temperature at 33 °C for 1 h and reared at ambient temperature for testing the expression of *SPIPm5* gene. Its gene expression was up-regulated under heat stress (Visetnan *et al.*, 2009). In this research, the *SPIPm5* gene was investigated and the *hsp70* gene was amplified as a positive control (Cottin *et al.*, 2010; Rungrassamee *et al.*, 2010). The results suggested that the *SPIPm5* transcripts increased after the shrimp expose to heat shock temperature at 24 and 48 h (**Fig. 3.14**). The *hsp70* transcipts were also increased slightly. Therefore, the *SPIPm5* transccripts were increased up to 48 h by heat shock temperature.

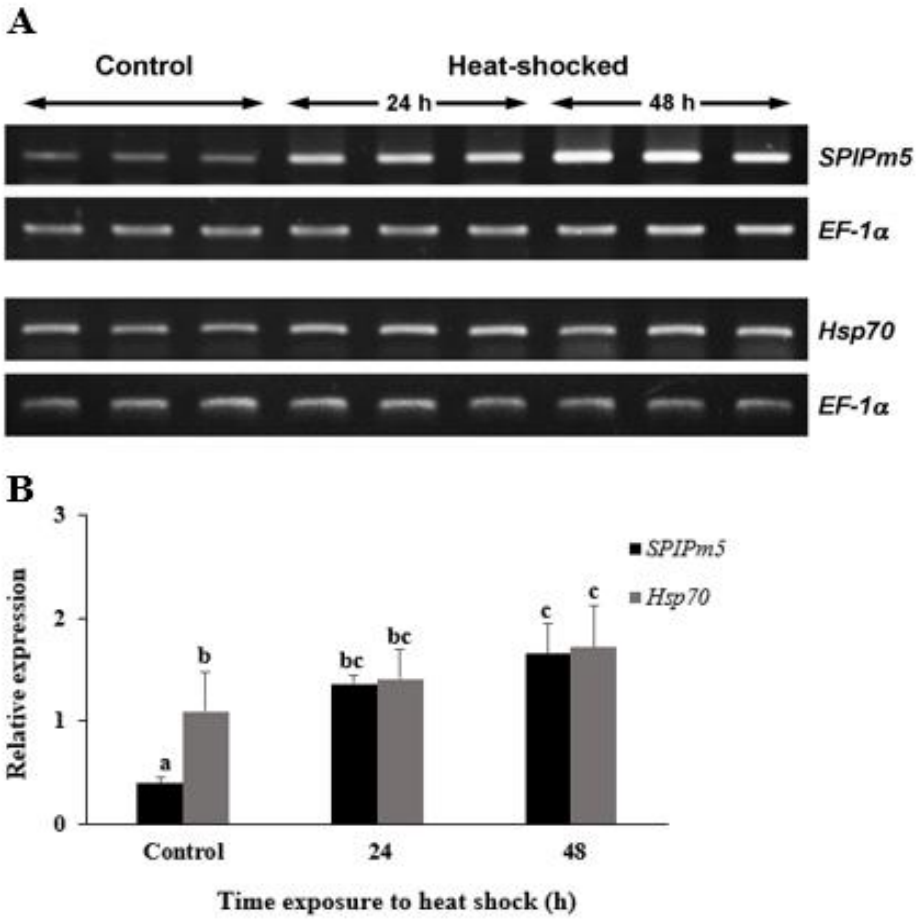

Figure 3.14. The *SPIPm5* and *Hsp70* transcripts were up-regulated under heat stress condition. (A) The normal shrimp were exposed to heat stress at 33 ˚C for 1 h and reared in ambient temperature. The hemolymph were collected at 24 and 48 h for prepare the total RNA and hemocyte cDNA synthesis. The control shrimp were also exposed to the same condition. The *SPIPm5* and *Hsp70* gene expression were detected by RT-PCR. (B) The relative expression of *SPIPm5* and *Hsp70* genes were analyzed. *EF-1α* gene was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

4.3. Expression of *SPIPm5* gene in response to WSSV or YHV infection

The *SPIPm5* clones are detected in the hemocyte cDNA libraries after WSSV and YHV infection (Pongsomboon *et al.*, 2011; Visetnan *et al.*, 2009). In this research, the *SPIPm5* transcripts were investigated after WSSV and YHV infections in shrimp. The hemolymph was collected at 3, 6, 12, 24 and 48 h for preparing the total RNA and cDNAs. The *SPIPm5* gene was analyzed by qRT-PCR. The results suggested that the *SPIPm5* transcripts were increased significantly at 3 and 12 h after the two viral infections (**Fig.3.15**).

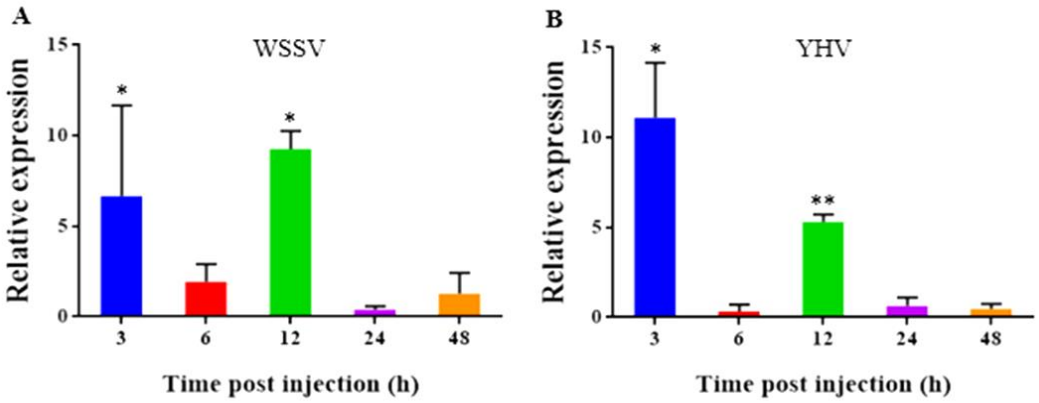

Figure 3.15. Expression of *SPIPm5* gene in response to WSSV and YHV injection. The normal shrimp were injected with WSSV and YHV dilution. The *SPIPm5* gene expression were investigated by qRT-PCR and the hemolymph were collected at various times. The results were means ± SDs of three independent amplification. The experiment was done in triplicate. The * and ** indicated significant differences ($P < 0.05$) over normal saline control. The expression of *EF-1α* was used as an internal control.

4.4. rSPI*Pm*5 protein expression

The rSPI*Pm*5 protein was over-produced for study the activity of the shrimp immune system. The rSPI*Pm*5 protein was expressed as soluble protein in *E. coli* host system and purified with Ni-NTA column (**Fig. 3.16**). Its molecular weight was about 17 kDa. The rSPI*Pm*5 protein was purified, subjected to SDS-PAGE (**Fig. 3.17, lane 1**) and detected by Western blot analysis using anti-SPI*Pm*5 antibody (**Fig. 3.17, lane 2**).

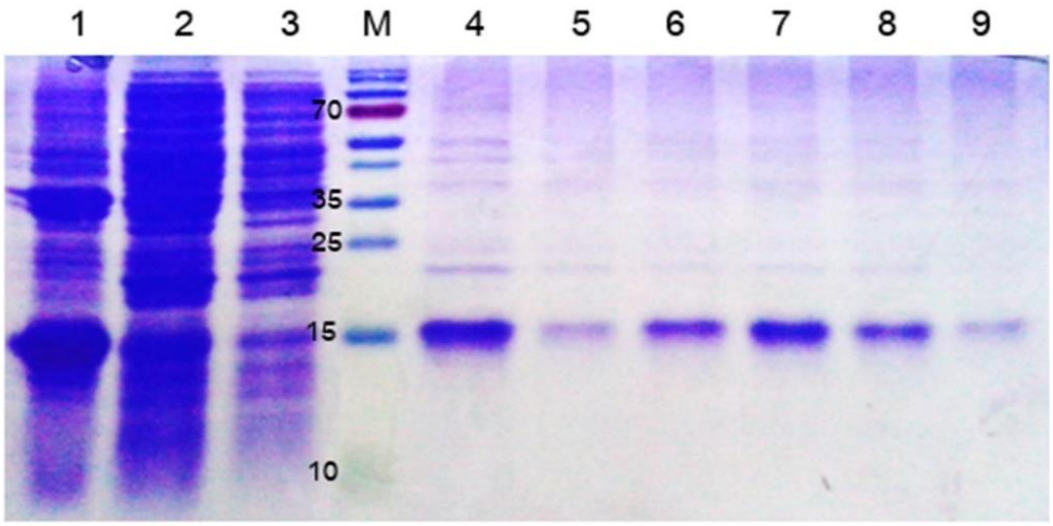

Figure 3.16. The recombinant SPI*Pm*5 proteins was expressed in *E. coli* BL21 (DE3) CodonPlus. After induced the cell by IPTG, the rSPI*Pm*5 was produced as soluble protein. Then, the protein was purified by Ni-NTA column, eluted with imidazole and dialyzed against PBS. The rSPI*Pm*5 protein was analysed by SDS-PAGE. Lane 1 = crude protein, Lane 2 = Wash fraction, Lane 3 = Flow through, Lane 4 = Elution fraction 1, Lane 5 = Elution fraction 2, Lane 6 = Elution fraction 3, Lane 7 = Elution fraction 4, Lane 8 = Elution fraction 5, Lane 9 = Elution fraction 6. Lanes M = protein standard marker.

