ปฏิสัมพันธ์ของแอนติลิโพพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 จากกุ้งกุลาดำ *Penaeus monodon* กับโปรตีนจากเชื้อไวรัสตัวแดงดวงขาว

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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INTERACTION OF ANTI-LIPOPOLYSACCHARIDE FACTOR ISOFORM 3 FROM BLACK TIGER SHRIMP *Penaeus monodon* WITH WHITE SPOT SYNDROME VIRUS PROTEINS

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ธนชัย เมธาธรรม : ปฏิสัมพันธ์ของแอนติลิโพพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 จากกุ้งกุลาดำ *Penaeus monodon* กับโปรตีนจากเชื้อไวรัสตัวแดงดวงขาว. (INTERACTION OF ANTI-LIPOPOLYSACCHARIDE FACTOR ISOFORM 3 FROM BLACK TIGER SHRIMP *Penaeus monodon* WITH WHITE SPOT SYNDROME VIRUS PROTEINS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. กุลยา สมบูรณ์วิวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. อัญชลี ทัศนาขจร, 136 หน้า.

เพปไทด์ต้านจุลชีพมีบทบาทสำคัญในการต่อสู้เชื้อจากจุลชีพ แอนติลิโพพอลิแซกคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 (ALF*Pm*3) เพปไทด์ต้านจุลชีพที่ถูกค้นพบในกุ้งกุลาดำ *Penaeus monodon* เป็นเพียงชนิดเดียวเท่านั้นที่สามารถต่อต้านเชื้อไวรัสตัวแดงดวงขาว (WSSV) ได้ อย่างไรก็ตามกลไกที่เกี่ยวข้องกับการต่อต้านเชื้อไวรัสของ ALF*Pm*3 ยังไม่ทราบอย่างแน่ชัด ดังนั้นเพื่อให้เกิดความเข้าใจเกี่ยวกับการตอบสนองต่อเชื้อไวรัสตัวแดงดวงขาวของโปรตีน ALF*Pm*3 ในงานวิจัยก่อนหน้านี้ได้ใช้เทคนิค Yeast two-hybrid assay ในการจำแนกโปรตีนของเชื้อไวรัสตัวแดงดวงขาว ได้แก่ โปรตีน WSSV186 WSSV189 WSSV395 WSSV458 และ WSSV471 ที่สามารถเกิดปฏิสัมพันธ์กับโปรตีน ALF*Pm*3 ได้ และยืนยันการเกิดปฏิสัมพันธ์ระหว่างโปรตีนรีคอมบิแนนท์ ALF*Pm*3 (rALF*Pm*3) กับโปรตีนรีคอมบิแนนท์ WSSV189 (rWSSV189) และ WSSV471 (rWSSV471) ด้วยเทคนิค *in vitro* pull-down แล้วและในการศึกษานี้ได้ยืนยันปฏิสัมพันธ์ระหว่างโปรตีน rALF*Pm*3 กับโปรตีนรีคอมบิแนนท์ WSSV458 (rWSSV458) ด้วยเทคนิค *in vitro* pull-down เช่นเดียวกัน และเมื่อบ่มโปรตีนรีคอมบิแนนท์ของไวรัส (rWSSV189, rWSSV458, rWSSV471) กับโปรตีน rALF*Pm*3 พบว่าการจับของโปรตีนรีคอมบิแนนท์ของไวรัสไปรบกวนแอคติวิตีในการต่อต้านเชื้อไวรัสตัวแดงดวงขาวของโปรตีน rALF*Pm*3 ซึ่งดูได้จากเปอร์เซ็นต์การรอดชีวิตของกุ้งกุลาดำกลุ่มที่ถูกฉีดด้วยเชื้อไวรัสตัวแดงดวงขาวที่บ่มกับโปรตีน rALF*Pm*3 ซึ่งผสมกับโปรตีนรีคอมบิแนนท์ WSSV แต่ละตัวที่ลดลงเมื่อเปรียบเทียบกับกุ้งกลุ่มที่ถูกฉีดด้วยเชื้อไวรัสตัวแดงดวงขาวที่บ่มกับโปรตีน rALF*Pm*3 เพียงอย่างเดียว อย่างไรก็ตามข้อมูลของโปรตีน WSSV189, WSSV458 และ WSSV471 ยังมีจำกัด ในที่นี้จึงศึกษาการแสดงออกของโปรตีน WSSV189, WSSV458 และ WSSV471 ในเหงือกและในเม็ดเลือดของกุ้งที่ติดเชื้อไวรัสตัวแดงดวงขาว ซึ่งพบว่ามีการแสดงออกของโปรตีนไวรัสทุกตัวเพิ่มขึ้นเมื่อติดเชื้อไวรัสตัวแดงดวงขาว ก่อนหน้านี้มีรายงานว่าโปรตีน WSSV458 เป็นโปรตีนโครงสร้างในส่วน tegument ของเชื้อไวรัสตัวแดงดวงขาว ในการศึกษานี้ใช้เทคนิค Western blot ศึกษาหาตำแหน่งของโปรตีน WSSV189 และ WSSV471 บนเชื้อไวรัสตัวแดงดวงขาวและบนโปรตีนของเชื้อไวรัสตัวแดงดวงขาวส่วน envelope และส่วน nucleocapsid โดยผลการทดลองแสดงว่าโปรตีนของไวรัสทั้งสองชนิดนี้อยู่ในส่วนของ envelope และเทคนิค Immunoelectron microscopy ซึ่งใช้แอนติบอดีที่จำเพาะต่อ WSSV189 และ WSSV471 ยืนยันตำแหน่งโปรตีนของไวรัสทั้งสองชนิดในส่วน envelope ของเชื้อไวรัสตัวแดงดวงขาว จากผลการทดลองแสดงว่ากลไกในการต่อต้านเชื้อไวรัสตัวแดงดวงขาวของโปรตีน ALF*Pm*3 เกี่ยวข้องกับการจับกับโปรตีนโครงสร้างของเชื้อไวรัสตัวแดงดวงขาว ได้แก่ WSSV189, WSSV458, WSSV471 และอาจจะรวมถึงโปรตีนของไวรัสชนิดอื่นๆ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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THANACHAI METHATHAM: INTERACTION OF ANTI-LIPOPOLYSACCHARIDE FACTOR ISOFORM 3 FROM BLACK TIGER SHRIMP *Penaeus monodon* WITH WHITE SPOT SYNDROME VIRUS PROTEINS. ADVISOR: ASST. PROF. KUNLAYA SOMBOONWIWAT, Ph.D., CO-ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., 136 pp.

Antimicrobial peptides (AMPs) play a vital role in combating microbial pathogens. Among AMPs identified in *Penaeus monodon*, only anti-lipopolysaccharide factor isoform 3 (ALF*Pm*3) has been reported to exhibit activity against white spot syndrome virus (WSSV); however, the mechanism(s) involved are still not clear. To better understand the ALF*Pm*3 function in WSSV response, ALF*Pm*3-interacting proteins including WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471 proteins from WSSV have been previously identified by yeast two hybrid assay. *In vitro* pull-down assay has been confirmed the interactions between the recombinant ALF*Pm*3 protein (rALF*Pm*3) and the recombinant WSSV189 (rWSSV189) and recombinant WSSV471 (rWSSV471) proteins. In this study, the binding of the rALF*Pm*3 protein and the recombinant WSSV458 protein (rWSSV458) was also confirmed. Pre-incubation of rWSSV189, rWSSV458, rWSSV471 proteins with the rALF*Pm*3 protein interfered the neutralization effect of the rALF*Pm*3 protein on WSSV *in vivo* was revealed. The decrease in the % survival of shrimp injected with WSSV pre-treated with the mixture of each rWSSV protein and rALF*Pm*3 protein compared to those injected with WSSV pre-treated with the rALF*Pm*3 protein only, was observed. However, the information on WSSV189, WSSV458 and WSSV471 proteins are limited. Here, the expression of the WSSV189, WSSV458 and WSSV471 proteins were determined in gills and hemocytes of WSSV-infected shrimp and the increase in their expression upon WSSV infection was observed. WSSV458 has been identified recently as a WSSV tegument protein. In the present study, Western blot analysis was employed to study localization of WSSV189 and WSSV471 proteins in the WSSV virion, fractions of envelope and nucleocapsid proteins of WSSV. The results indicated that both of them were envelope proteins. Immunoelectron microscopy using WSSV189 and WSSV471 specific antibodies was also performed and approved the location of both WSSV proteins on the WSSV envelope. Taken together, the results indicated that the ALF*Pm*3 performs its anti-WSSV action by binding to the WSSV structural proteins, WSSV189, WSSV458 and WSSV471 proteins and possibly others.

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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Shrimp aquaculture in Thailand

Thailand is one of the top countries in shrimp aquaculture production because the Thai shrimp product shared up to 75% of the world shrimp trade (Source: FAO databases, 2007). In Thailand, the shrimp species mainly produced are the black tiger shrimp (*Penaeus monodon*) and the white shrimp (*Litopenaeus vannamei*). Since 1990, Thailand had been the world leader in exporting shrimp production and became the biggest exporter of the black tiger shrimp. The industry has been valued approximately 300,000-400,000 metric tons annually providing an income of about 85,000 million bath yearly for the country (Source: Office of Agricultural Economics in cooperation with the Customs Department). The increase of shrimp farming came together with several problems on culture of shrimp such as the waste water from the farm, the outbreaks of shrimp diseases (Mohan et al., 1998). The black tiger shrimp production in Thailand has rapidly decrease since 2006 because it seriously affected by the disease outbreaks mostly caused by viruses such as white spot syndrome virus (WSSV), Taura syndrome virus (TSV) and yellow head virus (YHV) (Flegel, 2006) and *Vibrio* bacteria (Bachère et al., 2000). So, the production of the black tiger shrimp in Thailand was substantially dropped while the western white shrimp, *L. vannamei* has become more popular than black tiger shrimp. The

white shrimp species, a genetically improved species, has become an alternative shrimp species owing to its specific pathogen resistance over the black tiger shrimp. Moreover, it has rapid growth, high stocking density tolerance, low salinities and temperatures tolerance. However, the *L. vannamei* may have certain disadvantages because it is a non-native species for Thailand. Its broodstocks must be imported from the Hawaii Marine Institute. From these reasons, the shrimp farming of the native rather than the non-native species should be considered as necessary for Thailand. However, the black tiger shrimp; *P. monodon* is sill more advantageous because they are fast growing and tolerant to a wide range of salinity. Since 2012, the outbreak of the early mortality syndrome or Acute hepatopancreatic necrosis syndrome (AHPNS) in Thailand, caused the decrease of shrimp production more than 50% (200,000 metric tons to about 90,000 tons). As a result, the volume of Thai shrimp exports dropped by 30-40%. (Source: Thai Frozen Food Assn President). In this case, both *P. monodon* and *L. vannamei* were also affected by EMS/AHPNS outbreaks. So far, the knowledge on this new emerging disease is limited. Therefore, the outbreaks are uncontrollable. Not only the new emerging disease, virus infection caused by WSSV is still a major problem. Therefore, to maintain the shrimp farming industry in Thailand, the understanding of shrimp immunity and development of agriculture technology should be intensively studied.

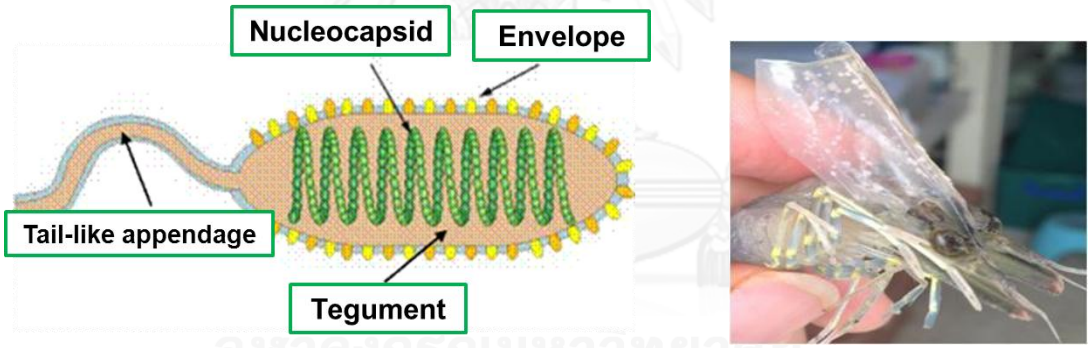
1.2 White spot syndrome disease

The major causes of shrimp diseases are mainly by viral pathogens. There are 7 families of viral pathogens including Parvoviridae, Bacuroviridae, Iridoviridae, Picornaviridae, Rhabdoviridae, and Togaviridae identified in penaeid shrimp (Alvarez et al., 1998; Jittivadhna, 2000). The WSSV and YHV are the major of viral pathogens of *P. monodon* (Boonyaratpalin et al., 1993; Wongteerasupaya et al., 1995a). WSSV has caused severe mortality and big economic losses to the shrimp aquaculture and shrimp farming industry for more than a decade (Escobedo-Bonilla et al., 2008; Lo et al., 2005) . In this study, we thus focused only on WSSV.

WSSV is a bacilliform, non-occluded enveloped large circular double stranded DNA virus containing about 292 to 305 kb for the Thailand and China isolates, respectively (Huang et al., 2005; van Hulten et al., 2001; Yang et al., 2001). The WSSV virions range between 210 and 380 nm in length and 70-167 nm in maximum width are symmetrical and ellipsoid to bacilliform in shape similar to baculovirus. The structure of intact virion consist of envelope, tegument, nucleocapsid and tail-like appendage (Fig. 1.1). The viral envelope is 6-7 nm thick and has the structure of an obviously lipidic bilayer membrane. The nucleocapsid are tightly packed within the virion and composed of globular protein subunit. The intact virion is a large enveloped rod-shaped particle (Inouye and Kawata, 1994; van Hulten et al., 2001; Wang et al., 1995).

WSSV was first discovered in Taiwan in 1992 (Chou et al., 1995). In 1994, WSSV was reported in Thailand (Lo et al., 1996). The WSSV infection in shrimp has a 100% mortality rate within 7-10 days and results in enormous economic losses in the shrimp farming industry (Flegel, 1997; Lightner, 1996a). The WSSV has a wide host range of decapods crustaceans including shrimp, crab and crayfish (Wang et al., 1998; Zhang et al., 2004b) and more than 93 species of arthropods have been reported as hosts or carriers of WSSV (Flegel and Alday-Sanz, 1998; Flegel, 1997; Lo et al., 1996). WSSV infects and causes disease in many shrimp species worldwide including *P. monodon*, *L. vannamei*, *Marsupenaeus japonicas*, *Feneropenaeus chinensis* and *Litopenaeus stylirostris.* In the host, WSSV infects a wide variety of cells from ectodermal and mesodermal origin. Histological changes are seen in the gill epithelium, antennal gland, haematopoeitic tissue, nervous tissue, connective tissue and intestinal epithelial tissue. Infected cells have prominent intranuclear occlusions that initially stain eosinophilic, but become basophilic with age; hypertrophied nuclei with chromatin margination; and cytoplasmic clearing (Wongteerasupaya et al., 1995b). Pathogenesis involves widespread tissue necrosis and disintegration. White spots on the shell of infected shrimp under scanning electron microscope appear as large, dome-shaped spots on the carapace measuring 0.3 to 3 mm in diameter. Smaller white spots of 0.02 to 0.1 mm appear as linked spheres on the cuticle surface. Chemical composition of the spots is similar to the

carapace, calcium forming 80–90% of the total material and it is suggested to have derived from abnormalities of the cuticular epidermis (Wongteerasupaya et al., 1995a). Clinical signs of the disease are a rapid reduction in food consumption, lethargy, a loose cuticle, a pink to reddish-brown discoloration and appearing white spots of 0.5-2 nm on the carapace of the cephalothorax (Chou et al., 1995; Lightner, 1996b). The virus severely damages the stomach, gills, subcuticular epithelial cells, lymphoid organs, antennal gland, and hemocyte (Lightner, 1996a; PohShing et al., 1996).