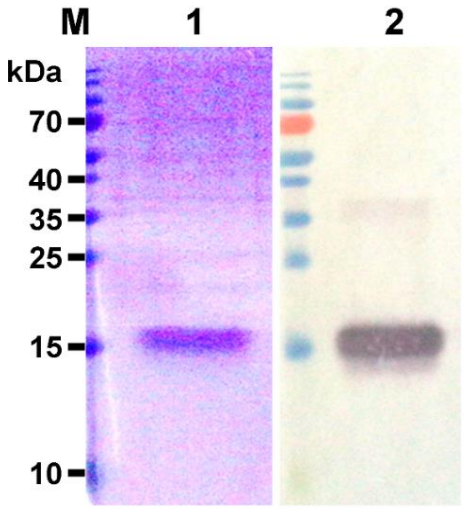

Figure 3.17. The purified rSPI*Pm*5 protein, it was purified by Ni-NTA column, analyzed by SDS-PAGE gel (lane1) and also investigated by Western blot analysis (lane 2). The rSPI*Pm*5 protein was detected using anti-SPI*Pm*5 as mouse polyclonal antibody. Lane M is protein standard marker.

4.5. Effect of SPI*Pm*5 injection on other immune genes

4.5.1 rSPI*Pm*5 injection

The *SPIPm5* transcripts were up-regulated after viral infection. The SPI*Pm*5 protein injection may influence the gene expression of other immune genes in blood circulation system against the viral infection. The rSPI*Pm*5 protein was injected into the normal shrimp to imitate the viral infected shrimp. The expression of immune genes: *crustinPm1*, *penaeidin3*, *penaeidin5*, *SPIPm2*, *SPIPm5* and *hsp70*, were investigated. The results showed that the expression of *crustinPm1* gene was induced but not *penaeidin3*. The *penaeidin5* gene was also slightly increased whereas the *hsp70* was decreased. Moreover, the rSPI*Pm*5 injection had no effect on the KPI, *SPIPm2* and *SPIPm5* gene expression (**Fig. 3.18**). The results suggested that the SPI*Pm*5 protein injection might positively influence the expression of *crustinPm1* and *penaedin5* genes which were under the regulation of Toll pathway. It meant

that the SPI*Pm*5 protein could activate the innate immune system against the viral infection.

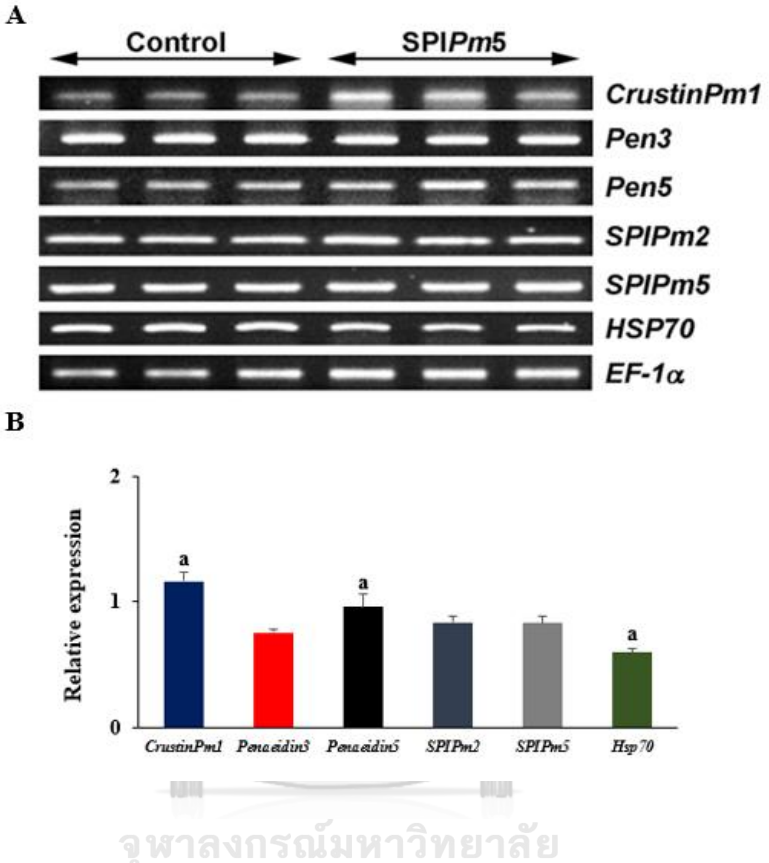

Figure 3.18. Effect of rSPI*Pm*5 protein injection experiment induced the other immune genes. (A) The rSPI*Pm*5 protein was injected into the normal shrimp and collected the hemolymph at 24 h. Gene expression of *crustinPm1*, *penaeidin3*, *penaeidin5*, *SPIPm2*, *SPIPm5* and *Hsp70* were detected by semi-quantitative RT-PCR. The control shrimp were also injected with 1×PBS. (B) The relative expression of all genes were analyzed. *EF-1α* was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

4.5.2 The (10×) rSPI*Pm*5 injection

Like the rSPI*Pm*5 injection, the higher amount of rSPI*Pm*5 were also investigated the effect on other related immune genes in shrimp. In this study, the 5 (10×) nmol amount of rSPI*Pm*5 protein was injected into the normal shrimp and the expression of immune genes was observed. After the rSPI*Pm*5 protein injection, the hemolymph was collected at 24 h. The total RNA and cDNAs were prepared. The expression of selected genes was analyzed by RT-PCR with specific primers. The results suggested that the *crustinPm1*, *penaeidin*3, *penaeidin5*, *SPIPm2*, *SPIPm5* and *hsp70* were all down-regulated (**Fig 3.19**).

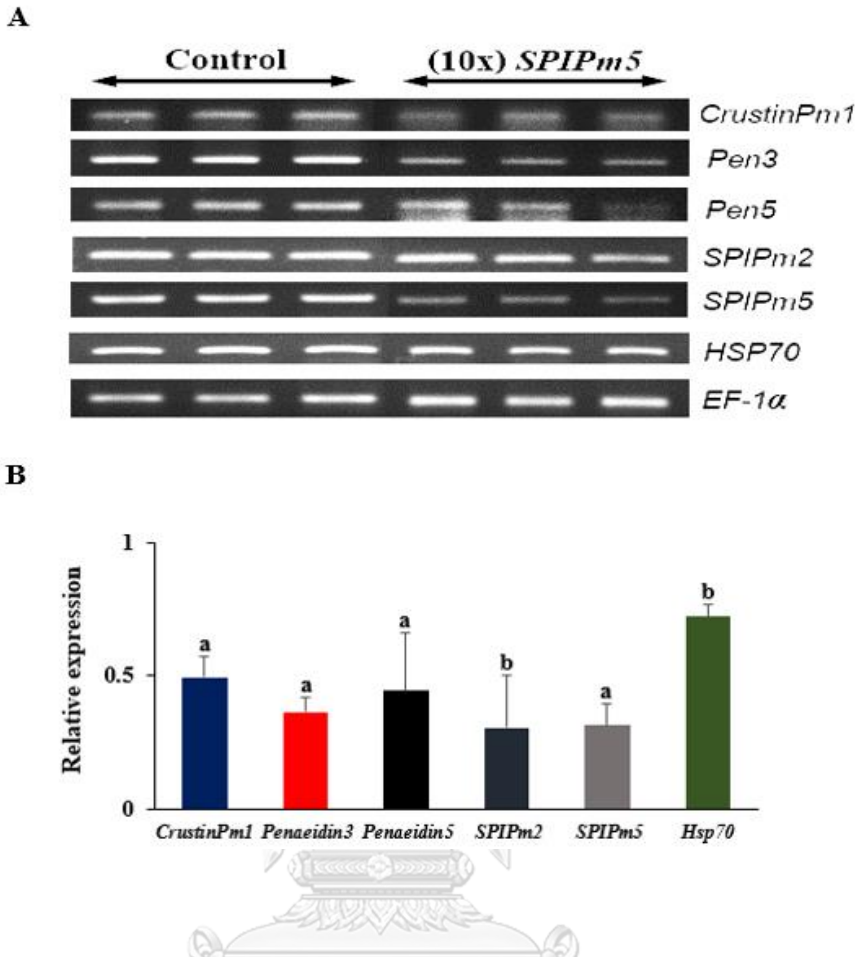

Figure 3.19. Effect of 5 (10×) nmol rSPI*Pm*5 injection experiment on the other related immune genes. (A) The rSPI*Pm*5 protein was injected into the normal shrimp. The hemolymph was collected at 24 h. Gene expression of *crustinPm1*, *penaeidin3*, *penaeidin5*, *SPIPm2*, *SPIPm5* and *Hsp70* was detected by semi-quantitative RT-PCR. The control shrimp were injected with 1×PBS. (B) The relative expression of all genes were analyzed. *EF-1α* was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