(Source: www.vetres.org)

Figure. 1.1 White spot syndrome virus (WSSV) virion.

The morphogenesis of WSSV and route of virus entry have been characterized and are directly related to the development of cellular lesions (Durand et al., 1997; Escobedo-Bonilla et al., 2008; Tsai et al., 2006; Wang et al., 1999). They have several stages of development (Fig. 1.2). At the early stage, the host cells invaded by WSSV

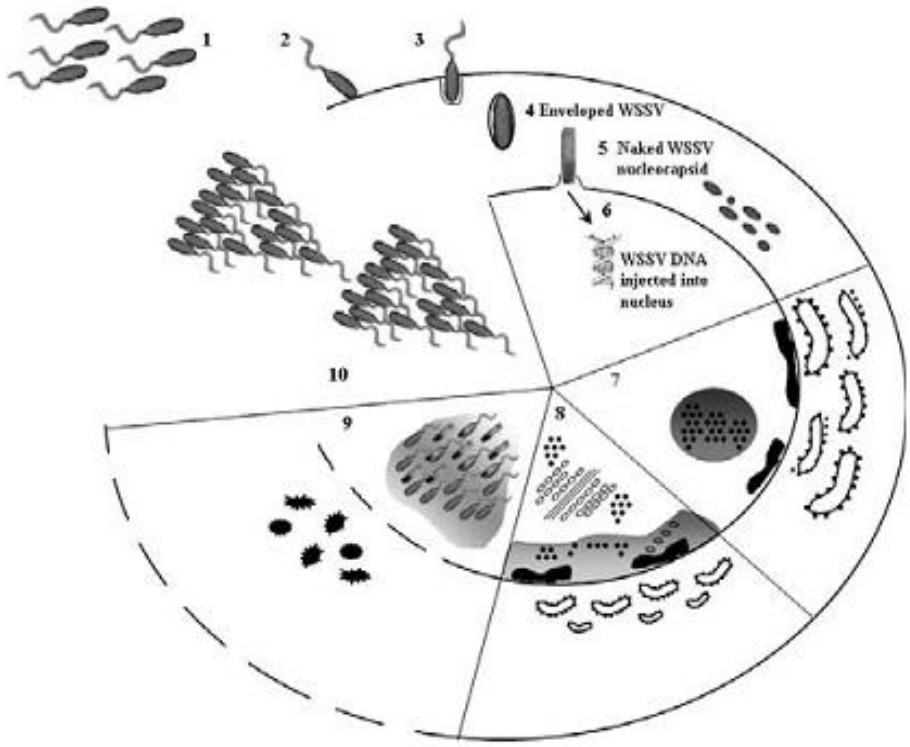
particles. Then, the virion attaches to a susceptible cell using its envelope proteins binding with the cell attachment motifs. The third stage, WSSV recognizes and enters into the cell. Next, WSSV envelope probably fuses with the endosome allowing the naked nucleocapsid to transport the viral DNA into the nucleus of the host cell in the same way as baculoviruses. After that, the naked WSSV nucleocapsid binds to the nuclear membrane and releases the genome into the nucleus. Afterwards, the replication of WSSV genome proceeds. Then, the early virogenic stroma appears consisting of the viral material in the nucleus. Accumulation of the cellular chromatin is observed near the nuclear membrane and the rough endoplasmic reticulum (RER) becomes enlarged and active. Next, the dense chromatin of host cell is observed (shaded area) in contrast to the less dense virogenic stroma. The vesicles are probably formed with membranous material found in the ring zone as in baculoviruses. The virogenic stroma is stored the viral nucleosome that contained nucleocapsid proteins. Then, the assembly of new WSSV virions within an electron-dense inclusion is observed. The nucleocapsids are filled into the empty envelopes. The nuclear membranes of the host cell are disupted. At the late stage, the intact WSSVs are released from the disrupted cells to begin the new cycle in other susceptible cells.

Proteomic methods have helped to identifiy a total of 58 structural proteins, over 30 of which are recognized as envelope proteins (Li et al., 2007a; Tsai et al.,

2006; Xie et al., 2006; Zhang et al., 2004b). Some of the WSSV envelope proteins involved in shrimp infection have been identified (Liang et al., 2005), (Vaseeharan et al., 2006). More than 40 structural proteins have been characterized from WSSV (Escobedo-Bonilla et al., 2008) (Table 1.1). Structural proteins were located in virion or nucleocapsid (Escobedo-Bonilla et al., 2013; Huang et al., 2002b; Li et al., 2005; Tsai et al., 2004; Xie et al., 2006). For the non-structural proteins, they involve in several biological functions such as transcriptional regulation (i.e. VP9), virus proliferation (i.e. WSV021), and DNA replication (i.e. WSV477) (Han et al., 2007; Ponprateep et al., 2013).

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

Protein name	Genbank accession number	Size (aminoacid residues)	Apparent size (kDa)	Putative function	Location in WSSV virion (references)
VP9	2GJIA	79	9	Transcriptional	Non-structural[18]
VP11	AAL89262	433	11	Unknown	Not determined[8]
VP12A (VP95)	AF402996	95	11	Structural	Tegument[8, 9, 21]
VP12B (VP68)	AF411464	68	7	Structural	Envelope[8, 12, 13]
VP13A	AAL89207	100	13	Energy metabolism	Not determined[8]
VP13B (VP16)	AAL89245	117	13	Structural	Envelope[21]
VP14	AAL89217	97	11	Structural	Envelope[21]
VP15	AAL89137	80	15	DNA binding protein	Nucleocapsid/core[8, 11]
VP19	AAL89341	121	19	Structural	Envelope[8, 9, 11]
WSV021	AAL33025	200	23	Regulation virus replication	Non-structural[19]
VP22 (VP184)	AAL89227	891	100	Unknown	Not determined[8]
VP24 (VP208)	DQ902656	208	24	Structural	Nucleocapsid[8, 10, 13]
VP26	EF534253	204	26	Structural	Tegument[8, 10]
VP28	EF534254	204	28	Structural	Envelope[8, 9, 10]
VP31	AY897235	261	31	Cell attachment	Envelope[6, 8, 9]
VP32	AAL89121	278	32	Structural	Envelope[8, 21]
VP35	AY325896	228	26	Structural	Nucleocapsid[1]
VP36A	AAL89002	297	36	Cell attachment	Tegument[8, 9]
VP33 (VP281)	EF534251	281	32	Cell attachment	Envelope[2, 8, 12, 21]
VP38A	AAL89182	309	35	Structural	Envelope[8, 9, 21]
VP38B	AAL89317	321	38	Endonuclease	Not determined[8]
VP39A	AAL89230	419	39	Structural	Tegument[8, 9]
VP39B	AY884234	283	32	Structural	Envelope[8, 15, 21]
VP41A (VP292)	AF411636	292	33	Structural	Envelope[2, 8, 13]
VP41B (VP300)	AF403003	300	34	Structural	Envelope[8, 21]
VP51A	AAL89162	486	51	Structural	Envelope[8, 17, 21]
VP51B (VP384)	AAL89179	384	46	Structural	Envelope[8, 9, 21]
VP51C (VP466)	AAL89232	466	50	Structural	Nucleocapsid[3, 8, 12]
VP53A (VP150)	AAL88935	1301	144	Structural	Envelope[8, 9, 21]
VP53B	AAL89039	968	53	Signal transduction pathway	Not determined[8]
VP53C	AAL89192	489	53	Unknown	Not determined[8]
VP55 (VP448)	AAL88919	448	55	Unknown	Not determined[8]
VP60A (VP56)	AAL89249	465	60	Structural	Envelope[21]
VP60B (VP544)	AAL89342	544	60	Adenovirus fibre-like protein	Nucleocapsid[8, 9, 13, 21]
VP75	AAL89256	786	75	Structural	Nucleocapsid[16]
VP76 (VP73)	AAL89143	675	76	Class 1 cytokine receptor	Nucleocapsid[4, 8, 17, 21]
VP90	AAL89251	856	96	Structural	Envelope[21]
VP95	AAL89370	800	89	Structural	Tegument[21]
VP110	AAL88960	972	110	Cell attachment	Envelope[8, 21]
VP124	AAL89139	1194	124	Structural	Envelope[8, 14, 21]
VP136A	AAL89194	1219	136	Cell attachment	Nucleocapsid[8, 21]
VP136B	AAL89392	1243	136	Unknown	Not determined[8]
VP180 (VP1684)	AAL88920	1684	169	Collagen-like protein	Envelope[8]
VP187	AAL89132	1606	174	Structural	Envelope[7, 21]
VP190	AAL33291	1565	174	Structural	Nucleocapsid[21]
WSV477	DQ121373	208	30	DNA replication	Non-structural[20]
VP664	AAL89287	6077	664	Cell attachment	Nucleocapsid[5, 8, 9]
VP800	AAL02264	800	90	Unknown	Not determined[8]



(Source: Escobedo-Bonilla et al., 2008)

Figure. 1.2 A model of the morphogenesis of white spot syndrome virus (WSSV)

Several preventive and curative measures have been developed though not successfully implemented in shrimp farms such as vaccination (Rout et al., 2007; Satoh et al., 2008) immunostimulants (Balasubramanian et al., 2008; Chotigeat, 2013) direct neutralization by antiviral proteins (Dupuy et al., 2004; Tharntada et al., 2009) and RNAi (Ongvarrasopone et al., 2008). In addition, innate immunity in shrimp has been studied intensively to understand the response of shrimp to viral infection (Liu et al., 2009b). Nevertheless, the host-viral interaction and the mechanism of WSSV entry into the shrimp cell and of the spread of the virus in the crustacean host are

still not clear. It is believed that the envelop proteins of virus might be important roles in viral infections (Wu et al., 2005; Zhang et al., 2004a).

The pattern recognition proteins (PRPs) have been isolated and characterized from crustaceans. Most pattern recognition proteins were found in shrimps and other crustaceans including β-glucan-binding protein (BGBP also abbreviated βGBP) (Duvic and Söderhäll, 1990) (Cerenius and Söderhäll, 2004), lipopolysaccharide- and glucan-binding protein (LGBP) (Lee et al., 2000), some masquerade-like proteins/serine proteinase homologues (SPHs) (Huang et al., 2000; Lee et al., 2001) and a large number of lectins. The LGBPs and the BGBPs will bind β-1,3-glucans and after this binding they will trigger immune reactions such as proPO-activation (Cerenius and Söderhäll, 2004; Duvic and Söderhäll, 1990; Lee et al., 2000). SPHs in crustaceans have mainly been implicated in pattern recognition and as opsonins. The masquerade-like protein, the first SPH in crustacean, bind to gram-negative bacteria and trigger a proteolytic processing of the protein (Lee et al., 2001). A broad and diverse category of lectin-like proteins have also been described within the crustacean and are considered to represent another class of PRP. C type lectins all contain one or two carbohydrate recognition domains (CRDs) and have a well-defined structure maintained by two or three pairs of disulphide bonds. Within the penaeid shrimp different lectins have been described from hepatopancreas and/or haemocytes and have been shown to be transcriptionally up-regulated in response

to infection with some viruses, including WSSV (Luo et al., 2003; Ma et al., 2007; Yang et al., 2007a), and Gram-negative bacteria (Luo et al., 2006) .

1.3 Shrimp immunity

In the last decade, a series of papers around shrimp immunity were published and a batch of related data accumulated, which are very useful for understanding the interaction between shrimp and pathogens to enrich the immune theory of invertebrates. Recently, several review papers summarize the achievements in shrimp immunity including EST sequencing and database construction (Leu et al., 2011), microarray analysis of shrimp immune response (Aoki et al., 2011), shrimp molecular responses to viral pathogen (Flegel and Sritunyalucksana, 2011), the cationic antimicrobial peptides in penaeid shrimp (Tassanakajon et al., 2010) and immune molecules and their crucial functions in shrimp immunity (Tassanakajon et al., 2013). Obviously understanding the shrimp immunology is necessary to develop an effective strategy for disease control. Fig. 1.3 summarizes the shrimp immune response against pathogen infection.

The shrimp immunity consist of two major responses including cellular mediated and humoral innate immune responses to fight the pathogen (Fig 1.3). Compared with humoral immunity, studies on the processes that govern the cellular immune response are very limited (Williams, 2007).The cellular response involves phagocytosis, apoptosis, nodule formation and encapsulation of pathogens (Iwanaga

and Lee, 2005; Little, 2006). It was suggested that cellular responses of phagocytosis and apoptosis played a more important role in the shrimp response to WSSV infection than did the humoral proPO system (Wang and Zhang, 2008). The humoral responses include the prophenoloxidase (proPO) system, the clotting cascade and a wide array of antimicrobial peptides (AMPs). The hallmark of the humoral response is the synthesis and secretion of AMPs. Two distinct pathways regulating the expression of the AMP gene families, the Toll and the Immune deficiency (IMD) pathways were identified and clearly studied (Leclerc and Reichhart, 2004; Naitza and Ligoxygakis, 2004; Tanji and Ip, 2005). Here. We described the key molecules involved in humoral immune response of penaeid shrimp. The antimicrobial peptide is further reviewed in section 1.6.

Toll, IMD and JAK/STAT pathways are main pathways regulating the immune response in shrimp (Li and Xiang, 2013a). Toll pathway of shrimp responds not only to Gram-positive bacteria, Gram-negative bacteria, but also to WSSV (Labreuche et al., 2009; Wang et al., 2009a; Wang et al., 2011b). Components of IMD pathway such as IMD, Relish and TRAF6 were isolated in shrimp (Li et al., 2009; Wang et al., 2009b; Wang et al., 2009c). The expression of Tumor necrosis factor receptor (TNFR) – associated factor 6 (TRAF6), from *L. vannamei* (LvTRAF6) was changed after *Vibrio alginolyticusand* and WSSV challenges. Dual luciferase reporter assays in *Drosophila* S2 cells revealed that LvTRAF6 could activate the promoters of antimicrobial peptide

genes (AMPs). It was indicated that LvTRAF6 might play a crucial role in antibacterial and antiviral responses via regulation of AMP gene expression (Wang et al., 2011a). The transcription of STAT in shrimp was modulated after WSSV infection, suggesting that a putative JAK/STAT pathway might exist in shrimp and be very important to virus infection (Chen et al., 2008a).

Hemolymph coagulation is part of the crustacean innate immune response; it prevents leakage of hemolymph from sites of injury and dissemination of invaders such as bacteria throughout the body. It has been shown that polymerization of clottable proteins (CPs) by the hemocyte transglutaminases (TG) triggers hemolymph clotting in crustaceans (Sritunyalucksana and Söderhäll, 2000). TG promotes the cross-linking of coagulin with hemocytesurface antigens called proxins and may facilitate the formation of a physiological barrier to invading pathogens (Osaki et al., 2002). The shrimp coagulation is believed to rely on the formation of a clottable protein (CP) polymer that is catalyzed by the Ca^{2+} dependent covalent linkage of the large dimeric CP by transglutaminase (TG) into long chains. In shrimps, TG has been found in *P. monodon* (Chen et al., 2005; Yeh et al., 2006) *F. chinensis* (Liu et al., 2007b), *M. japonicas* (Yeh et al., 2006) and *L. vannamei* (Yeh and Chen, 2009). Hemolymph CPs have been identified in *M. japonicus*, *L. vannamei* (Cheng et al., 2008) , *F. paulensis* (Perazzolo et al., 2005) and *P. monodon* (Kang et al., 2007; Yeh et al., 1999).

The proPO-activation pathway is a phenoloxidation cascade comprising of pattern recognition proteins, several serine proteases, and their inhibitors and terminates with the zymogens, proPO. Serine proteases can cleave proPO to generate the active enzyme, phenoloxidase. The serine proteinase that carries out the proteolysis of the proPO precursor has been variously named as the proPO activating factor (PPA) (Sritunyalucksana and Söderhäll, 2000). After the pathogen infection, the proPO cascade was induced to activate step by step by pro-enzymes. The final product melanin kills and limits the pathogen invasion. To prevent the harmful of over activation, serine proteinase inhibitors plays important role in regulating proPO system. In penaeid shrimp, enzymes in the proPO system are localized in the semigranular and granular cells (Perazzolo and Barracco, 1997). This is in accordance with the former studies showing that *P. monodon* proPO and PPAE mRNAs as well as a *L. vanamei* proPO mRNA are expressed only in hemocytes (Ai et al., 2009; Amparyup et al., 2009; Sritunyalucksana and Söderhäll, 2000; Charoensapsri et al., 2009, 2011). Previous reports revealed that RNAi-mediated silencing of two *P. monodon* proPO genes (*Pm*prpPO1 and *Pm*proPO2) and two *P. monodon* PPAE genes (*Pm*PPAE1 and *Pm*PPAE2) significantly decreased the total PO activity, leading to an increase in the bacterial number in *Vibrio harveyi*-infected shrimp and also enhanced their the mortality rate after infection (Amparyup et al., 2009; Charoensapsri et al.,

2009; Charoensapsri et al., 2011). These results indicated that proPO and PPAE are important to proPO system as well as the innate immune response in shrimp.

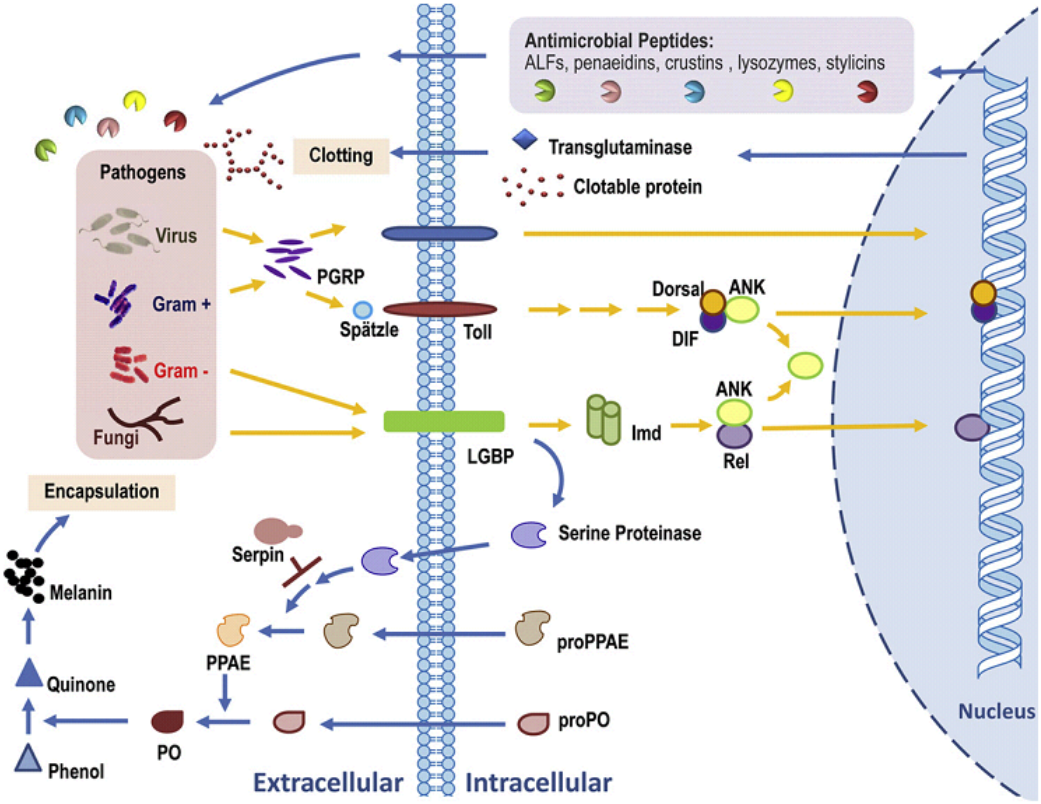


Figure. 1.3 A schematic model of the shrimp immune system (Tassanakajon et al., 2013).

1.4 Shrimp antiviral immunity

As stated before, virus infection is still the major problem of shrimp aquaculture and no effective method is available. Moreover, the knowledge in shrimp antiviral immunity is limited. Like other microbes, shrimp recognize viral components, like genomic DNA and RNA or dsRNA generated in virally infected cells

by host pattern recognition receptors (PRRs) after which the appropriate antiviral response is triggered (Liu et al., 2009a). Such antiviral responses have been review in shrimp, crayfish and other crustaceans. The higher antiviral activities of apoptosis (Wang et al., 2008) and phagocytosis than that of prophenoloxidase (proPO) system observed in *M. japonicus*, suggest that cellular responses play important role in the immune defense of invertebrates against WSSV infection (Wang and Zhang, 2008). However, in the WSSV-resistant *P. japonicus* apoptosis was not found to play an important role (Wu and Muroga, 2004). Antiviral-related proteins/genes in crustaceans or antiviral substances have been isolated from the tissue extracts of shrimp, blue crab, and crayfish. These can bind to various DNA and RNA viruses. Nevertheless, the mechanism of this inhibitory activity remains unclear (Pan et al., 2000).

The signaling pathways such as Toll pathway, IMD pathway and JAK/STAT have been reported to be triggered by virus infection. Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular patterns and play a critical role in innate immune responses. It has been shown to be involve in anti-virus response in shrimp. The first Toll receptor was reported in *Drosophila melanogaster* as a gene whose products control dorsoventral polarity during embryogenesis (Belvin and Anderson, 1996; Stein et al., 1991). Toll receptors have now been identified in *Penaeus monodon* (Arts et al., 2007), *Penaeus japonicus*, (Mekata et al., 2008), *P. vannamei* (Yang et al., 2007b) and *P. chinensis* (Yang et al., 2008). In mammals a suite

of 13 Toll-like receptors (TLRs) are reported with a variety of identified ligands, including PAMPs originating from bacteria, fungi and viruses (West et al., 2006). The recent study of (Han-Ching Wang et al., 2010), however, demonstrated that the RNA inhibition (RNAi) of Toll transcription had no effect on survival to WSSV infection in the *P. vannamei* although did lead to an increase in shrimp mortality following infection with *V. harveyi*, suggesting a role for the Toll receptor in response to Gram-negative bacterial infection but not in response to viruses. WSSV immediate early gene (ie1) was shown to employ shrimp STAT as a transcription factor to enhance its expression resulting in its high promoter activity in the host cells (Liu et al., 2007a). Another study showed cytokine activation mediated antiviral response in which shrimp STAT was activated in response to WSSV infection and that WSSV does not disrupt JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway but benefits from STAT activation in shrimp (Chen et al., 2008b). In another study, the dsRNA silencing of shrimp STAT resulted in reduction of both WSSV ie1 gene expression and WSSV genome copy number. The significant increase in the activation of shrimp STAT and rapid increase in WSSV copy number has been observed in WSSV infected brooders, which indicated that the spawning stress in brooders triggers WSSV replication via the activation of shrimp STAT (Lin et al., 2012). The transcription of STAT in shrimp was modulated after WSSV infection, which suggested that a putative JAK/STAT pathway might exist in shrimp and be very

important to virus infection (Li and Xiang, 2013b). Pelle from *L. vannamei* (LvPelle) revealed 24–40% identity with IRAK (IL-1 receptor-associated kinase) family of proteins, which are the central regulators of the TLR-mediated NF-kB pathway. In response to WSSV infection, *LvPelle* was found to be upregulated in gill, intestine and in the hepatopancreas at 3 h post-WSSV infection. *LvPelle* was further shown to interact with *L. vannamei* tumour necrosis factor receptor-associated factor 6 (*LvTRAF6*). In an another study, *LvTRAF6* was observed to be up regulated at 3 h in gills and hepatopancreas post-WSSV infection, indicating that it may play a crucial role in antiviral response (Wang et al., 2011b). Relish in IMD pathway and dorsal in Toll pathway of shrimp were both involved in the immune response of shrimp to bacteria and virus infections, which implied that these two pathways are not completely separated during the immune response of shrimp.

In shrimp, many studies have reported that virus-inhibiting proteins such as ALF, Beta-integrin, *Pm*AV, Syntenin SPIPm2 etc (Liu et al., 2009a). The recombiant *Pm*AV protein, has a C-type lectin-like domain (CTLD), showed a strong antiviral activity in inhibiting virus-induced cytopathic effect in fish cells *in vitro*. Further experiment showed that *Pm*AV did not bind to the WSSV implying that the antiviral mechanism of this protein was not due to inhibition of the attachment of virus to the target host cell (Luo et al., 2003). Beta-Integrins are transmembrane proteins of *M. japonicas* that recognize a large variety of extracellular and cell surface proteins,

have emerged as receptors or co-receptors for a large number of viruses. Recently, there was a report revealed that the β-integrin could bind to a VP187 and might be partly involved in WSSV infection (Li et al., 2007a).

In crustaceans, several Kazal type serine proteinase inhibitor (KPIs) are proposed to be involved in innate immune response to pathogenic infections although their actual functions are not known for certain (Rimphanitchayakit and Tassanakajon, 2010). Usually, the immune responsive KPIs are up-regulated when the animals are challenged with viral pathogens. For examples, a four-domain Kazal-serine proteinase inhibitor PAPI 1 from *Pacifastacus leniusculus*, a two-domain KPI gene from *Fenneropenaeus chinensis* and two two-domain KPI genes from red swamp crayfish, *Procambarus clarkii* are up-regulated in response to WSSV challenge (Kong et al., 2009; Li et al., 2010; Liu et al., 2006a). The SPI*Pm*2 gene from the black tiger shrimp *Penaeus monodon* is no exception. It is also up-regulated in response to YHV and WSSV challenges (Donpudsa et al., 2010; Pongsomboon et al., 2011; Prapavorarat et al., 2010). In response to WSSV challenge, the SPI*Pm*2 producing cells in the shrimp are reduced by more than half as they secrete the inhibitor into the circulation. It is assumed that the SPI*Pm*2 somehow perform its defensive role by inhibiting the viral proteinase. Injection of the recombinant SPI*Pm*2 (rSPI*Pm*2) into the shrimp before WSSV challenge attenuates the WSSV replication. Moreover, it helps prolong the lives of WSSV-infected shrimp (Donpudsa et al., 2010). The rSPI*Pm*2 was

able to temporarily and dose-dependently neutralize the WSSV and protect the hemocytes from viral infection judging from the substantially less expression of WSSV late gene VP28. The antiviral activity was very likely due to the binding of SPI*Pm*2 to the components of viral particle and hemocyte cell membrane (Ponprateep et al., 2011).

RNA interference (RNAi) has been proven to be an alternative and more specific approach for the antiviral mechanism in shrimps as this effect has been confirmed by injection of WSSV specific dsRNA/siRNA targeting WSSV proteins (Kim and Rossi, 2007; Liu et al., 2009a; Robalino et al., 2005). Effect of injection of WSSV specific dsRNA/siRNA can block viral disease progression. This effect has been confirmed with different unrelated viruses (Xu et al., 2007).

1.5 Antimicrobial peptides

Antimicrobial peptides (AMPs) function as a first line of defense against many microorganisms (Hancock et al., 2006). They have been identified in multicellular organisms. AMPs are typically small size, generally less than 150-200 amino acid residues, and have amphipathic structure and cationic property. Although the anionic peptides also exist. These peptides are active against a broad spectrum of microorganisms such as bacteria, virus, yeast, parasite, and fungi. AMPs may also exhibit an anti-tumor activity (Cruciani et al., 1991; Hancock and Diamond, 2000; Krepstakies et al., 2012). AMPs have also been founded in arthropods such as insects,

horseshoe crabs, and shrimp (Bachère et al., 2004; Bulet et al., 1999; Tassanakajon et al., 2011; Vizioli and Salzet, 2002). Moreover, depending on their distribution, the expression of antimicrobial peptides appears to be regulated by different pathways and these effectors may consequently participate in either a local or a systemic reaction. Some of AMPs are constitutively expressed in the secretory cells; whereas, others are induced upon microbial stimulation (Hancock and Diamond, 2000). In 2004, Bulet et al. evaluated that over 1000 AMPs have been isolated and characterized from multi-cellular organisms at the level of their primary structure and most of them has been identified in insects (Bulet et al., 2004).

AMPs can be separated into sub-groups on the basis of their amino acid compositions and structures. First sub-group is anionic peptides. For example maximin H5 from amphibians have the molecular weight of about 721.6-823.8 Da and are small anionic peptide rich in glutamic and aspartic acids. The second sub-group is linear cationic **α**-helical peptides such as cecropin, magainin, pleurocodin, CAP18 and LL37. The third group is cationic peptide enriched for specific amino acids. This sub-group includes the bactenecins, hymenoptaecin, coleoptercin and indolicidin. The fourth sub-group is anionic and cationic peptides containing cysteine residues that can form disulfide bonds such as defensin, protegrin and brevinin. At last, anionic and cationic peptide fragments of larger proteins including lactoferricin and casocidin I (Brogden, 2005).

The antibacterial mechanism of AMPs can be devided into two mechanism; one is transmembrane pore-forming and another is intracellular antimicrobial peptide activity (Brogden, 2005; Jenssen et al., 2006b). Most AMPs appear to act on bacteria using a transmembrane pore-forming mechanism via interaction with the cell membrane components leading to pore formation and leakage of the bacterial cytoplasmic contents. Now, categorized by the pore forming mechanisms, there have been 4 distinct models as follows; aggregate, barrel-stave, carpet and toroidal-pore models. Moreover, the AMPs may pass through the cell membrane and enter the cells, they might bind to macromolecules and interrupt the synthesis of vital components, such as DNA, RNA and proteins, and resulted in cell death.