4.5.3. The 0.5 (1×) and 5 (10×) nmol rSPI*Pm*5 injection under heat shock condition

The heat stress can activate the innate immune genes in shrimp and the rSPI*Pm*5 injection were also up-regulated. The heat stress and rSPI*Pm*5 injection may have influence on the other immune genes. In this study, the 0.5 (1×) (**Fig 3.20**) and 5 (10×) (**Fig 3.21**) nmol amounts of rSPI*Pm*5 protein were injected into the shrimp. Then, the shrimp were exposed to heat stress at 33 °C for 1 h. For studying the effect of rSPI*Pm*5 injection along with under the heat shock temperature, the AMPs transcripts, *SPIPm2*, *5* and *hsp70* genes were also investigated. The PBS injection was used to inject the control group. The results suggested that the higher amount of rSPI*Pm*5 could affect the selected genes transcripts: the expression of *SPIPm5* was down-regulated but not those of other immune genes, *SPIPm2*, *hsp70*, *crustinPm1*, *penaeidin3* and *5*, was gradually expressed comparing with control groups. However, the minimal rSPI*Pm*5 injection with heat stress, the expression of all genes: *SPIPm2*, *SPIPm5*, *hsp70*, *crustinPm1*, *penaeidin3* and *5*, was also expressed like the higher protein injection.

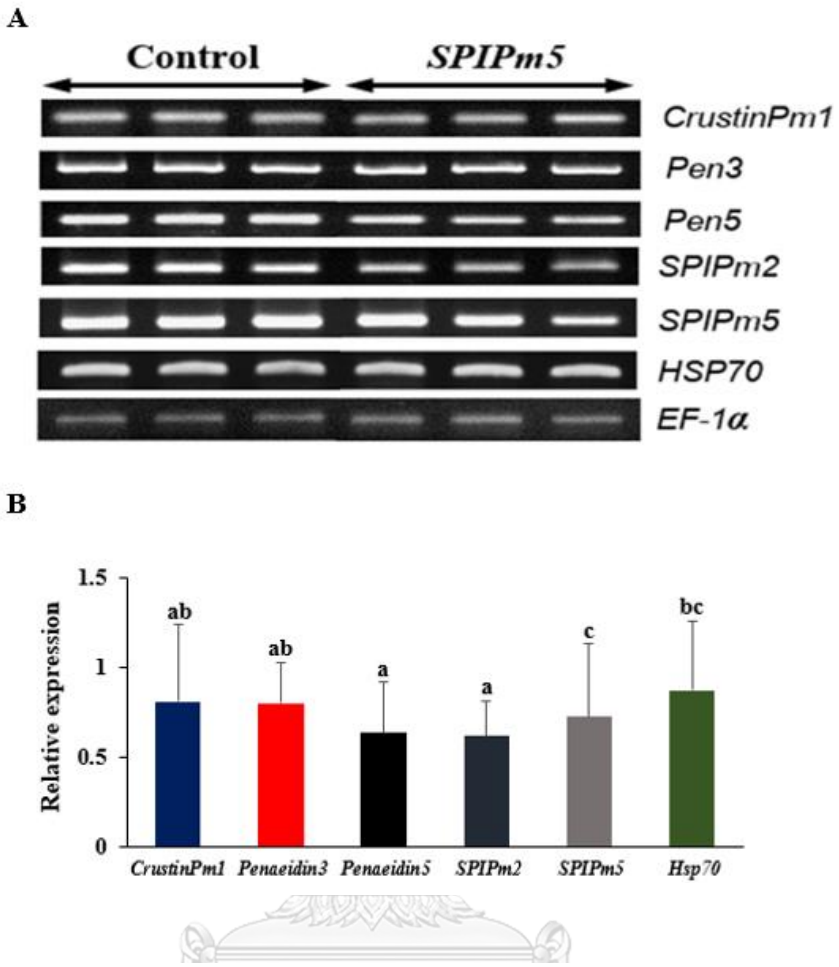

Figure 3.20. Effect of 0.5 (1×) nmol rSPI*Pm*5 injection into the normal shrimp on the related immune genes under heat shock temperature. (A) The rSPI*Pm*5 protein were injected into the normal shrimp. The related immune gene expression was also tested and collected the hemolymph at 24 h after rSPI*Pm*5 injection and heat stress. (B) The relative expression of *crustinPm1*, *penaeidin3*, *penaeidin5*, *SPIPm2*, *SPIPm5* and *Hsp70* genes were analyzed. *EF-1α* was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

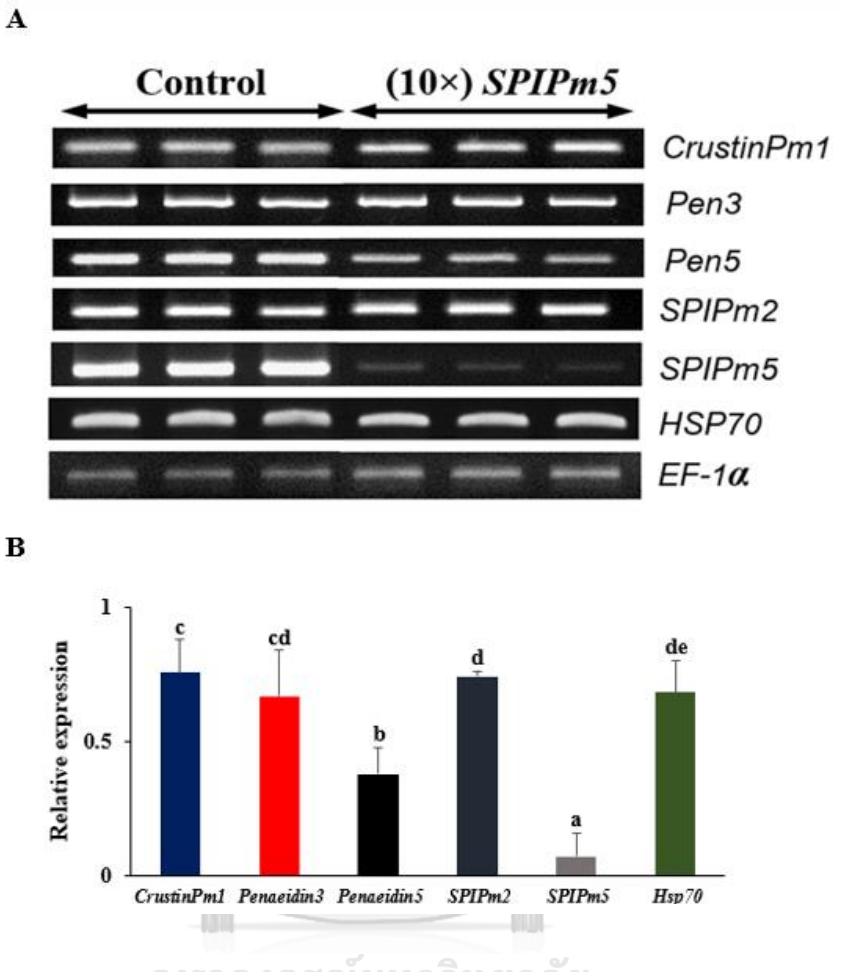

Figure 3.21. Effect of 5 (10×) nmol rSPI*Pm*5 injection into the normal shrimp on the related immune genes under heat shock temperature. (A) The rSPI*Pm*5 protein were injected into the normal shrimp. The related immune gene expression was also tested and collected the hemolymph at 24 h after rSPI*Pm*5 injection and heat stress. (B) The relative expression of *crustinPm1*, *penaeidin3*, *penaeidin5*, SPIPm2, *SPIPm5* and *Hsp70* genes were analyzed. *EF-1α* was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

4.5.4 Effect of *SPIPm5* dsRNA injection

From above experiment, the *crustinPm1* and *penaeidin5* were slightly induced after injection with the minimal rSPI*Pm*5 protein. The effect on other immune gene expression were tested using the gene silencing. Double stranded RNA (dsRNA) of *SPIPm5* and GFP genes were synthesized using *in vitro* T7 RiboMAX™ Express Large Scale RNA Production System (Promega). The normal shrimp were divided into three groups, injection with 5 µg/g shrimp of *SPIPm5* dsRNA and *GFP* dsRNA. The normal saline was injected as a control group. In the preliminary experiment, we injected with 10 µg/g shrimp of *SPIPm5* dsRNA, all shrimp died after injection for about 10 h.