AMPs in shrimp attract a lot of attention due to their function in killing bacteria or virus, suggesting their potential use in aquaculture. Different types of antimicrobial peptides or proteins have been reported in shrimp such as penaeidins, crustin, ALFs, and stylicins. The review reports on AMPs and their immune functions in shrimp. Shrimp AMPs are primarily expressed in hemocytes, which migrated to infection sites and AMPs are secreted into the circulation as well as site of infection to fight against pathogen invasion (Tassanakajon et al., 2010). The penaeidins is a family of antimicrobial peptides acting against gram-positive bacteria and fungi. Penaeidins were found in many species of shrimp which are *L. vanamei*, *Litopenaeus stylirostris*, *Litopenaeus schmitti*, *Farfantepenaeus brasiliensis*, *F. chinensis*,

Fenneropenaeus penicillatus, *Farfantepenaeus subtilis*, *Farfantepenaeus paulensis*, *Litopenaeus setiferus* and *P. monodon* (Destoumieux et al., 1997; Gross et al., 2001; Supungul et al., 2004; Tassanakajon et al., 2010). Penaeidin 5 from the *P. monodon* which was found to play a possible role in protection against viral infection (Woramongkolchai et al., 2011). Crustins such as crustin*Ls*, crustin*Lv* and crustin*Pm* have been identified in the penaeid shrimp, *L. setiferus*, *L. vannamei* and *P. monodon*, respectively (Bartlett et al., 2002; Tassanakajon et al., 2010). They possess sequence identify with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Therefore, crustins play a role against Gram-positive and Gram-negative bacteria and also inhibit proteinase activity (Krusong et al., 2012; Tassanakajon et al., 2010). Peptide fragment derived from hemocyanin of *L. vannamei*, *L. stylirostris* and *P. monodon* possessing antiviral activity has also been identified and its activity against Gram positive bacteria was reported (Destoumieux-Garzón et al., 2001; Patat et al., 2004; Zhang et al., 2004a). Anti-lipopolysaccharide factors (ALFs) are AMPs identified in crustaceans. They have broad antimicrobial activities towards gram-positive and gram-negative bacteria, filamentous fungi and viruses (Li et al., 2008; Liu et al., 2006a; Somboonwiwat et al., 2005). The activities of ALFs against marine pathogens such as *V. harveyi* and WSSV have been reported (Liu et al., 2006a; Ponprateep et al., 2009; Tharntada et al., 2009). rLs-Stylicin1 was able to agglutinate *Vibrio penaeicidae* in vitro in agreement with its potent LPS-binding

activity on immobilized LPS of *V. penaeicidae* (Rolland et al., 2010). In conclusion, AMPs as products of immune response, play important roles in killing or cleaning the infected pathogens directly.

1.6 Cationic antiviral peptides

The success of viruses in evolution has been assured by four general attributes: genetic variation, variety in means of transmission, efficient replication within host cells, and the ability to persist in the host (Wagner et al., 1999). Due to these attributes, the control of viral diseases has not been an easy task. Despite the existence of antiviral drugs, there is a need to explore novel antiviral compounds in order to control emerging viral pathogens. Several cationic antiviral peptides from various sources have been isolated since the 1980s (Table 1.2) and they have shown strong potential for novel therapeutic drugs against many viral infections. Due to the promiscuity of these peptides, it is possible to verify a broad spectrum of antiviral activities within the same peptide. Moreover, this promiscuous activity can be extended to simultaneous cytotoxic activity against tumor cells (Fig. 1.4). The first study reporting an antiviral role of a cationic peptide was published in 1986, in which the activity of **α**-defensin was described as inhibiting a number of viruses including herpes simplex virus types 1 and 2 (HSV), cytomegalovirus (CMV) as well as inhibiting the vesicular stomatitis virus with human neutrophil peptide 1 (HNP1) *in vitro* (Daher et al., 1986; Findlay et al., 2013). Since then, many reports have shown the antiviral

activity of cationic host-defense peptides such as α-, β-, and θ-defensins, and the use of effective antiviral therapy with cathelicidins, as previously reviewed (Findlay et al., 2013). It is very promising that in the last years many new antiviral peptides have been either identified or synthesized in order to aid the development of new therapeutic antivirus therapies (Mulder et al., 2013).

Table. 1.2 Cationic antiviral peptides from different sources, their application, and their mechanisms of action (Mulder et al., 2013).

Peptide	Source (s)	Group	Application	Mechanism of action	References
HUMAN HEALTH					
Alloferon 1 Alloferon 2	*Calliphora vicina*	Insect	IAV	Immunomodulatory activity	Chernysh et al., 2002
Brevinin-1	*Rana brevipoda*	Frog	HSV	Viral inactivation	Yasin et al., 2000
Caerin 1.1 Caerin 1.9 Maculatin	–	Amphibian Skin	HIV	Disrupts the integrity of the virion membrane	Vancompernolle et al., 2005
CAP37	*Homo sapiens*	Human leococytes	HSV-1 AdV	Disrupts the envelope and/or capsid	Gordon et al., 2009
Cecropin	*Hyalophora cecropia*	Insect	JV HSV HIV	Suppresses viral protein synthesis Cellular target Suppresses viral gene expression	Wachinger et al., 1998; Albiol Matanic and Castilla, 2004
Circulin A	*Chassalia parvifolia*	Plant	HIV		Daly et al., 1999
Defensin	*Homo sapiens*	Human	HSV IAV HCMV VSV HIV AdV	Interacts with glycosaminoglycans Inactivates viral particle Cellular target Unknown	Daher et al., 1986; Nakashima et al., 1993; Gropp et al., 1999; Yasin et al., 2000; Bastian and Schafer, 2001; Sinha et al., 2003
Dermaseptin	Genus *Phyllomedusa*	Frog	HIV HSV	Disruptis viral membrane	Belaid et al., 2002
Didemnins A Didemnins B	Genus *Tridemnum*	Tunicate	HSV Parainfluenza Dengue virus	Inhibits RNA and DNA viral replication	Rinehart et al., 1981; Aneiros and Garateix, 2004
HNP-1 HNP-3	*Homo sapiens*	Human	HSV AdV	Blocks early steps of viral replication	Ganz et al., 1985; Bastian and Schafer, 2001; Hook et al., 2006
Hp1090	*Heterometrus petersii*	Scorpion	HCV	Disrupts viral membrane integrity	Yan et al., 2011
Indolicidin	*Bos taurus*	Bovine	HIV HSV	Inhibits integrase Targets viral glycosaminoglycans	Robinson et al., 1998
Lactoferricin	*Homo sapiens* *Bos taurus*	Human, Bovine	HCMV HIV HSV Papilloma	Activity at virus-cell interface Blocks heparan sulfate	Andersen et al., 2001; Jenssen et al., 2004; Mistry et al., 2007
LL-37	*Homo sapiens*	Human	HSV IAV	Viral receptor-based mechanisms	Yasin et al., 2000; Barlow et al., 2011
Magainin	*Xenopus laevis*	Frog	HSV HIV	Suppresses viral gene expression	Aboudy et al., 1994; Albiol Matanic and Castilla, 2004
Mellitin	*Apis mellifera*	Insect	HSV JV	Cellular target	Wachinger et al., 1998; Yasin et al., 2000; Albiol Matanic and Castilla, 2004
Microspinosamide	*Sidonops microspinosa*	Marine sponge	HIV	Inhibits cytopathic effect of HIV-1 infection	Rashid et al., 2001

Peptide	Source (s)	Group	Application	Mechanism of action	References
Pa-MAP	*Pleuronectes americanus*	Fish	HSV	Interacs with viral envelope	Migliolo et al., 2012
PAP	*Phytolacca americana*	Plant	HIV HBV HSV	Inhibits viral protein synthesis	Kaur et al., 2011
Polyphemusin	*Tachypleu tridentatus*	Horseshoe crab	HIV	Binds gp120 and CD4	Nakashima et al., 1992; Tamamura et al., 1996
Protegrin	*Homo sapiens*	Human	HIV HSV	Unknown Viral inactivation	Yasin et al., 2000; Steinstraesser et al., 2005
Tachyplesin	*Tachypleus tridentatus*	*Horseshoe crab*	HIV HSV VSV IAV	Virus-cell fusion Viral inactivation Viral envelope	Morimoto et al., 1991; Murakami et al., 1991; Yasin et al., 2000
θ-defensin	*Homo sapiens*	Human	HIV HSV	Binds glycosylated gp120 Binds gB and blocks viral attachment	Cole et al., 2002; Yasin et al., 2004
ANIMAL HEALTH					
Cecropin B CF17	*Hyalophora cecropia*	Insect Synthetic	IHNV VHSV SHRV IPNV	Disrupts the viral envelope Disintegrates the viral capsids	Chiou et al., 2002
Epinecidin-1 TH 1-5 cSALF	*Oreochromis mossambicus* *Penaeus monodon*	Fish Shrimp	NNV	Agglutinates NNV virions into clump	Chia et al., 2010
Pleurocidin MDPle	*Limanda limanda*	Fish	VHSV	Disrupts the viral membrane via toroidal pore formation model	Falco et al., 2009
AGRICULTURE					
Potide-G PAP	*Solanum tuberosum L* *Phytolacca americana*	Plant Plant	PVYO TMV CMV CaMV	Unknown Inhibit viral protein synthesis	Tripathi et al., 2006 Chen et al., 1991
Indolicidin	*Bos taurus*	Bovine neutrophils	TMV	Unknown	Bhargava et al., 2007
Peptamine	*Pseudomonas chlororaphis O6*	Bacteria	TMV	Unknown	Park et al., 2012
Analogs of melittin	*Apis mellifera*	Synthetic	TMV	Cellular target	Marcos et al., 1995

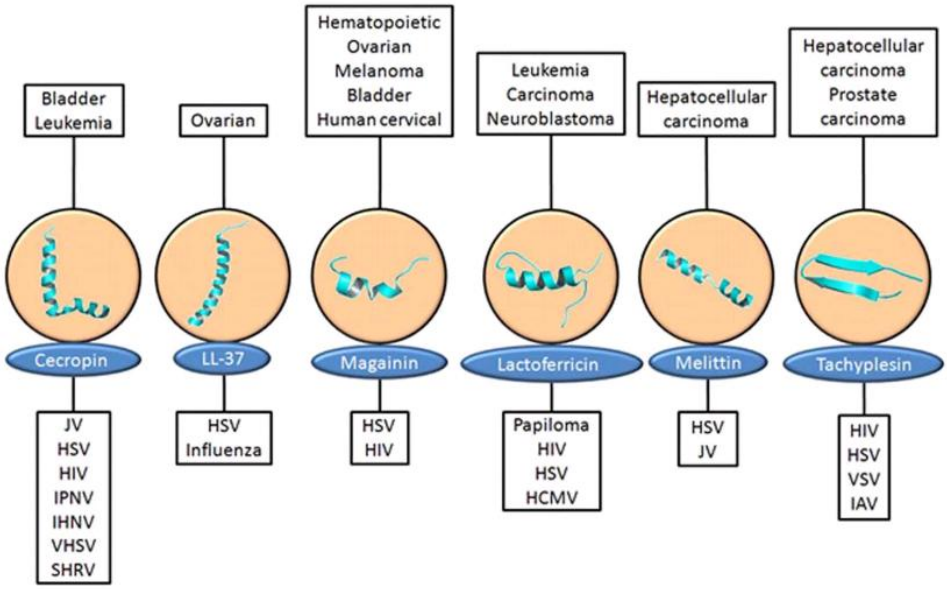


Figure. 1.4 Promiscuous cationic peptides with antitumor and antiviral activities. Each promiscuous peptide and their various tumors against which present cytotoxic activity (top) and viruses against which present antiviral activity (bottom). PDB from left to right, 2IGR, 2K6O, 2LSA, 1Z6V, 2MLT, 1WO1. HCMV, human cytomegalovirus; HIV, Human immunodeficiency virus; HSV, herpes simplex virus; IAV, influenza; IHNV, infectious hematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; Jv, Junin virus; SHRV, snakehead rhabdovirus; VHSV, viral hemorrhagic septicemia; VSV, vesicular stomatitis virus (Mulder et al., 2013).

The mechanisms of how antiviral peptides act against viruses have been studied. Three major mechanisms involve targeting (1) cell surface components for examples Glycosaminoglycan, CXCR co-receptor and ATPase protein, (2) intracellular molecules and (3) viral proteins (Fig. 1.5).

When AMPs binds to the cell surface targets, the virus cannot attach to the cells resulting in blocking the viral entry. The activity of Lfcin has been claimed to be

attributed to the affinity of this peptide for carbohydrates, which are viral binding sites on the cell membrane, such as heparin sulfate (HS) and glycosaminoglycans (GAGs), thereby blocking viral entry (Andersen et al., 2003). The antiviral activity of LfcinB and LfcinH (human lactoferricin) against HSV has been verified with the ability of this peptide to interact with HS and block viral entry. It has been found that the positive net charge of the peptides are critical for affinity with HS which is due to the many negatively-charged sulfate groups present in the molecule (Jenssen et al., 2004). Together with Lfcin, the peptides human α-defensin, LL-37 and magainin have also been reported to bind to GAGs in order to perform their respective activities (Fig. 1.5) (Jenssen et al., 2006a). Not only the positive-charged group, a stabilized secondary structure is important for antiviral activity for both LfcinB and LfcinH. The higher potency of LfcinB against some viruses compared to LfcinH is attributed to the β-sheet conformation of LfcinB in solution compared with the α-helical structures of LfcinH (Jenssen et al., 2004). Lactoferrin (Lf), the protein from which the peptide Lfcin is derived, shows antiviral activity against a number of viruses as much as seven times greater than that of Lfcin, proposing that either the size of the molecule is important or that other regions of LF contribute to the antiviral activity (Andersen et al., 2003; Gifford et al., 2005). The positive charge of Lf was found to be important for antiviral activity against human HCMV (Valenti and Antonini, 2005). When negatively-charged groups were added to Lf by succinylation, the antiviral potency was mostly

decreased, whereas the addition of positive charges to Lf through amination of the protein resulted in increased anti HCMV activity (Harmsen et al., 1995). On the other hand, when tested against HIV-1, a 4-fold stronger antiviral effect of Lf was observed when negatively-charged groups were added (Harmsen et al., 1995). The proposed mechanism of action for anti-HIV activity was that Lf and the charged-modified protein bind strongly to the V3 loop of the gp120 envelope protein, increasing the net negative electric charge of viral particles, and resulting in inhibition of virus-cell fusion and entry of the virus into cells (Puddu et al., 1998).

AMPs with the antiviral activity can also bind to the intracellular targets and inhibit the biological processes. A proposed mechanism of action has suggested that melittin suppresses cell fusion mediated by HSV-1 syncytial mutants probably by interfering with the activity of the Na^+ K^+ ATPase, a cellular enzyme involved in the membrane fusion process (Albiol Matanic and Castilla, 2004). Analysis of the effect of melittin on the production of HIV-1 transcripts was assayed in acutely infected T-cells cultured with various concentrations of melittin. Levels of all HIV-1 transcript classes were suppressed (reduction of～30% when compared with cells without melittin) in a dose-dependent manner (Wachinger et al., 1998). Another mechanism whereby melittin interferes with viral gene expression has been proposed and involves intracellular immunization against HIV (Fig. 1.5). Melittin interfere in the process of cellular signal transduction, such as the activation of phospholipase A2 for instance,

and the decrease in activities of calmodulin and protein kinase C (Fisher et al., 1994; Gravitt et al., 1994; Sharma, 1993). These properties may therefore change the balance and activities of cellular stimulators of HIV transcription (NFkB, AP-1 and NFAT) or induce inhibitory factors (interferon-induced cellular inhibitor) (Wachinger et al., 1998).