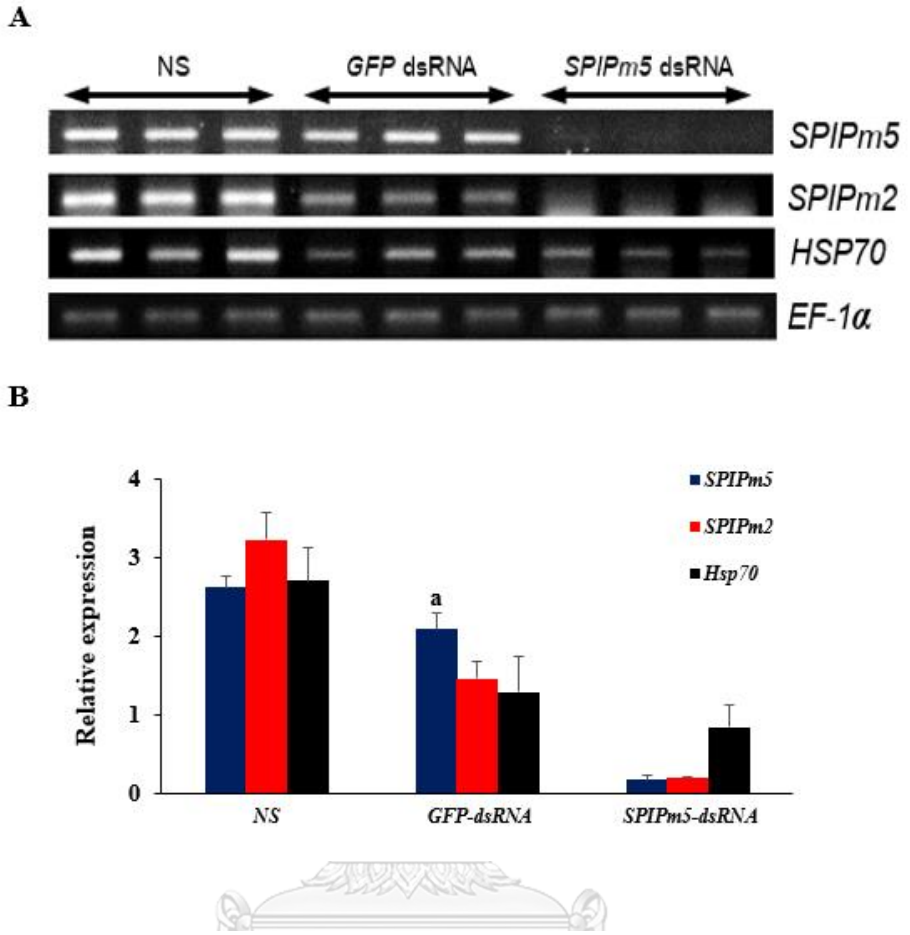

Figure 3.22. The *SPIPm5* dsRNA injection affected the expression of *SPIPm2*, *5* and *hsp70* genes. (A) The normal shrimp were injected with 5 µg/g shrimp of *SPIPm5* dsRNA and *GFP* dsRNA. The normal saline was injected as control group. After injection, the shrimp were exposed to heat shock temperature for1 h and reared at ambient temperature. The hemolymph was collected at 24 h for the total RNA and cDNA preparation. The effect gene silencing was analyzed by RT-PCR. (B) The relative expression of *SPIPm5*, *SPIPm2* and *Hsp70* genes were analyzed. *EF-1α* was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

After dsRNA injection, the shrimp were exposed to heat shock temperature and reared at ambient temperature. The hemolymph was collected at 24 h for total RNA and cDNA preparation. The expression of *SPIPm2, 5* and *hsp70* was investigated by RT-PCR. The results suggested that the *SPIPm5* dsRNA injection could affect to the expression of *SPIPm2* and *5* but showed less effect on *hsp70* gene (**Fig. 3.22**). It indicated that the gene silencing experiment showed the complicated results that the *SPIPm5* dsRNA injection inhibited both the expression of *SPIPm5* and *SPIPm2* genes. It could not be used for the study of other related immune genes.

4.5.5. Effect of anti-*SPIPm5* antibody injection on other immune genes

The anti-SPI*Pm*5 antibody injection might influence the related immune gene expression. The antibody would inhibit directly the SPI*Pm*5 protein in blood circulating system and affected the other immune gene expression. In this study, the normal shrimp were injected with crude and 1/10 dilution of anti-SPI*Pm*5 antibody, respectively. The BSA was also injected as a control group. The hemolymph was collected at 24 h from the anti-SPI*Pm*5 antibody injected shrimp. Total RNA and cDNAs were prepared for the amplification of related-immune genes by RT-PCR. The results suggested that the crude anti-SPI*Pm*5 antibody inhibit affect to AMP and KPI genes especially *SPIPm2* and *5* genes and then the crude protein injection could effect to the *crustinPm1*, *penaeidin3* and *5* but not *hsp70* gene (**Fig. 3.23**). However, the anti-SPI*Pm*5 antibody dilution injection was also affect to the related immune genes: *SPIPm2*, *SPIPm5*, *hsp70*, *crustinPm1*, *Penaeidin3* and *5*. All genes expression was up-regulated compare with control group. The antibody injection experiment could not use for study the effect of the related-immune genes.

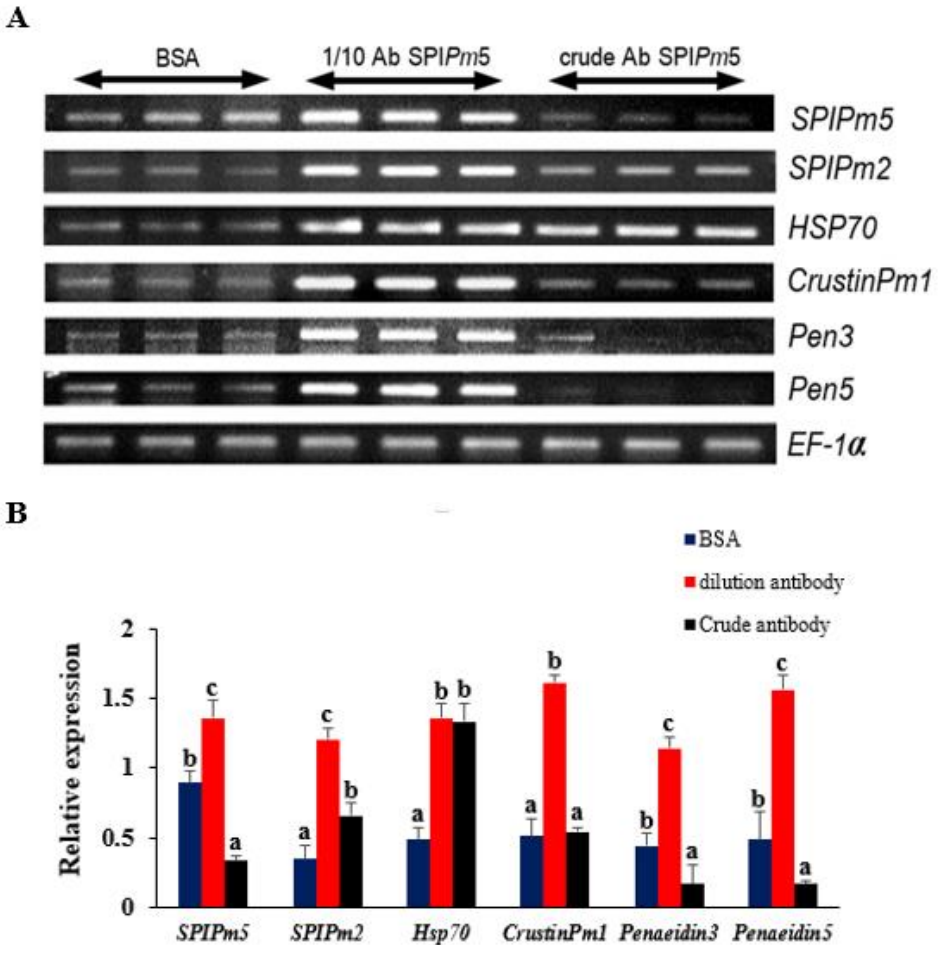

Figure 3.23. Effect of anti-SPI*Pm*5 antibody injection on the expression of related immune genes. (A) The normal shrimp were divided into three groups and injected with the crude and 1/10 dilution of anti-SPI*Pm*5 antibody. The BSA was injected into shrimp as control group. The hemolymph was collected at 24 h from the anti-SPI*Pm*5 antibody injected shrimp. Total RNA and cDNAs were prepared for RT-PCR. (B) The relative expression of *SPIPm5*, *SPIPm2*, *Hsp70*, *crustinPm1*, *penaeidin3* and *penaeidin5* genes were investigated. *EF-1α* was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

4.6. Inhibition of rSPI*Pm*5 protein on proPO activity

The proPO defense system is activated after microbial infection for protection of the host cells. In this study, the rSPI*Pm*5 protein might affect the hemolymph proPO activity. The various amounts of rSPI*Pm*5 were tested. The results showed that the 0.5 nmol rSPI*Pm*5 had minimal inhibitory affect on the proPO activity of hemolymph. Higher amounts of rSPI*Pm*5 protein inhibited the proPO activity better (**Fig. 3.24**).

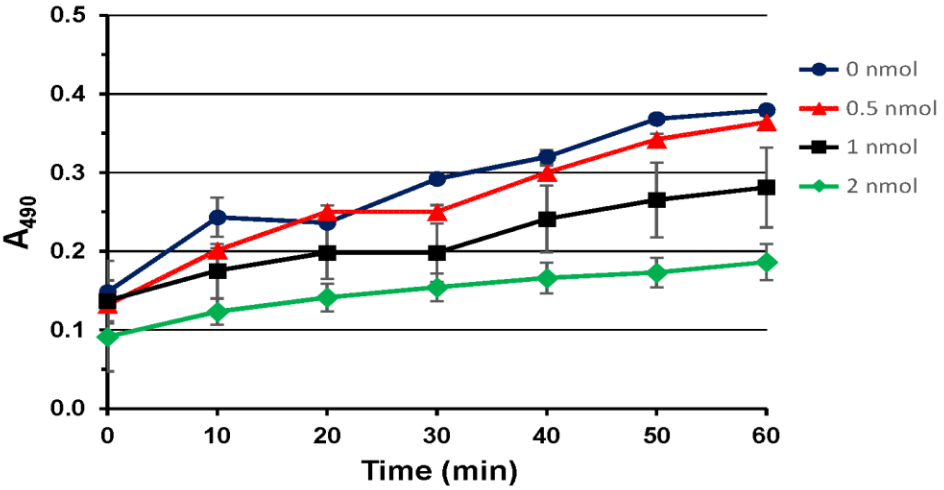

Figure 3.24. Inhibition on proPO activating system by rSPI*Pm*5. The rSPI*Pm*5 protein was pre-incubated with 200 µg of shrimp hemolymph and LPS. The reaction was started by adding the chromogenic substrate, L-3, 4- dihydroxyphenylalanine (L-DOPA). Inhibition of proPO activating system with the 0, 0.5, 1 and 2 nmol amount of rSPI*Pm*5 protein were detected the A_{490} every 10 min up to 60 min by microplate reader. The experiment was done in triplicate.

4.7. Mortality assay of microbial infected shrimp

4.7.1. rSPI*Pm*5 injection, heat stress and WSSV infection

Several innate immune genes in defense system in shrimp; AMPs, heat shock protein and prophenoloxidase system, were induced by heat stress. The *SPIPm5* transcripts in shrimp was also up-regulated under heat stress temperature. In non-heat stress, the rSPI*Pm*5 injection may activate the shrimp immune system as well as the pathogen infection. The SPI*Pm*5 is able to prolong life of shrimp after WSSV infection. It is possible that the rSPI*Pm*5 injection may help the infected-shrimp fight against the WSSV infection.

For study the inhibitory activity, the normal shrimp were divided into five groups injected with PBS plus heat-shock, WSSV plus heat-shock, WSSV, rSPI*Pm*5 and rSPI*Pm*5 plus WSSV. The mortality rate was observed. The results showed that the rSPI*Pm*5 help prolong the life of WSSV-infected shrimp for about 8 days. The effect of SPI*Pm*5 injection in shrimp was similar to the heat condition. The mortality rate of PBS plus heat shock and rSPI*Pm*5 injection groups were minimal (**Fig. 3.25A**)

4.7.2 Effect of rSPI*Pm*5 injection on the mortality of WSSV-and YHV-infected shrimp

From the survival experiment above, the protective effect of SPI*Pm*5 against WSSV infection was shown. In this study, the inhibition activity of rSPI*Pm*5 injection was also tested against the YHV compared with WSSV infection. The normal shrimp were observed after injection with WSSV, rSPI*Pm*5 plus WSSV, YHV, rSPI*Pm*5 plus YHV and PBS. The results showed that the rSPI*Pm*5 was able to prolong the life of WSSV- and YHV-infected shrimp longer than the infected-shrimp control from 50% survival rate of 3-4 days to 6 days (**Fig. 3.25B**).

4.7.3 Effect of rSPI*Pm*5 injection on the mortality of *Vibrio*-infected shrimp

From the viral infection in survival experiment, the rSPI*Pm*5 injection could help prolong the life of infected-shrimp. Like the viral infection, the bacterial infection was also tested. The bacterial strains, VP_{AHPND} and *V. harveyi*, were tested along with the rSPI*Pm*5 injection. The VP_{AHPND} causes EMS (Dangtip *et al.*, 2015; Thitamadee *et al.*, 2016). The *V. harveyi* causes luminous vibriosis in infected shrimp (Dash *et al.*, 2017).

Like other survival experiments, the normal shrimp were injected with *V. harveyi*, rSPI*Pm*5 plus *V. harveyi*, VP_{AHPND}, rSPI*Pm*5 injection plus VP_{AHPND} immersion and PBS injection as control groups. The survival rate was observed. The rSPI*Pm*5 protein injection helped prolong the life of infected-shrimp a little longer from 50% survival rate of 4–5 days to 6–7 days (**Fig. 3.25C**).

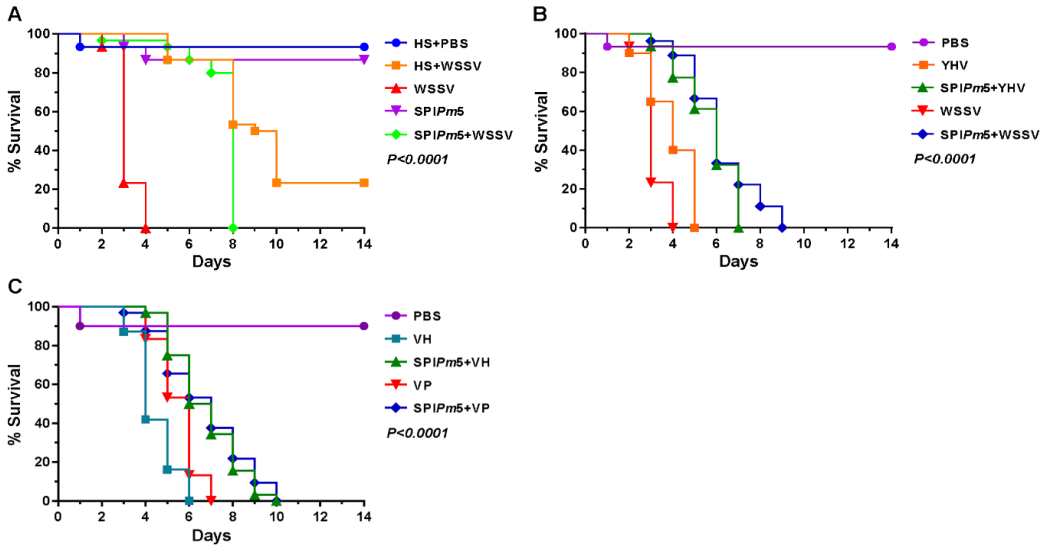

Figure 3.25. The Kaplan-Meier survival plots showing the protective effect of rSPI*Pm*5 protein on the pathogen infected shrimp. The survival experiment were tested (A) heat stress and antiviral activity of rSPI*Pm*5 on the WSSV-infected shrimp (B) on the WSSV- and YHV-infected shrimp and (C) antibacterial activity of rSPI*Pm*5 on the VP_{AHPND} immersion and *V. harveyi*-injection in shrimp. The experiment was done in triplicate. The cumulative mortality was observed for 14 days. The *P* value calculated by log-rank test in the GraphPad Sigma Plot indicates significant difference.

4.8. rSPI*Pm*5 possesses inhibitory effect on the expression of viral genes

From the survival experiment, the inhibition activity of SPI*Pm*5 responded to the viral-infection more than the bacterial-infection. The SPI*Pm*5 might inhibit their viral-replication processes. In this study, the normal shrimp were challenged with various amounts of rSPI*Pm*5 along with WSSV or YHV injection and collected the hemolymph at 24 h after the two viral challengeing. The expression of *VP28* or *YHV* genes was analyzed by RT-PCR. The results showed that the expression of *VP28* and *YHV* genes were down-regulated after injection with the higher amounts of rSPI*Pm*5. The SPI*Pm*5 affected the *VP28* more than *YHV* gene expression (**Fig. 3.26**).