Direct interaction of AMPs on viral proteins results in inhibition of adsorption/virus-cell fusion process. Lectins have been reported to be able to that bind to the protein gp120 of human immunodeficiency virus type 1 (HIV-1) with high affinity, blocking its entry into the host cell (Fig. 1.5) (Münk et al., 2003). Antimicrobial peptide interactions with glycoproteins in the viral envelope have been proposed to influence the viral entry process. θ-Defensin (retrocyclin 2) interacts with the HSV-2 glycoprotein B with high affinity, thus protecting the cells from HSV-2 infection (Yasin et al., 2004). The closely related retrocyclin-1 binds HIV gp120 with high affinity, as long as the envelope protein is glycosylated, probably resulting in an anti-HIV activity. This makes the θ-defensin the first antimicrobial peptide isolated from vertebrates with a lectin-like character (Wang et al., 2003). The polyphemusin analogue T22 has been demonstrated to inhibit fusion between the HIV envelope and the host cell membrane (Nakashima et al., 1992) through specific binding of the viral envelope protein gp120 and the T-cell surface protein CD4 (Tamamura et al., 1996). Another human-derived peptide which targets envelope/membrane is the

promiscuous peptide CAP37, first isolated from the granule fractions of human PMNs. It has been shown to have potent activity against viruses (HSV-1 and Adenovirus), bacteria (*Pseudomonas aeruginosa*, *E. coli*, and *Enterococcus faecalis*), and fungi (*Candida albicans*). Its structure/function differs from the others peptides here mentioned. It is known that cystine residues forming intramolecular disulfide bridges are necessary for the antibacterial function of CAP37 but are not required for its antiviral activity, which has been suggested to be involved in the rupture of the envelope and/or capsid (Gordon et al., 2009). The same is applied to indolicidin, a peptide isolated from the cytoplasmic granules of bovine neutrophils (Hsu et al., 2005). However, besides its DNA-binding ability, it has also shown activity against HIV and HSV through a membrane mediated antiviral mechanism (Robinson et al., 1998). One example is the promiscuous peptide *Pa*-MAP isolated from the fish *Pleuronectes americanus*, which beyond having shown broad antimicrobial activity against bacteria (*E. coli* and *S. aureus*), fungi (*Candida parapsilosis*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*) and also has activity against viruses (HSV-1 and HSV-2). The antiviral mechanism of this peptide has been suggested to involve its interaction with the viral envelope (Migliolo et al., 2012; Teixeira et al., 2013).

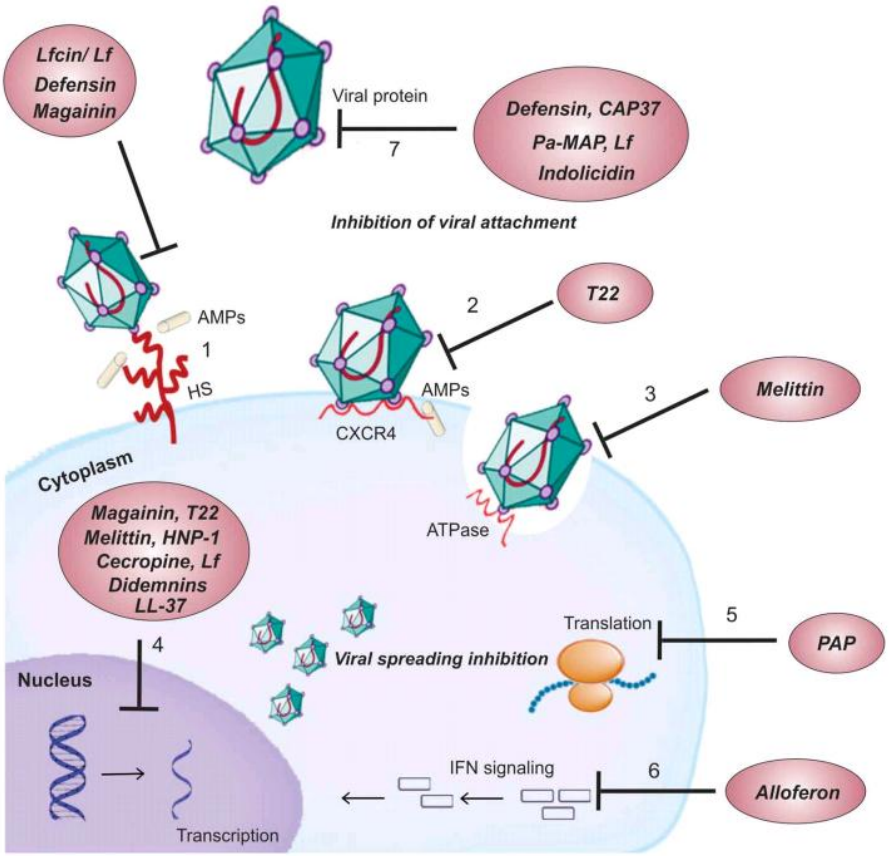


Figure. 1.5 Mechanisms of action of cationic antiviral peptides. Cell surface targets: (1) Interaction of peptides with different glycosaminoglycan (e.g., HS) present on the cell surface competing with the virus for cellular binding sites. (2) Blocking of viral entry into the cell by binding the peptide to viral CXCR4co-receptor required for its entry. (3) Suppression of cell fusion by interfering with the activity of ATPase protein. Intracellular targets: (4) Suppression viral gene expression. (5) Inhibition of peptide chain elongation by inactivating the ribosome. (6) Activation of an immune modulatory pathway by induction of NK and IFN. Viral protein targets: (7) Binding of peptides to viral proteins causing inhibition of adsorption/virus-cell fusion (Mulder et al., 2013).

1.7 Anti-lipopolysaccharide factor

1.7.1 ALF classification

Anti-lipopolysaccharide factor (ALF) is the family of AMPs isolated from the hemocytes of the horseshoe crabs, *Tachypleus tridentatus* and subsequently in many crustaceans, including shrimps, lobsters, crayfish and crabs (Tassanakajon et al., 2014). ALFs are composed of 114–124 amino acid residues with a short signal peptide sequence of 16–26 residues. The molecular mass of the mature peptide is about 11 kDa. The theoretical p*I* of the mature peptide and LPS-BD of ALFs ranges from 5.35–10.35 and 4.37–10.95, respectively.

The ALF was classified into two groups according to their p*I* as cationic ALFs and anionic ALFs (Tassanakajon et al., 2014).

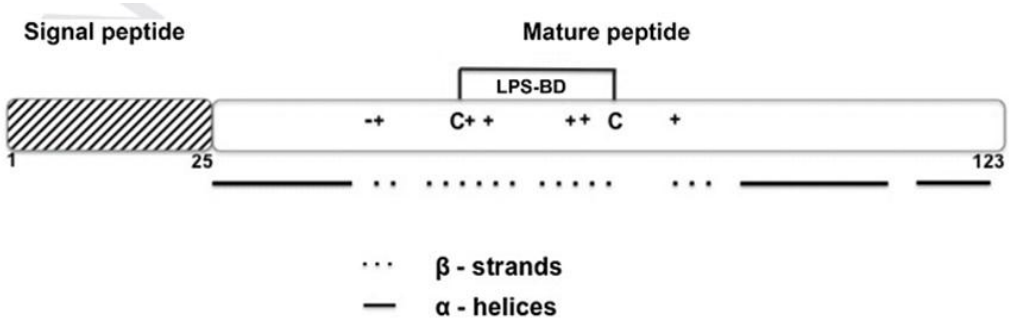
ALFs are amphipathic peptides that contain two-highly conserved-cysteine residues that form a stable disulfide loop habouring a highly conserved cluster of positively charged (Lys and Arg) residues. Typically, ALFs are highly hydrophobic at the N-terminal region and contain a conserved three-dimensional structure, consisting of three α–helices packed against a four-stranded β-sheet. ALFs are amphipathic peptides containing a LPS-binding domain (LPS-BD), which is a β-hairpin structure linked by a conserved disulfide bridge. This stable disulfide loop harbors either a highly conserved cluster of positively charged (Lys and Arg) residues or negatively charged (Glu and Asp) and hydrophobic residues.

Cationic LPS-BDs have been found in both basic and anionic ALFs while anionic LPS-BDs have been found in anionic ALFs only. The 3D structure revealed that the activity of cationic ALF depends on the positively charged cluster within the disulfide loop, which is in accord with explaining their highly conserved nature, and that the amphipathic disulfide loop corresponds to the binding site for the lipid A component of bacterial cells. Many residues of the LPS binding site of anionic ALF were absence leading to the lack of antimicrobial activity of the ALF but the cationic ALF is not (Rosa et al., 2013).

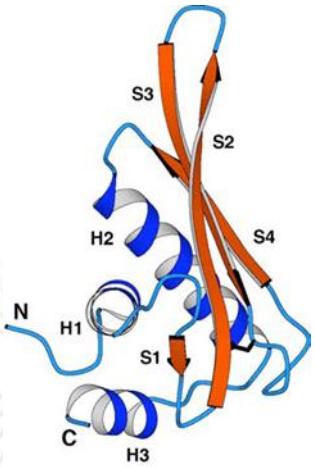
Only the structures of LALF and ALF*Pm*3 have been resolved (Hoess et al., 1993; Yang et al., 2009). Based on the NMR structure of ALF*Pm*3 (a cationic ALF), it can recognize LPS, a major cell wall component of Gram-negative bacteria, through seven amino acid residues; six positively-charged residues and one negatively-charged residue located in the LPS-BD and in the flanking β-strands (Fig. 1.6).

1.7.2 ALF structure

A)



B)



(Source: Yang et al., 2009)

Figure. 1.6 The primary and NMR structure of an ALF*Pm*3 from horseshoecrabs and shrimps. Schematic illustration of the primary structure of ALF (A). Based on the resolved 3D-structure of ALF*Pm*3, ALF contains three α-helices (solid lines) and a four-stranded β-sheet (dashed line). ALF has a signal peptide of about 25 amino acid residues and a mature peptide of 98 amino acid residues. The signature of ALF is the cluster of positive charged amino acids (+) within the disulfide bridge stabilized LPS-BD and the positive charged residues within the flanking β-strands that are responsible for LPS recognition (Tassanakajon et al., 2014). The NMR-structure resolved by Yang et al, 2009 is shown (B).

1.7.3 Expression and antibacterial activity

In *P. monodon*, several isoforms of ALF have been identified from the express sequence taq (EST) database (Tassanakajon et al., 2011). The 124 EST clones represented ALFs were found in hemocyte libraries from normal, pathogen (WSSV or *V. harveyi*) challenged and heat-stressed shrimp. These ESTs exhibited 57% and 65%

amino acid homology with those of the horseshoe crab *T. tridentatus* and *L. polyphemus*. At least 6 different isoforms (ALF*Pm*1-6) (Ponprateep et al., 2012; Prapavorarat et al., 2010; Supungul et al., 2004) have been identified. The different in LPS-BD sequences probably correlate with their antimicrobial activity (Ponprateep et al., 2012). ALF*Pm*3 is the most abundant isoform found in the hemocytes of the black tiger shrimp. It has been expressed in the yeast *Pichia pastoris* expression system. The recombinant ALF*Pm*3 (rALF*Pm*3) protein exhibits antimicrobial activity against both Gram-negative and Gram-positive bacteria as well as fungi (Somboonwiwat et al., 2005). ALF*Pm*3 transcript is up-regulated upon *V. harveyi* infection revealing its roles in shrimp bacterial response (Somboonwiwat et al., 2008). ALF*Pm*3 is able to bind to Gram-negative and Gram-positive bacterial cells and their major cell wall components, lipopolysaccharide (LPS) and lipoteichoic acid (LTA), respectively. The antimicrobial action of ALF*Pm*3 against Gram-negative bacteria is involved in bacterial membrane disruption (Jaree et al., 2012). From the three-dimensional structure of ALF (Fig. 1.6), the amphipathic disulfide loop binds to lipid A and neutralizes the biological effect of LPS (Hoess et al., 1993; Pristovšek et al., 2005; Yang et al., 2009). Moreover, In previous studies, seven isoforms of ALF were identified from *F. chinensis* (Li et al., 2013; Liu et al., 2005) and one of them, named *ALFFc*, showed strong inhibitory effects on the growth of Gram-positive and Gram-negative bacteria as revealed by the activity of the synthetic peptides corresponding

to its LPS-BD sequence (Guo et al., 2014). By detecting antimicrobial activities of sequence-modified peptides from ALFFc-LBD, they found that the disulfide bond across the LBD domain, lysine and arginine residues inside the LBD domain were important to antibacterial activities. However, SpALF1 and SpALF2 from *Scylla paramanosain* have no effect of the membrane integrity of *E. coli*, even for those SpALFs that are also active against *E. coli* (Liu et al., 2012a). On the other hand, very anionic ALF is devoid of antimicrobial activity (Rosa et al., 2013). The absence of a positively charged amino acid cluster in the anionic LPS-BD causes the deficient LPS binding ability leading to the lack of antimicrobial activity of the ALF. Therefore, we noted here that the more positively charged amino acids in the LPS-BD, the better the antimicrobial activity of the ALF.

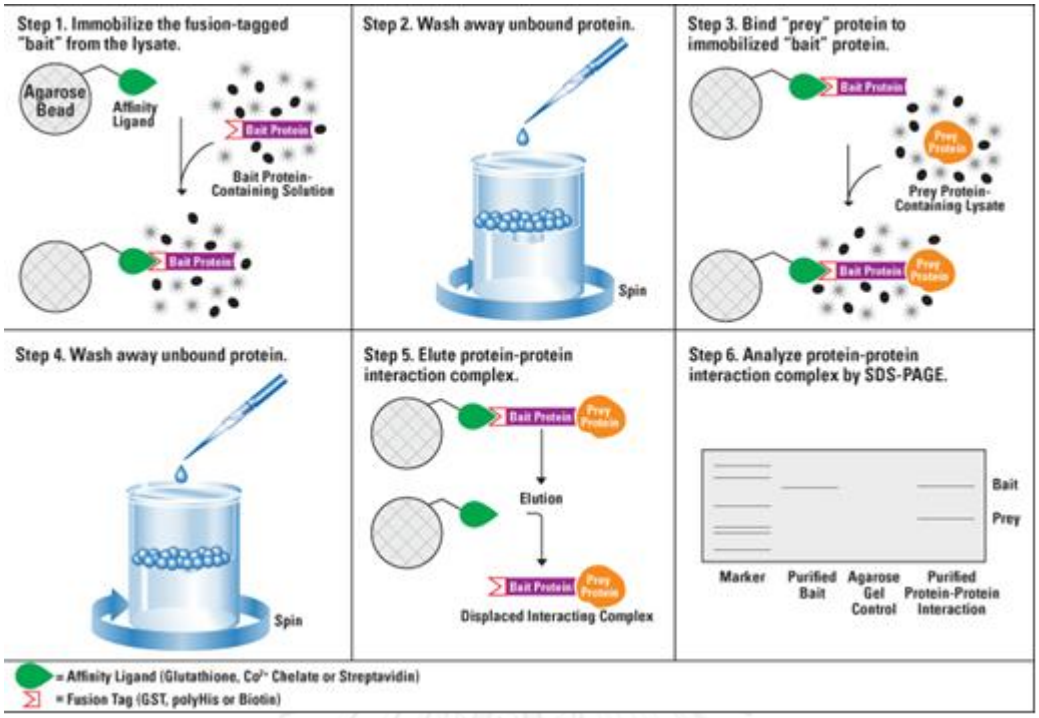
1.7.4 Antiviral activity

ALF from crayfish was firstly reported for its involvement in WSSV infection response (Liu et al., 2006a). ALF*Pm*3 was tested to neutralize WSSV and reduce WSSV infectivity in shrimp (Tharntada et al., 2009). The ALF*Pm*3 is the only shrimp AMP that are reported to exhibit an anti-WSSV activity. The transcript of ALF*Pm*3 up regulated upon *V. harveyi* but also WSSV infection revealing its roles in the shrimp immune response (Ponprateep et al., 2012; Somboonwiwat et al., 2008). Silencing of ALF*Pm*6 in *P. monodon* led to increased shrimp mortality after WSSV infection (Ponprateep et al., 2012). The rALF*Pm*3 can efficiently neutralize the bacteria's pathogenicity to

shrimp and protect *P. monodon* from WSSV infection (Tharntada et al., 2009). Antiviral activity of ALF and its derivatives have been reported. The cyclic synthetic fragment of ALF has been shown to exhibit antiviral activity against nervous necrosis virus (NNV), a fish non-envelope viral pathogen, by agglutinating the NNV virion into a clump (Chia et al., 2010). Also, synthetic ALF based on the LPS-BD of *Limulus* ALF block the viral entry of human pathogenic viruses such as HIV-1, HCV, and HSV1 and 2 by binding to the docking molecule on the host cell surface (Hoffmann et al., 2014; Krepstakies et al., 2012). *In vivo*, synthetic peptide of LPS-BD of *Fc*ALF could reduce the propagation of WSSV, while only the lysine residues in LPS-binding domain is indispensable in the anti-WSSV property (Guo et al., 2014). However, the antiviral mechanism(s) of ALF against WSSV are not understood. The recombinant protein of ALF, rALF*Pm*3, showed apparent antiviral activity against herpes simplex virus type 1 and human adenovirus respiratory strain (Carriel-Gomes et al., 2007). A recent report showed that the expressions of ALFs in *F. chinensis* were up-regulated when the WSSV infection process transformed from latent infection period to acute infection period (Li et al., 2013). The *nLvALF1*was specifically expressed in lymphoid organ (Oka) of shrimp. Its transcriptional level was significantly up regulated after white spot syndrome virus (WSSV) challenge, suggesting that *nLvALF1* might participate in the defense against WSSV in *Litopenaeus vannamei* (Liu et al., 2014). All these results suggested that ALF might play important roles in defense against WSSV infection.