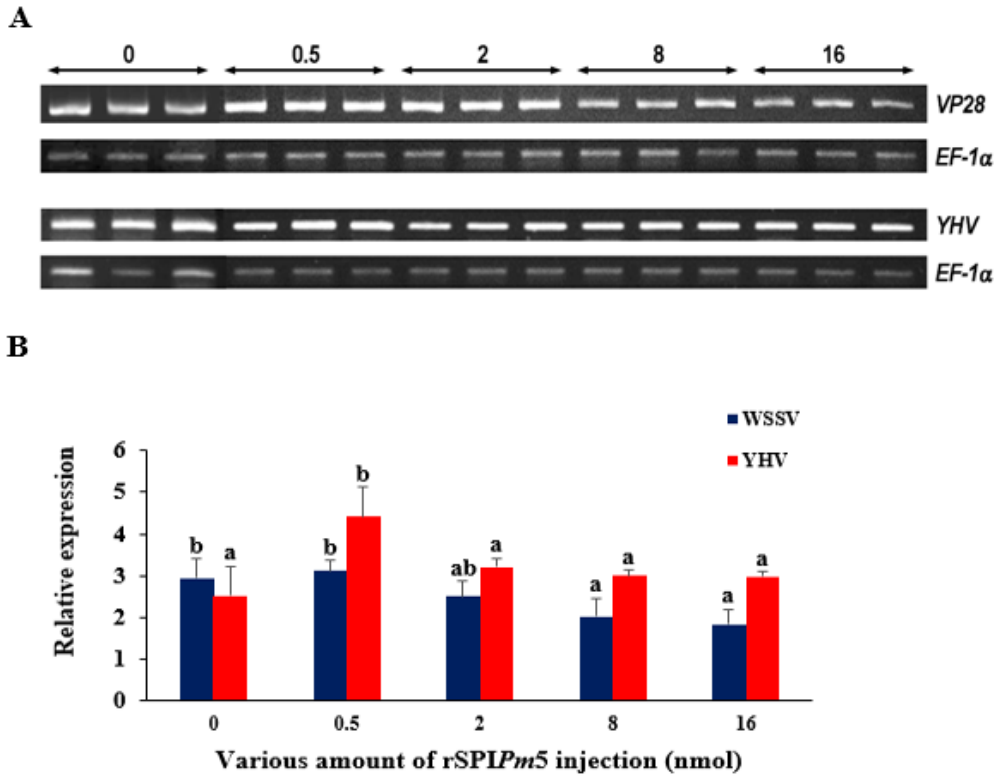

Figure 3.26. To increase the dose dependent of rSPI*Pm*5 response to the viral infection. (A) The 0.5, 2, 8 and 16 nmol of rSPI*Pm*5 plus WSSV and YHV dilution were injected into the shrimp. The hemolymph were collected at 24 h after post injection. The *VP28* and *YHV* gene expression were investigated by semi-quantitative RT-PCR and investigated the target genes by agarose gel electrophoresis. (B) The relative expression of *VP28* and *YHV* were analyzed. *EF-1α* was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

4.9. rSPI*Pm*5 reduced the growth of *Vibrio* bacteria in shrimp

From the *Vibrio* infection in the survival experiment, the rSPI*Pm*5 injection delayed the mortality rate of *Vibrio*-infected shrimp. The SPI*Pm*5 could reduce the bacterial growth. In this study, the VP_{AHPND} and *V. harveyi* in TSB culture were tested along with the 0.5 and 5 nmol amount of rSPI*Pm*5 and also detected the growth of bacteria with spectrophotometer by measuring the OD_{600} every 1 h up to 4 h. The results showed that the rSPI*Pm*5 protein did not act alone in inhibiting the growth of VP_{AHPND} and *V. harveyi*. The rSPI*Pm*5 inhibition activity did not directly against the two *Vibrio* bacteria.

For the bacterial clearance assay, the inhibitory activity of SPI*Pm*5 injection were tested along with two *Vibrio* strains infection. The normal shrimp were injected with the various amount of rSPI*Pm*5 plus *V. harveyi* and VP_{AHPND} immersion. The hemolymph were collected from the bacterial infected-shrimp at various times: 5, 15, 30, 60 and 180 min. The hemolymph was diluted, aliquoted and dropped on TCBS agar plates. The colonies were counted. The results showed that the numbers of colonies were increased at various times but decreased with the higher amount of rSPI*Pm*5 injection. The colonies of *V. harveyi* decreased more than VP_{AHPND} infection after the infected-shrimp were treated along with rSPI*Pm*5. The *V. harveyi* was more sensitive to the inhibitory activity of SPI*Pm*5 protein than VP_{AHPND} (**Fig. 3.27**).

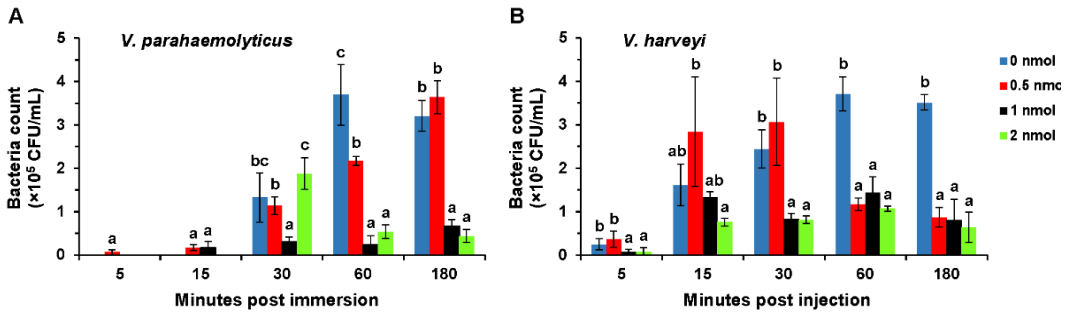

Figure 3.27. The bacterial clearance assay from the hemolymph of *Vibrio*-infected shrimp on TCBS agar. The normal shrimp were injected with 0, 0.5, 1 and 2 nmol amount of rSPI*Pm*5 plus *V. harveyi* (B) and VP_{AHPND} immersion (A). The hemolymph were collected at 5, 15, 30, 60 and 180 min after post injection. The number colonies of two *Vibrio*-bacteria were counted on TCBS agar plates. The experiment was done in triplicate. The results are means ± SDs. Significant differences are accepted at $P < 0.05$.

4.10. Expression of AMP genes in SPI*Pm*5- and bacteria-injected shrimp

The bacterial clearance assay concluded that the growth of *V. harveyi* and VP_{AHPND} infection were inhibited by SPI*Pm*5. The effect of SPI*Pm*5 was tested as early as 60 min after challenging with the *V. harveyi* injection and VP_{AHPND} immersion. Both bacterial growths were reduced. It might be a result from the SPI*Pm*5 protein and its inhibitory activity that induced the expression of AMP genes. Their AMP expression might inhibit the growth of *Vibrio*-bacteria.

In this study, the normal shrimp were injected with various amounts of rSPI*Pm*5 plus *V. harveyi* or VP_{AHPND} immersion. The hemolymph was collected at 1 h for the detection of the AMP gene expression by RT-PCR. The results showed that the expression of AMP genes: *ALFPm3*, *crustinPm1*, *crustinPm7*, *penaeidin3*, p*enaeidin5* and *lysozyme*, was down-regulated compared with the

control groups (**Fig. 3.28**). It showed that the SPI*Pm*5 did not activate the AMP gene expression but it might influence other immune defense system to protect the infected-shrimp.

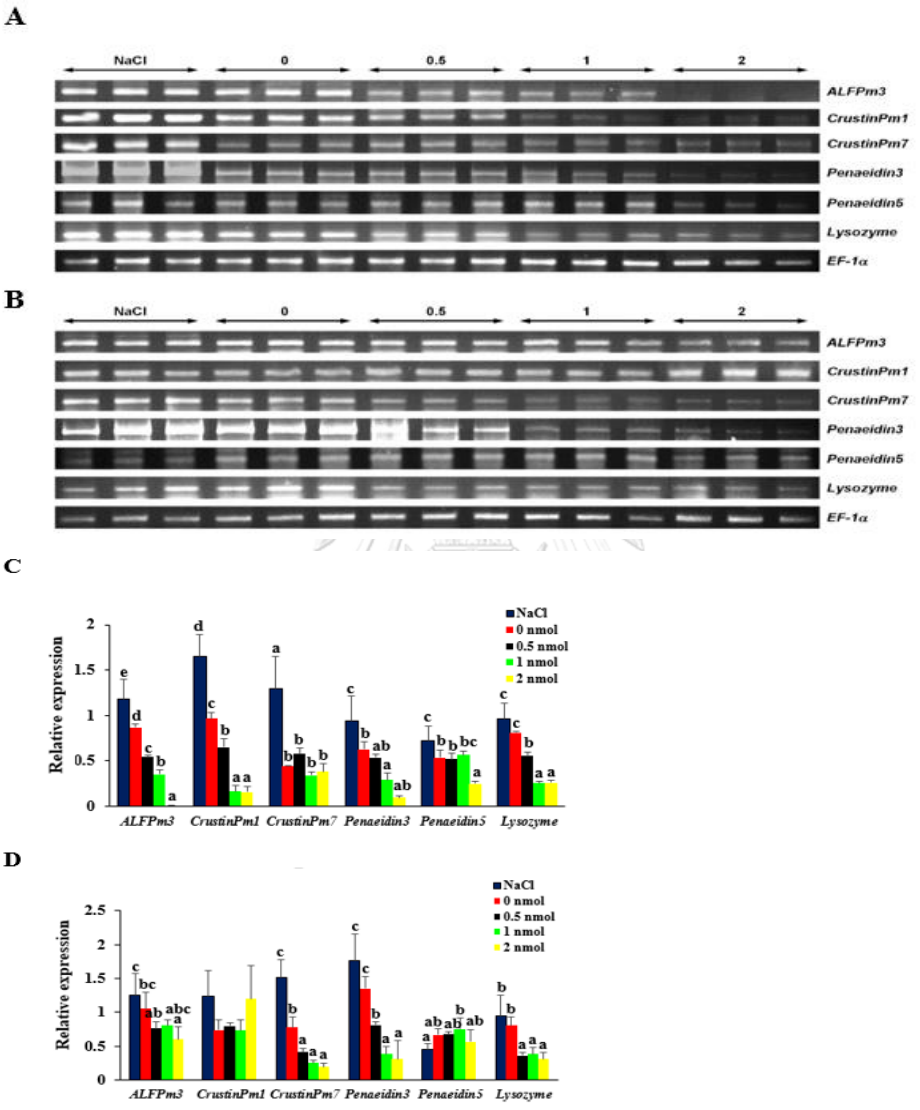

Figure 3.28. The AMPs gene expression were detected after challenge with the rSPI*Pm5* plus *V. harveyi* injection (A) and VP_{AHPND} immersion (B). The hemolymph were collected at 1 h. The gene expression of AMPs, *ALFPm3*, *crustinPm1*, *crustinPm7*, *penaeidin3*, *penaeidin5* and *lysozyme*, were investigated by RT-PCR. (C, D) The relative genes expression was analyzed from *V. harveyi*- and *V. parahaemolyticus*-infected shrimp, respectively. *EF-1α* gene was used as an internal control. Letters indicate significant differences above the control at $P < 0.05$.

CHAPTER IV

DISCUSSION

The pathogen infection causes the major problem in shrimp aquaculture. After the microorganism invasion in shrimp, the innate immune system is activated. The hemocyte tissues store and secrete many types of immune components for fighting the pathogen infection (Rosa & Barracco, 2010; Tassanakajon *et al.*, 2013). The infected cells are involved in phagocytosis, nodule formation, encapsulation, wound healing, clotting and proPO activation system. In addition, they also induce the production of antimicrobial peptides (AMPs), proteinase inhibitors and cytokine-like protein (Cerenius & Soöderhäll, 2004; Iwanaga & Lee, 2005; Tassanakajon *et al.*, 2013). These immune components in the hemocytes can inhibit the pathogen.

For the shrimp immune major pathway, Toll and Imd pathway, that response to the pathogen infection. The Toll pathway responds to Gram-bacteria and fungi whereas the Imd pathway responds to Gram-negative pathway (Li *et al*., 2013). Herein, we focus on Spätzle protein activation to Toll pathway. After challenge with the pathogen, the signaling infection recognizes by pattern recognition protein (PRP) and the unknown protease cascades were activated and cleaved the proSpätzle protein into active Spätzle protein. Its active protein binds to the Toll receptor, transmit the infection signal into the nucleus and activate the synthesis of AMPs gene for inhibition the growth of invader (Jang *et al*., 2006; Arnot *et al*., 2010).

For another protein, several types of serine proteinase inhibitors are required for many biological process innate immunity. They control the various proteinase-mediated biological processes such as complementation system, blood coagulation melanization and apoptosis (Iwanaga *et al*., 2005; Jiravanichpaisal *et al*., 2006). Their gene expression are also increased and in

response to the pathogen infection. For the KPIs in *P. monodon*, the 9 types of KPIs were identified from the EST database. The SPI*Pm*5 is one of KPIs, its gene expression responds to the viral infection and heat stress temperature (Visetnan *et al*., 2009; Pongsomboon *et al*., 2011).

In this research, we focus on a Spätzle protein and a Kazal type serine proteinase inhibitor. The Spätzle protein relays the infection signal through the Toll pathway into the nucleus for the synthesis of AMPs. For the Kazal type serine proteinase inhibitor, SPI*Pm*5, we investigated its possible function in black tiger shrimp for it might regulate the immune defense system. Spätzle is an extracellular protein. Its active domain activates the Toll pathway for the embryonic dorsal-ventral patterning and the antimicrobial peptide proteins synthesis (Jang *et al*., 2006). The Spätzle proteins from several arthropod have been studied, for example *Drosophila* (*Dm*Spz1) (Weber *et al*., 2003), *M. sexta* (Spz1A) (An *et al*., 2010), *L. vannamei* (*Lv*Spz1, *Lv*Spz2 and *Lv*Spz3) (Wang *et al*., 2012), *B. mori* (*Bm*Spz1) (Wang *et al*., 2007) and *F. chinensis* (*Fc*Spz) (Shi *et al*., 2009), etc. All *Spätzle* genes isoforms from *P. monodon* have not been identified before.

Three isoforms of *LvSpz* genes have been characterized from *L. vannamei* (Wang *et al*., 2012). The *Lv*Spz amino acid sequences are closely related to those of *Pm*Spz. There may be three isoforms equivalent to the *LvSpz* genes. The *PmSpz* genes were analysed from the *P. monodon* EST database and Genbank (accession no. KM225226) and shown to be equivalent to the *LvSpz1* and *3* genes. For the *PmSpz2* gene, the specific primers were designed from *LvSpz2* gene sequences. The *PmSpz2* gene showed less expression in hemocytes but highly in gill tissues. The results concluded that there were three isoforms of *Spätzle* gene in *P. monodon*, namely, *PmSpz1*, *2* and *3*.

From previous study, the expression of *PmSpz1* gene was increased in response to Gram-positive bacteria and fungi infection. For this research, the

PmSpz1 transcript was amplified by RT-PCR to show that it was expressed in all tissues tested and also in response to the WSSV infection. Like the *P. monodon PmSpz1*, the gene expression of *FcSpz* gene, equivalent to *LvSpz3* gene, is also detected in all tissues tested and in response to WSSV and *V. anguillarum* (Shi *et al.,* 2009). In the oak silkworm, *Antheraea pernyi*, *ApSpz* transcripts are up-regulated after infection with fungus and Gram-positive bacteria, *Enterococcus pernyi* but not Gram-negative bacteria, *E. coli* (Sun *et al.,* 2016).

It is believed that the *Pm*Spz1 protein is synthesized in the hemocytes. It is secreted into the blood circulation of shrimp as pro-protein and, upon infection, is cleaved to active Spätzle protein. In this study, the *Pm*Spz1 protein in plasma was investigated by Western blot analysis. The result is interesting that the large amount of active *Pm*Spz1 protein was found in hemocytes lysate and plasma, even at zero-time point of WSSV infection. Small amount of the *Pm*Spz1 pro-protein was detected in the plasma. The presumed active *Pm*Spz1 protein detected might not be ready for the Toll activation process. We hypothesized that to activate the Toll pathway in infected shrimp, the certain factor(s) from pathogen infection might be needed for the ac*Pm*Spz1 and Toll receptor binding.

The open reading frame and encoded amino acid sequences of *Pm*Spz1 clone was analyzed. The *Pm*Spz1 protein was 237 amino acids in length with 25 amino acids of signaling peptide. The *Pm*Spz1 mature protein had a Spätzle conserved domains at its C-terminal amino acid sequence position 116-208. The Spätzle cleavage sites of insects have been reported. The Spätzle was cleaved by the serine protease Easter during dorsal-ventral patterning and Spz-processing enzyme after the pathogen infection. The active domain of Spätzle protein activates the Toll signaling pathway (Ferrandon *et al.,* 2004; F. Li & Xiang, 2013). In this study, the alignment of Spätzle amino acid sequences of

*Pm*Spz1, *Dm*Spz1, *Ms*Spz1A and *Bm*Spz1 were done to predict the cleavage site of *Pm*Spz1. The cleavage site was identified at the C-terminal of His109 of *Pm*Spz1. The *Pm*Spz1 cleavage site is equivalent to Arg195, Arg169 and Arg152 of *Dm*Spz1, *Ms*Spz1A and *Bm*Spz1, respectively. The Spätzle proteins in other arthropods are supposedly cleaved by trypsin-like enzymes but the *Pm*Spz1 was not cleaved by trypsin-like enzyme.

The mature and active domain of *Pm*Spz1 proteins were over-produced in the *E. coli* host system for study their activities. In the survival experiment, the purified r*Pm*Spz1 protein was tested for the inhibitory activity against WSSV infection. The results showed that the r*Pm*Spz1 could prolong the life of WSSV-infected shrimp about 50% up to ten days. It was possible that the r*Pm*Spz1 injection activated the AMP synthesis against the WSSV infection. For *Drosophila*, the *Dm*Spz1 mature protein induces the synthesis of drosomycin in the downstream signaling process (Weber *et al.*, 2003).