1.8 *In vitro* pull-down assay

The pull-down assay is an *in vitro* method used to determine a physical interaction between two or more proteins. Pull-down assays are useful for both confirming the existence of a protein-protein interaction predicted by other techniques and as an initial screening assay for identifying previously unknown protein-protein interactions. In this technique, a bait protein is tagged and captured on an immobilized affinity ligand; for example, polyhistidine tag for Ni^{+} immobilized beads, and used to purify interacting proteins from a protein solution (Kaelin, 1999). The fusion protein and cell lysate are mixed in the presence of specific beads and incubated to allow protein associations to occur. The fusion proteins and any associated molecules are collected by centrifugation. The unbound proteins are washed out. The complexes are then eluted from the beads. Then, the protein-protein interactions are determined by SDS-PAGE (Fig. 1.7). The *in vitro* pull-down technique is especially useful for probing protein interactions in solutions that might go undetected in a membrane assay. This method of detecting interactions is determined by the availability of antibodies to the target protein.



(Source: http://www.piercenet.com)

Figure. 1.7 A schematic of *in vitro* pull-down assay

1.9 Objectives of the dissertation

ALF has been shown to interfere with WSSV replication in both *in vitro* cell cultures and *in vivo* in animals. However, the mechanism(s) of how the ALF neutralize WSSV are not known. Previously, five ALF*Pm*3-interacting proteins from WSSV have been identified including WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471 protein. It is possible that the direct binding between ALF*Pm*3 and WSSV proteins implied how the ALF*Pm*3 neutralizes WSSV. We aim to further confirm the interaction between ALF*Pm*3 protein and WSSV proteins as well as study the effect of the binding on ALF*Pm*3 anti-WSSV activity. Like WSSV189 and WSSV471 proteins, in this study, the interactions between the recombinant ALF*Pm*3 (rALF*Pm*3) and the recombinant WSSV458 (rWSSV458) was confirmed by *in vitro* pull-down assay. The effect of WSSV proteins (rWSSV189, rWSSV458, and rWSSV471) binding to ALF*Pm*3 on

WSSV-neutralizing activity of the rALF*Pm*3 was determined. The identified ALF*Pm*3-interacting proteins such as WSSV189 and WSSV471 were further characterized. The localization of WSSV189 and WSSV471 proteins was identified by Western blot analysis and immunoelectron microscopy (IEM).

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

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

Autoclave model # MLS-3750 (SANYO E&E Europe (UK Branch) (UK Co.)

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

ÄKTA Prime Plus FPLC Purification System (GE Healthcare)

Balance PB303-s (Mettler Teledo)

Biophotometer (eppendrof)

Centrifuge 5804R (eppendrof)

Centrifuge Avanti™ J-301 (Beckman Coulter)

Centrivap Concentrator (LABCONCO)

-20°C Freezer (Whirlpool)

-80°C Freezer (Thermo Electron Corporation)

Force mini centrifuge (Select BioProducts)

Gel Documention System (GeneCam FLEX1, Syngene)

GelMate2000 (Toyobo)

Gene pulser (Bio-RAD)

Incubator 30°C (Heraeus)

Incubator 37°C (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

Laminar Airflow Biological Safety Cabinets ClassII Model NU-440-400E (NuAire, Inc., USA)

Nickel Grids, 300 mesh, G300Ni (EMS)

Copper Grids, 300 mesh, G300Cu (EMS)

Microcentrifuge tube 0.6 ml and 1.5 ml (Axygen®Scientific, USA)

Minicentrifuge (Costar, USA)

Mini-PROTEAN® 3 Cell (Bio-RAD)

Nipro disposable syringes (Nissho)

Optima™L-100 XP Ultracentrifuge (Beckman Coulter)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendrof AG, Germany)

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

pH-meter pH 900 (Precisa, USA)

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

Power supply, Power PAC3000 (Bio-RAD Laboratories, USA)

Refrigerated incubator shaker (New Brunswick Scientific, USA)

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Sonicator (Bandelin Sonoplus, Germany)

SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices)

Touch mixer Model#232 (Fisher Scientific)

Trans-Blot®SD (Bio-RAD Laboratories)

Transmission electron microscope (Jeol Japan JEM-2100)

Water bath (Memmert)

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

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

2.1.2 Chemicals and Reagents

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

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

5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Fermentas)

Absolute alcohol, C_2H_5OH (Hayman)

Acetic acid glacial, CH_3COOH (Merck)

Acrylamide, C_3H_5NO (Merck)

Agarose, low EEO, Molecular Biology Grade (Research Organics)

Agar powder, Bacteriological (Hi-media)

Aquacide I (Calbichem®)

Alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson Immuno Research Laboratories, Inc.)

Ammonium persulfate, $(NH_4)_2S_2O_8$ (Bio-Rad)

Anti-β-actin antibody (Sigma)

Anti-His antibody (Merck)

Biotin (Sigma-Aldrish)

Bovine serum albumin (Fluka)

Bromophenol blue (Merck, Germany)

Casein Enzyme Hydrolysate, Type-I, Tryptone Type-I (Hi-media)

Casein Peptone (Hi-media)

Chloroform, $CHCl_3$ (Merck)

Chemiluminescence HRP (Perkin Elmer, Inc)

CL-XPosure™ Film (5×7 inches) (Thermo scientific)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

D-Glucose anhydrous (Ajax)

Developer solution (Kodak Scientific)

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

Di-Potassium hydrogen phosphate anhydrous (Sigma)

Ethylene diamine tetraacetic acid disodium salt, EDTA (Ajax)

Ethidium bromide (Sigma)

GeneRuler™ 100bp DNA ladder (Fermentas)

GeneRuler™ 1kb DNA ladder (Fermentas)

Glycerol, $C_3H_8O_3$ (Ajax)

Glycine, USP Grade, NH_2CH_2COOH (Research organics)

Fixer solution (Kodak Scientific)

Hybond^{TM}-ECL membrane (GE Healthcare)

Hydrochloric acid (HCl) (Merck)

HiTrap SP HP column (GE Healthcare)

Imidazole (Fluka)

Isopropanol, C_3H_7OH (Merck)

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

Magnesium chloride, $MgCl_2$ (Merck)

Methanol, CH_3OH (Merck)

0.22 μM and 0.45 μM Millipore membrane filter (Millipore)

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

N, N´-methylenebisacrylamide, $C_7H_{10}N_2O_2$ (USB)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Page Ruler™ unstained protein Ladder (Thermo Scientific)

Page Ruler™ Prestained Protein Ladder (Thermo Scientific)

Paraformaldehyde (Sigma)

Peptone (Sigma)

Phenol:chloroform:isoamyl alcohol (Sigma)

Phosphoric acid (Labscan)

Potassium dihydrogen phosphate (Sigma)

Skim milk powder (Mission)

Silver nitrate (Merck)

Sodium carbonate anhydrous (Carlo Erba)

Sodium chloride, NaCl (Ajax)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

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

Sodium hydrogen carbonate, $NaHCO_3$ (BDH)

Sodium dihydrogen orthophosphate, $NaH_2PO_4.H_2O$ (Ajax)

di-Sodium hydrogen orthophosphate anhydrous, NaH_2PO_4 (Ajax)

Sodium hydroxide, NaOH (Merck)

Triton® X-100 (Merck)

TriReagent® (Molecular Research Center)

Tris (Vivantis)

Tryptic soy broth (Difco)

Tween™-20 (Fluka)

Urea (Affy Metrix USB)

Yeast extract (Sigma)

YNB (Difco)

2.1.3 Kits

High-speed plasmid mini kit (Geneaid)

Nucleospin® Extract II kit (Macherey-Nagel)

RevertAID™ first strand cDNA synthesis kit (Fermentas)

2.1.4 Enzymes

Taq DNA polymerase (RBC Bioscience)

2.1.5 Antibiotics

Ampicillin (BioBasic)

Chloramphenicol (Sigma)

G418 sulfate (Gibco®)

2.1.6 Bacterial, yeast and virus strains

Bacillus megaterium

Escherichia coli strain XL-1-Blue

E. coli strain 363

E. coli strain BL21(DE3)

E. coli strain BL21-CodonPlus(DE3)-RIL

Pichia pastoris strain KM71

White spot syndrome virus (WSSV)

2.2 General protocols

2.2.1 Agarose gel electrophoresis

The agarose gel was prepared by dissolving the agarose gel powder in 1× TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). Then the agarose gel was melted and the solution was cooled down until 50 °C. The solution was poured into a tray equipped with comb for a well-forming. After the gel was polymerized, it was loaded into the chamber with 1× TBE running buffer. The DNA samples mixed with 1X loading dye (final concentration) (50 mM Tris-HCl, 2.5 mg/ml

bromophenol blue, 2.5 mg/ml xylene cyanol, 60% glycerol at pH 7.6) were loaded into the wells. Next, the standard DNA markers (100 bp or 1 kb DNA ladders) were loaded into the well. The DNA was separated by electrophoresis at 100 volts for 25 min in 1× TBE buffer. The gel was stained with ethidium bromide solution for 1 minute and de-stained in the water for 20 min. The DNA bands were visualized under the UV transilluminator.

2.2.2 Protein analysis

2.2.2.1 Analysis of recombinant protein by SDS-PAGE

The gel solutions for 15% separating gel (2.50 ml of 30% acrylamide, 1.27 ml of 1.5 M Tris-HCl pH 8.8, 50 µl of 10% SDS, 50 µl of 10% APS, 2 µl of TEMED and 1.13 ml of distilled water) and 5% stacking gel (500 µl of 30% acrylamide, 380 µl of 1 M Tris-HCl pH 6.8, 30 µl of 10% SDS, 30 µl of 10% APS, 3 µl of TEMED and 2.06 ml of distilled water) were prepared. The separation gel solution was poured into the glass plates with 1 mm spacer and covered on top with distilled water. After 30 min, the gel was completely polymerized. Then, the stacking gel solution was poured on top of the separating gel and comb was immediately put between the glass plates. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with distilled water to remove excess un-polymerized acrylamide. The protein samples were prepared by resuspending the proteins in 1× SDS loading buffer (12 mM Tris-HCl, pH 6.8, 5% glycerol, 0.4% SDS, 0.02% bromophenol blue, and 2.88 mM 2-mercaptoethanol). The samples were then boiled for 5 min and spun down. The samples and protein standard marker were loaded into gel. Electrophoresis was run in 1× SDS running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 0.1% (w/v) SDS) with a constant current of 25 mA per gel until the dye font search the end of the gel. After electrophoresis, the gel was stained in the Coomassie brilliant blue R250 staining solution (0.1% (w/v) Coomassie brilliant blue R250, 10% (v/v) acetic

acid, 45% (v/v) methanol) at room temperature with gentle shaking for 1 h. Next, the gel was destained using the destaining solution (10% (v/v) acetic acid, 10% (v/v) methanol) and shaken at room temperature until the gel background was clear.

2.2.2.2 Protein detection by Western blot analysis

To detect the expected protein, the Western blot analysis was performed using a specific antibody. After protein separation by SDS-PAGE, the gel was soaked in the transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol). The nitrocellulose membrane and two pieces of thick filter papers whose size is same as the gel size, were soaked in the transfer buffer for 30 min. Then, the first filter paper nitrocellulose membrane, the gel, and the second filter paper nitrocellulose membrane, respectively were placed onto the platform of Trans-Blot® SD (Bio-Rad) and the air bubble was removed by rolling over the surface of filter paper by pipette. Then, the protein was transferred at constant 110 mA for 90 min. Then, the nitrocellulose membrane was blocked in a blocking solution (5% (w/v) skim milk in 1× PBS buffer and 0.05% (v/v) Tween™-20 at pH 7.4 (PBS/Tween™-20)) at room temperature with gentle shaking for 1 h. Then, the membrane was washed for 3 times 10 min each with 1×PBS/Tween™-20. After that, the membrane was incubated with the primary antibody that was diluted at the appropriate concentration in 1×PBS/Tween™-20 containing 1% (w/v) skim milk for 3 h at 37 °C with gentle shaking. Subsequently, the membrane was washed for 3 times 10 min each with PBS/Tween™-20 again to remove the unbound protein. The secondary antibody conjugated with alkaline phosphatase or horseradish peroxidase (HRP) (Jackson Immuno Research Laboratories, Inc.) was diluted in 1×PBS/Tween™-20 containing 1% (w/v) skim milk at room temperature for 1 h. After washing, the substrate NBT and BCIP (Fermentas) at the final concentration of 375 and 188 μg/ml, respectively, in 100 mM Tris- HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5 was

incubated with membrane for alkaline phosphatase detection. When the bands of protein were detected, the reaction was stopped by washing the membrane with distilled water. For HRP detection, Western Lightning® Plus-ECL, enhanced chemiluminescence substrate (Perkin Elmer, Inc) was used. The chemiluminescence reagent was prepared by mixing an equal volume of the Enhanced Luminol Reagent and the Oxidizing Reagent. Then, the membrane was incubated in the chemiluminescence reagent (0.125 ml of Chemiluminescence reagent per cm^2 of membrane) for 1 min with gentle agitation. Next, the protein band was visualized after removing the excess chemiluminescence reagent by placing the membrane into a cassette. The membrane was then exposed to CL-XPosure™ Film (5×7 inches) (Thermo scientific) at the optimum time intervals. Then, the film was developed in developer solution (Kodak Scientific) for 1 min, washed in the water for 1 min and placed in fixer solution (Kodak Scientific) to stop reaction.

2.2.2.3 Determination of protein concentration

The protein concentration was measured based on the method of Bradford (Bradford, 1976) using bovine serum albumin (Fluka) as a standard protein. Once of Coomassie brilliant blue G250 dye binds to proteins, the red color of reaction was converted to the blue solution, and protein was detected by monitoring the absorption at 595 nm. A sample solution (100 µl) was mixed with 1 ml Bradford working buffer and incubated for 10 min at room temperature, A_{595} was subsequently measured.

2.3 Animal

The black tiger shrimp, *Penaeus monodon*, each weighing of about 3-5 grams were purchased from local shrimps farm at Chachoengsao province and Suratthani provinces, Thailand. They were used for cumulative mortality experiment. The

pathogen-free white shrimp, *Litopenaeus vannamei*, each weighing of about 10-15 grams were supported by Charoen Pokphand Group, Thailand. The white shrimps were used to produce WSSV stock. All shrimp were acclimatized in the laboratory aquaria and let them adapt to new environment at 15 ppt salinity at least 1 week before used in each experiment.