For the active Spätzle protein, it could activate the antimicrobial proteins, for example, the active form of *Drosophila*, *Dm*Spz C-106, induces the drosomysin gene expression bypassing the immunity challenge (Weber *et al.*, 2003). Like *Drosophila*, Chinese shrimp, the *Fc*Spz1 C-114 induces the expression of *crustin2* gene but not *lysozyme*, *astacidin* and *crustin1* genes in crayfish (Shi *et al.*, 2009). For silkworm, the active *Bm*Spz1 protein activates the expression of eight AMP genes, *attacin-2*, *cecropin-A1*, *-B1*, *-D1*, *gloverin-A5*, *-B*, *lebocin-3* and *moricin-A1* (Wang *et al.*, 2007). In this study, the rac*Pm*Spz1 protein was injected into the shrimp to test its activity to induce the expression of AMP genes. All but *penaeidin5* showed the up-regulation of gene expression under the Toll pathway. From previous studies, the *penaeidin3* was up-regulated partly by the regulation of the Toll and Imd pathways whereas the *penaeidin5* was expressed under the Imd pathway (Visetnan *et al.*, 2009). The expression of *ALFPm3* gene was not under the control of the Imd pathway but this study was up-regulated under the Toll pathway. For *crustinPm1* and *7*

genes, Arayamethakorn (2007) found that the *crustinPm1* gene was expressed under the Toll pathway whereas the *crustinPm7* was under both the Toll and Imd pathways.

In crustaceans, the Kazal-type serine proteinase inhibitors (KPIs) are involved in the innate immune system against the pathogen infection because their trnascripts are changed in response to the microbial infection. From previous study, the KPIs from other arthropod have been reported that their gene expression are up-regulated in response to the invasion and the antimicrobial proteins was also induced, for example, the *Fc*SPI in Chinese shrimp (Kong *et al.*, 2009; Wang *et al.*, 2009), *hc*PcSPI1-4 in freshwater crayfish (Li *et al.*, 2009; Li *et al.*, 2010), *Pt*KPI in swimming crab (Wang *et al.*, 2012) and SPI*Pm*2 in black tiger shrimp (Donpudsa *et al*., 2009).

The SPI*Pm*5 protein is a two-domain KPIs from *P. monodon*. From previous studies, the *SPIPm5* transcripts were up-regulated in heat stress and response to YHV infection. The SPI*Pm*5 had proteinase inhibitory activity but lack bacteriostatic effect against bacteria. It also inhibits subtilisin and elastase. In this study, the expression of *SPIPm5* gene was shown to express in all shrimp tissues tested and its gene expression is up-regulated by heat stress, WSSV and YHV. In addition, the rSPI*Pm*5 protein was over-produced in *E. coli* host system for testing the antiviral and antibacterial activities.

Since the *SPIPm5* gene expression was up-regulated under heat stress, the rSPI*Pm*5 injection into the normal shrimp was considered to mimic the heat stress condition. The rSPI*Pm*5 injection might also affect the AMP transcripts and the related-innate immune genes in shrimp. The results showed that the rSPI*Pm*5 injection had more or less no effect on the expression of *penaeidin3*, *penaeidin5*, *SPIPm2* and *SPIPm5* genes. The expression of *crustinPm1* showed slightly up-regulated and that of *hsp70* was down-regulated. For the effect on proPO system, the various amounts of rSPI*Pm*5 was tested with hemolymph

(Ponprateep *et al.*, 2017). The results suggested that the proPO system was inhibited. It meant that the SPI*Pm*5 could control the proPO system.

The rSPI*Pm*5 protein was tested in the survival experiments. From previous study, the expression of *SPIPm5* gene was induced after infection with WSSV and YHV in shrimp. The expression of *SPIPm2* gene was also increased in heat stress (Tassanakajon *et al.*, 2006). The heat stress and the viral-infection were tested with the various amount of rSPI*Pm*5 in shrimp. The results showed that the infected shrimp could prolong life of shrimp more than the infected shrimp control groups. The rSPI*Pm*5 protein was also tested with the *Vibrio*-infected shrimp, *V. harveyi* injection and VP_{AHPND} immersion like the viral infection. The results showed that the *Vibrio*-infected shrimp also survive longer more than the control groups after the rSPI*Pm*5 protein injection.

Since the rSPI*Pm*5 showed the inhibitory activity to the viral and bacterial infected shrimp, it might inhibit the replication of viruses and bacterial growth. In this study, the various amounts of rSPI*Pm*5 were tested with the microbial infection in shrimp. The results showed that their replication was decreased after increasing the amounts of rSPI*Pm*5 injection. For testing the inhibitory activity, the rSPI*Pm*5 was not directly against the microbial growth *in vitro* (data not shown). The rSPI*Pm*5 activity might somehow activate the innate immune system such as AMP genes expression or other defense systems for protection of the infected shrimp via two main signaling pathways, Toll and Imd (Arayamethakorn *et al.*, 2017; Visetnan *et al.*, 2015). The rSPI*Pm*5 injection might activate the AMP genes expression. The results were surprising for the expression of AMP genes in infected-shrimp showed down-regulation after the various amounts of rSPI*Pm*5 injection increased. The results might indicate that the rSPI*Pm*5 protein activates the other innate immune system against the microbial infection. Thus, the SPI*Pm*5 plays crucial

roles in the innate immunity of shrimp besides its function as a proteinase inhibition (Visetnan *et al.,* 2009).

CHAPTER V

CONCLUSION

Spätzle is an extracellular protein in innate immune system. Its infection signaling relays through the Toll pathway into nucleus for the synthesis of antimicrobial proteins. Three *Pm*Spätzle isoforms are identified in *Penaeus monodon*. The *PmSpz1*, *2* and *3* are equivalent to *LvSpz1*, *2* and *3*, respectively. The *PmSpz1* and *3* were expressed fairly common in the hemocytes whereas the *PmSpz2* gene expression was hardly detected in hemocyte tissue but only in gill could it be detected. The *PmSpz1* gene was shown to express in all shrimp tissues tested and its gene expression is up-regulated by WSSV infection. The *Pm*Spz1 in hemolymph was found to mostly exist as an active form awaiting to activate the Toll pathway whereas the pro-protein was decreased in plasma as the infection progressed. The mature r*Pm*Spz1 and its active domain were over-produced in an *E. coli* expression system for activity studies. The r*Pm*Spz1 protein was tested for its function by injecting along with WSSV. It was found that the r*Pm*Spz1 protein rendered the shrimp less susceptible to the WSSV infection. The active r*Pm*Spz1 protein was also tested its activity to up-regulate the synthesis of AMPs. The rac*Pm*Spz1 protein activates the synthesis of *crustinPm1*, *crustinPm7*, *ALFPm3*, *penaeidin3* but not *penaeidin5*. It meant that the expression of all antimicrobial proteins but not *penaeidin5* was under the regulation of Toll pathway.

For another activated immune protein, the SPI*Pm*5 is a two domain KPI with the P1 residues Leu and Thr capable of inhibiting subtilisin and elastase. The *SPIPm*5 gene was shown to expression in all shrimp tissues tested and its gene expression is up-regulated by heat stress, WSSV and YHV. Injection of rSPI*Pm*5 protein had more or less no effect on the expression of *crustinPm1*, *penaeidin3*, *penaeidin5*, *Hsp70*, *SPIPm2* and *SPIPm5* genes. Like some other proteinase inhibitors, the rSPI*Pm*5 could inhibit the hemolymph proPO

activity. In survival experiment, the protective effect of SPI*Pm*5 prolongs the life of microbial infected shrimp. The increased endurance against microbial infection was due to the inhibitory effects presumably activated by rSPI*Pm*5 on viral replication and bacterial growth but not the expression of antimicrobial peptides. Therefore, the SPI*Pm*5 plays an important role in shrimp innate immunity against the viral and bacterial infection.

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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% Stacking gel

H_2O	2.7	ml
30%(w/v) Acrylamide solution	0.67	ml
1.5 M Tris (pH 6.8)	0.5	ml

10% SDS	0.04	ml
10% Ammonium persulfate	0.04	ml
TEMED	5	µl

5× Sample buffer

1.5 M Tris (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)-aminomethen	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

PUBLICATIONS

1. **Boonrawd S**, Mani R, Ponprateep S, Supungul P, Masrinoul P, Tassanakajon, Rimphanitchayakit V. (2017). Characterization of *Pm*Spätzle 1 from the black tiger shrimp *Penaeus monodon*. *Fish and Shellfish Immunology*, 65: 88–95.
2. **Boonrawd S**, Supungul P, Tassanakajon, Rimphanitchayakit V. (2018). Antimicrobial activity of a serine proteinase inhibitor SPI*Pm*5 from the black tiger shrimp *Penaeus monodon*. *Fish and Shellfish Immunology*, 77: 147–155.

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