2.4 WSSV challenged shrimp

2.4.1 Preparation of the purified WSSV for the experimental infection

WSSV stock for the experimental infection was firstly prepared according to the method (Xie et al., 2005a). The WSSV stock was diluted in the TN buffer (20 mM Tris-HCl pH 7.4 and 400 mM NaCl). *L. vannamei* were intramuscularly infected with 100 µl of WSSV solution and cultured until they are moribund. Gills were dissected from the moribund shrimp and then homogenized in the TNE buffer (50 mM Tris-HCl pH 8.5, 400 mM NaCl, 5 mM EDTA). After centrifugation at 5,000 × g for 5 min at 4 °C, the supernatant was filtered through 0.45 µM a sterile filter membrane and centrifuged at 30,000 × g for 30 min at 4 °C. The pellet was rinsed with the TM buffer (50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$) and the loose brown pellet was gently blowed out by pasture pipette. After centrifugation at 5,000 × g for 5 min at 4 °C, the first pellet of WSSV was collected and resuspended in the TM buffer and the supernatant were centrifuged at 30,000 × g for 30 min at 4 °C. After that, the supernatant was discarded and the second pellet of WSSV was collected and resuspended in the TM buffer. Finally, the purified virus was devided into aliquots and kept at -80 °C until used.

2.4.2 WSSV challenge

One microliter of WSSV stock was serial diluted in the TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4) to 10^{-9} dilution. Fifty microliters of the purified WSSV at

the dilution of 10^{-9} was injected into the second abdominal segment of the shrimp. This dosage resulted in 100% shrimp mortality within 5 days.

2.5 Preparation of the purified recombinant proteins

In this research, three recombinant proteins of ALF*Pm*3-interacting proteins from WSSV such as WSSV189 (rWSSV189), WSSV458 (rWSSV458) and WSSV471 (rWSSV471) and the recombinant ALF*Pm*3 protein (rALF*Pm*3) were expressed and purified. The rWSSV189, rWSSV458 and rWSSV471 proteins were expressed in the *Escherichia coli* system as described previously (Jaree, 2014; Suraprasit, 2012). On the other hand, the rALF*Pm*3 protein was expressed and purified from yeast *Pichia pastoris* according to the previous report (Somboonwiwat et al., 2005).

2.5.1 Expression of recombinant WSSV proteins

The glycerol stock of *E. coli* stain BL21-CodonPlus(DE3)-RIL containing recombinant plasmid pET-19b expressing rWSSV189 and rWSSV471 proteins were streaked on LB agar plate (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 1.5% agar) supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The glycerol stock of *E. coli* stain BL21 (DE3) containing recombinant plasmid pET-16b expressing rWSSV458 protein was streak on LB agar plate supplemented with 100 µg/ml ampicillin only. Each LB agar plate was incubated at 37 $^{\circ}$C for an overnight. As a starter culture, a single colony of each clone was inoculated into 50 ml of LB broth containing 100 µg/ml of ampicillin and 50 µg/ml of chloramphenicol for rWSSV189 and rWSSV471 proteins and LB broth containing 100 µg/ml of ampicillin for rWSSV458 protein and grown with shaking at 250 rpm, 37 $^{\circ}$C for overnight. The starter was inoculated into 500 ml of fresh LB broth (1:100 v/v) and cultured at 250 rpm, 37 $^{\circ}$C until the OD_{600} reached 0.4. The 1 M

isopropyl-β-galactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the recombinant protein expression. The cells were collected at 2 h after induction for rWSSV189 and 4 h after induction for rWSSV458 and rWSSV471 protein. The over-production of recombinant proteins was analyzed by 15% SDS-PAGE before further processes. Expression by centrifugation at 8,000 × g for 10 min at 4 $^{\circ}$C, the pellet of cell expressing the expect recombinant protein was resuspended with 40 ml of 1X PBS. The proteins were frozen-thawed for 3 times and the cells were lyzed by sonication (Bandelin Sonoplus, Germany). After that, the inclusion bodies and soluble fraction were separated by centrifugation at 10,000xg for 15 min and analyzed by 15% SDS-PAGE and Western blot analysis using polyclonal antiserums against rWSSV189, rWSSV458 and rWSSV471 protein as primary antibodies at the dilution of 1:3,000 and goat anti-mouse IgG as a secondary antibody at the dilution of 1:10,000. The crude proteins were kept at -80 $^{\circ}$C until they were subjected to purification.

2.5.2 Purification of recombinant WSSV proteins

2.5.2.1 Purification of the rWSSV189 protein

The rWSSV189 proteins was purified as reported previously (Suraprasit, 2012). The cells expressing the crude rWSSV189 protein in 1× PBS buffer were frozen and thawed for 3 times and the cells were lyzed by a sonicator (Bandelin Sonoplus, Germany). Then, the inclusion bodies of the rWSSV189 protein were collected. The rWSSV189 protein was purified under non-denaturing condition using nickel affinity chromatography. The solubilized crude proteins were dialyzed against 20 mM sodium carbonate buffer, pH 10, to refold protein then the protein was purified using Ni Sepharose 6 Fast Flow bead (GE Healthcare). The Ni Sepharose 6 Fast Flow bead was packed into the column and washed with 10 column volumes of distilled water and equilibrated with 10 column volumes of binding buffer (20 mM sodium carbonate

buffer, pH 10 containing 0.5 M NaCl, and 20 mM imidazole). The crude rWSSV189 protein was applied to the column and let it incubated with the beads at room temperature for 2 h. Then, the column was washed with 10 column volumes of binding buffer to remove unbound proteins. The bound protein was eluted with 5 column volumes of 20 mM sodium carbonate, 0.5 M NaCl buffer pH 10 containing 100 mM imidazole. The elution fractions were run on 15% SDS-PAGE to check the purity of the protein and Western blot analysis using the mouse anti-rWSSV189 polyclonal antiserum as a primary antibody and the alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody at the dilution of 1:3,000 and 1:5,000, respectively. The fractions containing expected recombinant protein were pooled and the imidazole was removed by dialysis one hundred thousand fold at 4 °C against 0.1× PBS, pH 7.4.

2.5.2.2 Purification of the rWSSV458 protein

The cells containing the crude rWSSV458 protein was frozen and thawed for 3 times and then lyzed by a sonicator (Bandelin Sonoplus, Germany). As described for rWSSV189 protein purification, the pellet of inclusion bodies was dissolved in 20 mM Tris-HCl pH 7.4 containing 6 M urea. On the other hand, the His_6-tag rWSSV458 protein was purified by nickel affinity chromatography using the denaturing condition. First, the Ni Sepharose 6 Fast Flow bead (GE healthcare) was equilibrated with the binding buffer (20 mM Tris-HCl supplement with 6 M urea, pH7.4 containing 0.3 M NaCl, and 20 mM imidazole). The protein was incubated with Ni bead at room temperature for 1 h. Then, the Ni-bead was washed by the binding buffer for 10 column volumes to remove unbound proteins. Next, the rWSSV458 protein was eluted by the elution buffer (20 mM Tris-HCl supplement with, 0.3 M NaCl and 6 M urea, pH 7.4) containing 50 mM imidazole, 100 mM imidazole, 150 mM imidazole, 250 mM imidazole or 500 mM imidazole. The purified fractions were

analyzed by 15% SDS-PAGE. The fractions containing the expected recombinant protein were pooled and the imidazole and urea were removed by dialysis one hundred thousand fold at 4 ℃ against 0.1× PBS, pH 7.4. The protein was concentrated by aquacide I (Calbichem®) and quantified using Bradford assay. The purified protein was analyzed by 15% SDS-PAGE and Western blot analysis using a polyclonal mouse anti-rWSSV458 antiserum as a primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody at the dilution of 1:3,000 and 1:5,000, respectively.

2.5.2.3 Purification of the rWSSV471 protein and antibody production

The cells containing the crude recombinant protein, rWSSV471, was frozen and thawed for 3 times and subjected to sonication using a sonicator (Bandelin Sonoplus, Germany). After that, the inclusion bodies were collected by centrifugation at 10,000 × g for 15 min. The rWSSV471 was solubilized with 20 mM Tris-HCl pH 7.4 containing 6 M urea. The rWSSV471 protein containing His_6-tag was purified by nickel affinity chromatography using the denaturing condition. First, the Ni Sepharose 6 Fast Flow bead (GE healthcare) was equilibrated with the binding buffer (20 mM Tris-HCl supplement with 6 M urea, pH7.4). The crude protein was incubated with Ni bead at room temperature for 2 h. Then, the Ni-bead was washed with 10 column volumes of the binding buffer or until the unbound proteins was removed. Next, the rWSSV471 protein was eluted using a serie of the elution buffer (20 mM Tris-HCl supplement with 6 M urea, pH 7.4) containing 50 mM imidazole, 100 mM imidazole, 150 mM imidazole and 500 mM imidazole. The fractions of the purified rWSSV471 protein were analyzed by 15% SDS-PAGE. The fractions containing expected recombinant protein were pooled and the imidazole and urea were removed by dialysis one hundred thousand fold at 4 ℃ against 0.1× PBS, pH 7.4. The

protein was concentrated by aquacide I (Calbichem®) and quantified using Bradford assay. The purified protein was analyzed by 15% SDS-PAGE and Western blot analysis using a polyclonal mouse anti-rWSSV458 antiserum as a primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody at the dilution of 1:30,000 and 1:10,000, respectively. Because the low immuno activity of the previous preparation of the mouse anti-WSSV471 polyclonal antibody, five hundred micrograms of purified WSSV471 protein was used to immunize a mouse in order to produce a new polyclonal antibody specific to rWSSV471 at the Biomedical Technology Research Unit, Chiangmai University, Thailand.

2.5.3 Expression of the recombinant ALF*Pm*3 protein

The glycerol stock of the yeast, *Pichia pastoris* strain KM71 containing ALF*Pm*3 expression cassettes (Somboonwiwat et al., 2005) was streaked on the YPD agar plate (1% yeast extract, 2% peptone, 2% dextrose and 1.5% agar) and incubated at 30 $^{\circ}$C for 3-5 days. A single colony was inoculated into YPD broth medium and incubated at 30 $^{\circ}$C, 280 rpm for overnight. The starter was inoculated at 1:100 ratio into 400 ml of fresh BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% yeast nitrogen base with ammonium sulphate without amino acid (YNB), 0.02% biotin, and 10% glycerol). The cells were cultured until OD_{600} reached 14 and harvested by centrifugation at 8,000 rpm for 10 min at 4 $^{\circ}$C. The cell pellet was resuspended in 40 ml BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6, 1.34% YNB, 0.02% biotin, and 5% glycerol). After that, the protein production was induced by adding 100% methanol every 24 h to maintain the final concentration at 0.5%. The secreted-rALF*Pm*3 protein in the supernatant was collected at 2 days of induction after centrifugation at 8,000 rpm for 10 min at 4 $^{\circ}$C. The crude rALF*Pm*3 protein was analyzed by 15% SDS-PAGE with Coomassie blue staining.

2.5.4 Purification of the recombinant ALF*Pm*3 protein

The crude rALF*Pm*3 protein was purified by a strong cation exchange chromatography, 5 ml HiTrap SP HP column (GE healthcare) using AKTA Prime Plus Purification System (GE healthcare). The crude protein was diluted 1:1 with the start buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7). The process of purification was controlled with the flow rate at 3 ml/min. First, the column was equilibrated with the start buffer 20 column volume. Then, the rALF*Pm*3 protein was loaded into the column and washed with the start buffer to remove unbound proteins until A_{280} decreased to zero. In the next step, the purified rALF*Pm*3 protein was eluted by the elution buffer (20 mM Tris-HCl, 1 M NaCl, pH 7). The rALF*Pm*3 protein containing fractions was collected and analyzed by Coomassie stained 15% SDS-PAGE. The purified protein was dialyzed one hundred thousand fold against distilled water at 4 °C. Next, The purified rALF*Pm*3 protein was concentrated by aquacide I (Calbichem®) for 30 min and quantified by measuring A_{280}. The ALF*Pm*3 concentration was calculated using the following equation (Somboonwiwat et al., 2005).

$$[\text{rALF}Pm3]\ (\text{M}) = \frac{A_{280} \times \text{dilution factor}}{33270}$$

2.5.5 Antimicrobial activity assay of the rALF*Pm*3 protein

The antimicrobial activity of the purified rALF*Pm*3 protein was tested against Gram-negative bacterium, *E. coli* 363, and a Gram-positive bacterium, *B. megaterium*. The minimum inhibitory concentration (MIC) values were used to determine the activity. The glycerol stock of *E. coli* 363 and *B. megaterium* were streaked on the LB agar plate. A single colony of *E. coli* 363 and *B. megaterium* were grown in 5 ml of the LB broth with shaking at 250 rpm, 37 °C for overnight. The overnight culture was

inoculated (1:100 (v/v)) into the fresh LB broth and incubated with shaking at 250 rpm, 37 °C until the OD_{600} reached 0.1. Then, the culture was diluted with the poor broth (1% tryptone type-1, 0.5% NaCl, pH 7.5) to an OD_{600} of 0.001. One-hundred-microliters of diluted *E. coli* 363 and *B. megaterium* cultures were mixed with 20 µl of rALF*Pm*3 at various concentrations in a 96-well microtiter plate. Twenty microliters of the distilled water mixed with 100 µl of the poor broth were used as a positive control. The reactions were cultured for overnight with shaking at 180 rpm, 30 °C. The growth of bacteria was measured at OD_{600} using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices). The MIC value was recorded as the range between the highest concentration of the peptide where bacterial growth was observed and the lowest concentration of the peptide that cause 100% of inhibition bacterial growth.

2.6 Protein-protein interaction assay between WSSV458 and ALF*Pm*3 by *in vitro* pull-down assay

The interaction between WSSV458 protein and ALF*Pm*3 protein was confirmed by *in vitro* pull-down assay. The purified recombinant protein of WSSV458 and ALF*Pm*3 were used for the investigation. The Ni Sepharose (GE healthcare) was used to trap a bait protein, 6X His-rWSSV458 protein then the interaction between rALF*Pm*3 and rWSSV58 was tested. Briefly, one-hundred-microliters of the suspended Ni Sepharose (GE Healthcare) were loaded into a spin column. The beads were equilibrated with 10 column volumes of the wash solution (1X Tris buffer saline (50 mM Tris-HCl, 150 mM NaCl) pH 7.2 containing 40 mM imidazole). The wash solution was discarded by centrifugation at 1,250 × g for 1 min at 4 °C. Then, the excess amount (55 µM) of rWSSV458 protein was incubated with the pre-equilibrated Ni beads at 4 °C for 1 h with gentle rocking motion on a rotating platform. Next, the rWSSV458 protein was removed from the column by centrifugation and it named the

bait flow-through fraction. After that, the Ni bead was washed with excess volume of the wash solution until the all the unbound protein was removed. In this step, the last fraction of wash solution was kept for checking on 15% SDS-PAGE. After that, 280 μM of the rALF*Pm*3 protein, a prey protein was incubated with the rWSSV458 bound-Ni bead at 4 $^{\circ}$C for 1 h with gentle rocking motion. Next, the unbound rALF*Pm*3 protein designated as the prey flow-through fraction was removed from the column by centrifugation. Then, the column was washed with wash solution until the unbound rALF*Pm*3 protein was completely removed. The last fraction of wash solution was kept for checking on 15% SDS-PAGE. Then, the protein complex of rALF*Pm*3 and rWSSV458 was eluted by adding 300 μl of the elution buffer (50 mM Tris-HCl, 150 mM NaCl) pH 7.2 containing 150 mM imidazole) and incubated with gentle rocking motion at 4 $^{\circ}$C for 1 h. Then, the elution fraction was collected after centrifugation. The protein complex was analyzed by 15% SDS-PAGE and Western blot analysis using polyclonal anti-rWSSV458 antiserum as a primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG as a secondary antibody at the dilution of 1:3,000 and 1:5,000, respectively. For rALF*Pm*3 detection, the polyclonal anti-rALF*Pm*3 antiserum was used as a primary antibody and alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody at the dilution of 1:5,000 and 1:10,000, respectively. The bait control column was the Ni beads incubated in the same condition as above except that the rALF*Pm*3 incubation was omitted. The prey control column was performed as same as the protein complex assay but the rWSSV458 was omitted.

2.7 Effect of the rWSSV proteins binding on WSSV-neutralizing activity of ALF*Pm*3

2.7.1 Determination of the dosage of WSSV used for cumulative mortality assay

To investigated the appropriate dosage of WSSV for cumulative mortality assay. Ten shrimps (3-5 grams body weight) were cultured individually in the box (30 cm×50 cm×30 cm) and acclimated for a 2- to 3-day period, in the 15 ppt salinity water (seawater; Marine Environment), and fed daily with commercial feed. Each box connected to a circulation system with spongefilter pump and air supply.

The purified WSSV was serially diluted in TN buffer to 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} dilutions. Equal volume (50 µl) of each diluted purified WSSV was incubated for 30 min at room temperature. Shrimp were injected at second abdominal segment with various concentrations of WSSV prepared. The shrimp cumulative mortality was observed and recorded every 12 h for the duration of 8 days. TN buffer was used as a control. The experiment was performed in triplicate. The 10^{-9} dilution of WSSV which caused 100% death of shrimp in 5 days was selected for cumulative mortality assay.

2.7.2 Determination of optimum concentration of the rALF*Pm*3 protein

In order to find an appropriate amount of rALF*Pm*3 that is effectively neutralized WSSV, various concentrations of the purified rALF*Pm*3 protein (100 µM, 200 µM and 280 µM) were determined for the WSSV-neutralizing activity. Shrimps were divided into five groups of 10 shrimp each (3-5 g body weight). The rALF*Pm*3 protein was incubated with WSSV at 10^{-9} dilution for 30 min at room temperature (Tharntada et al., 2009). After that, rALF*Pm*3 neutralized WSSV was injected at second abdominal segment of the shrimp. The cumulative mortality of shrimp was observed

and recorded every 12 h for the duration of 8 days. TN buffer and WSSV were used as a control. The experiment was performed in triplicate.

2.7.3 Cumulative mortality assay

In this experiment, 10 shrimps of 3-5 g body weight was used for each group. For the WSSV-infected control group, 50 µl of 10^{-9} diluted purified WSSV adjusted volume to 150 µl with the TN buffer was injected into the second abdominal segment of the shrimp. The percentage of this dosage decreased to 0% within 5 days. For the injection control group, 150 µl of TN buffer only was injected into the shrimp. The group showing WSSV neutralizing activity of rALF*Pm*3 was shrimp injected with 150 µl mixture of WSSV pre-incubated with 280 µM rALF*Pm*3 in the TN buffer for 30 min at room temperature. Same amount of WSSV as above was to study the effect of rWSSV protein (rWSSV189, rWSSV458 and WSSV471 proteins) binding on the WSSV-neutralizing activity of rALF*Pm*3. The rWSSV proteins at a concentration much lower or at least equal if compared to that of the rALF*Pm*3 protein, in this case at 1:4 (rWSSV189:rALF*Pm*3), 1:5 (rWSSV458:rALF*Pm*3) and 1:4 (rWSSV471:rALF*Pm*3) by mole, were used. For the test groups, 280 µM rALF*Pm*3 were incubated with each of the rWSSV proteins (77 µM of the rWSSV189 protein, 55 µM of the rWSSV458 protein and 70 µM of the rWSSV471 protein in TN buffer) for 10 min and, then incubated with WSSV for another 30 min before being injected into the shrimp. In order to prove that the rWSSV proteins used here have no effect on WSSV infection, shrimp were injected with a 150 µl mixture of WSSV pre-incubated with each of rWSSV proteins at the same concentration of the test groups for 30 min.

The glutathione-S-transferase (GST) protein was used as a control protein as of the test groups, but each of rWSSV protein was replaced by the GST protein. The

percentage of survival of shrimp was observed every 12 h post WSSV infection for the duration of 10 days. The experiment was performed in triplicate.

2.8 Confirmation of WSSV infection in shrimp

To confirms the effect of rWSSV proteins on WSSV replication. The black tiger shrimp, *P. monodon* (weighting of about 3-5 g) were divided into six groups of 12 shrimps each and injected with either WSSV only (control) or each of the rWSSV proteins (rWSSV189, rWSSV458 or rWSSV471 protein) treated WSSV. At 0, 24 and 48 h post-injection, the hemolymph was collected from 2 individuals. The total RNA was extracted and cDNA was synthesized. VP28 gene was subsequently checked by Reverse transcription polymerase chain reaction (RT-PCR).

2.8.1 Total RNA extraction and cDNA synthesis

2.8.1.1 Total RNA extraction

At 0, 24 and 48 h post-injection, hemolymph was individually collected. The hemolymph samples were briefly mixed with 500 µl of TriReagent® (Molecular Research Center). Then, 200 µl of chloroform were added. Each sample was vortexed for 15 sec and let it stand at room temperature for 10 min before centrifugation at 12,000 × g for 15 min at 4 °C. The colorless upper aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube. The total RNA was precipitated with 500 µl of cold isopropanol. The mixture was left at room temperature for 10 min and then centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was removed and the pellet of total RNA was washed in 1 ml of 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water and centrifuged at 13,500 × rpm for 15 min at 4 °C. The ethanol was completely removed by pipetting. The RNA pellet was briefly air-dried for 10 min. The total RNA was dissolved with an

appropriate amount of DEPC-treated water and kept on ice until it was completely dissolved. The purified total RNA was kept at -80 ℃ until used.

2.8.1.2 Determination of the quantity and quality of RNA samples

The quantity of total RNA was measured by spectrophotometer at A_{260} nm. The concentration of total RNA was determined using the formular:

$$[RNA] = A_{260} \times \text{dilution factor} \times 40$$

One A_{260} corresponds to 40 μg/ml of RNA (Sambrook, 1989). The relative purity of RNA samples was examined by measuring the ratio of $A_{260/280}$. The maximum absorption of nucleic acid, and protein is at 260, and 280 nm, respectively. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. An approximately ratio above 1.7 is generally accepted as pure RNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The quality was further investigated through agarose gel electrophoresis. The gel was stained with EtBr and visualized under UV light, respectively.

2.8.1.3 DNase treatment of total RNA samples

The obtained total RNA was further treated with RQ1 RNase-free DNase (Promega). The reaction contains 5 μg of total RNA in 1× RNase-free DNase buffer and 1 unit of RQ1 RNase-free DNase. The DNase treatment reactions were incubated at 37 ℃ for 30 min to remove the contaminating chromosomal DNA. Then, the total RNA was purified by TriReagent® as described in section 2.8.1.1. The quantity and quality of total RNA was examined as described in section 2.8.1.2.

2.8.1.4 First-strand cDNA synthesis

The first strand cDNA was synthesized from 1 μg of the total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific). According to the kit's instruction, the reaction was performed by mixing 1 μg of the total RNA, 0.5 μg of the oligo$(dT)_{18}$ primer and adjusted the volume to 12 μl by DEPC-treated water. The reaction was incubated at 65 °C for 5 min and spun down and chilled on ice for 5 min to allow the primer to anneal to RNA. After that, 4 μl of 5X reaction buffer, 1 μl of RiboLock™ RNase inhibitor (20U/μl), 2 μl of 10 mM dNTP mix and 1 μl (200U/μl) of RevertAid™ M-MuLV reverse transcriptase were added and gently mixed. The reaction mixture (20 μl) was incubated 42 °C for 1 h and finally heated at 70 °C for 15 min to terminate the reaction. The cDNA was stored at -20 °C until used.

2.8.2 Reverse transcription polymerase chain reaction (RT-PCR)

To confirm the WSSV infection in shrimp, we studied the expression of VP28 gene, which is the late gene of WSSV, by reverse transcription-PCR (RT-PCR). cDNA of each group of the *P. monodon* infected with WSSV and each of the rWSSV proteins (rWSSV189, rWSSV471 or rWSSV458 protein) that WSSV was used as a template. The elongation factor-1α (EF-1α) gene was generally used as an internal control. The PCR reaction in a 12.5 μl total volume was carried out. The reaction contained 1 μ of 2 fold diluted cDNA template, 1.25 μl of 10× PCR buffer, 0.125 μl of 10 mM dNTP, 0.125 μl of 10 μM VP28-F primer, 0.125 μl of 10 μM VP28-R primer, 8.8125 μl of ultrapure water, and 0.0625 μl of 5 U/μl RBC *Taq* polymerase (Bioscience). The PCR condition was pre-denaturation at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec, and the final extension at 72 °C for 10 min. The PCR condition for EF-1α gene was pre-denaturation at 94 °C for 1 min followed by 27 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 30 sec,

and the final extension at 72 °C for 10 min. The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis.

Table. 1. 3 Primer pairs used for PCR amplification

Gene	**Primer**	**Sequence***	**PCR product**
EF-1α	EF-F	5'-GGTGCTGGACAAGCTGAAGGC-3'	145 bp
	EF-R	5'-CGTTCCGGTGATCATGTTCTTGATG-3'	
VP28	VP28-F	5'-ATCGCCATGCTATTTATTGTGATT-3'	600 bp
	VP28-R	5'-TAGCGGATCCCTCGGTCTCAGTGC-3'	

2.9 Detection of native WSSV proteins expressed in shrimp tissues

2.9.1 Preparation of shrimp's hemocytes and gills lysate supernatant

To detect the expression of WSSV proteins after WSSV infected shrimp. The 3-5 g, *P. monodon*, were infected with 50 µl of 10^{-7} diluted purified WSSV. This dosage resulted in a 100% shrimp mortality rate within 3 days. Hemolymph was withdrawn and the gills were dissected from the WSSV-infected *P. monodon* at 0, 6, 24 and 48 hpi. Hemocytes were collected by centrifugation at 800 × g, 4 °C for 10 min and homogenized in 1× PBS. The hemocyte lysate supernatant (HLS) was separated by centrifuged at 10,000 × g for 10 min at 4 °C. Gills were homogenized in 1× PBS containing 200 mM Phenylmethylsulfonyl fluoride (PMSF). Then, the gill lysate supernatant (GLS) was separated by centrifuged at 10,000 × g for 10 min at 4 °C. The protein concentration of a HLS and GLS was determined by the Bradford assay.

2.9.2 Detection of WSSV proteins in expressed hemocytes and gills of WSSV-infected shrimp

One hundred micrograms of HLS and GLS were separated by 15% SDS-PAGE and the ALF*Pm*3 and WSSV proteins were detected by Western blot analysis. A purified polyclonal rabbit anti-ALF*Pm*3 antibody (1:5,000) and purified polyclonal mouse anti-rWSSV protein antibodies at the dilution of 1:3,000 for WSSV458 detection and 1:5,000 for WSSV189 and WSSV471 detection, were used as the primary antibodies to detect native proteins by Western blot analysis, respectively. The horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000) was used as the secondary antibody for ALF*Pm*3 detection and the horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000) as the secondary antibody for WSSV proteins (WSSV189, WSSV458 and WSSV471) detection. For the loading control, β-actin was detected using anti-β-actin antibody (Sigma) at 1:5,000 dilution was used as the primary antibody and the horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000) was used as the secondary antibody. Western Lightning® Plus-ECL, (Perkin Elmer, Inc) was applied for protein detection by chemiluminescence HRP as described in section 2.2.2.2.

2.10 Localization of WSSV189 and WSSV471 structural proteins on WSSV virion

The purified WSSV used in for the following experiments were provided by Dr. Chu Fang Lo, Institute of Zoology, National Taiwan University, Taipei, Taiwan. It was prepared from crayfish, *Procambarus clarkiii* (Xie et al., 2005a) .

2.10.1 Localization of WSSV189 and WSSV471 structural proteins by Western blot analysis

To localize the WSSV proteins, the intact WSSV virion was fractionated into 2 parts which are envelope and nucleocapsid parts by treatment with Triton X-100.

First, the purified intact WSSV was pelleted by centrifuged at 13,000 rpm for 30 min at 4 $^{\circ}$C. Then, the pellet was resuspended in 200 µl of TMN buffer (20 mM Tris-HCl, 150 mM NaCl, 20 mM $MgCl_2$ pH 7.5). Two microliters of Triton X-100 was added to a final concentration of 1% and gently shaked for 30 min at room temperature. Then, the pellet and supernatant were separated by centrifugation at 13,000 rpm, 4 $^{\circ}$C for 30 min. The supernatant containing the envelope protein was collected. The pellet was resuspended with 200 µl of TMN buffer containing 1% Triton X-100 and incubated with gentle shaking for 30 min at room temperature. After centrifugation, the pellets were resuspended with 50 µl of TMN buffer and this fraction was "nucleocapsid fraction". The envelope and nucleocapsid fractions were separated by 12.5% SDS-PAGE gel. The WSSV189 and WSSV471 proteins were detected by Western blot analysis. A purified polyclonal mouse anti-rWSSV189 antibody (1:5,000) and a purified polyclonal anti-rWSSV471 antibody (1:5,000) were used as the primary antibodies. The membrane was incubated with the primary antibody for overnight at 4 $^{\circ}$C. Then, it was incubated with the horseradish peroxidase-conjugated goat anti-mouse IgG, the secondary antibody, at the dilution of (1:5,000) for 1 h at room temperature. Western Lightning® Plus-ECL, (Perkin Elmer, Inc) was applied for protein detection by chemiluminescence HRP as described in section 2.2.2.2.

2.10.2 Localization of WSSV189 and WSSV471 proteins on WSSV virion by immunoelectron microscopy (IEM)

Ten microliters aliquots of purified WSSV virion suspension prepared from *P. clarkii* were adsorbed to Formvar-supported, carbon-coated nickel grids (300 mesh) for 5 min at room temperature, and then the excess solution was removed. The grids were blocked with the blocking buffer (5% bovine serum albumin, 5% normal goat serum, 0.1% cold-water fish skin gelatin (Aurion), 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 30 min, incubated with the incubation buffer (0.1% Aurion BSA-c, 15 mM NaN_3, 10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 5 min, and then

incubated for 2 h at room temperature with the purified mouse anti-WSSV189 polyclonal antibody or the purified mouse anti-WSSV471 polyclonal antibody diluted 1:40 in the incubation buffer. As a control, an additional grid containing WSSV virions was incubated with a dilution of preimmune mouse serum. After three washes with the incubation buffer, the grids were incubated for 1 h at room temperature with a goat anti-mouse secondary antibody conjugated with 18-nm-diameter gold particles (1:100 dilution in the incubation buffer). The grids were then washed three times with the incubation buffer, washed three times with distilled water to remove the excess salt, and stained with 1% phosphotungstic acid (pH 7.2) for 3 min. Specimens were examined with a transmission electron microscope (Hitachi–Science Technology H-7650).

CHAPTER III

RESULTS

3.1 Preparation of the purified recombinant proteins

The purified recombinant WSSV proteins of rWSSV189, rWSSV458 and rWSSV471 were prepared. The purified rWSSV458 protein was used for *in vitro* pull-down assay to confirm its interaction with ALF*Pm*3. All purified rWSSV proteins was used for study the effect of their binding to rALF*Pm*3 on the anti-WSSV activity of the rALF*Pm*3 protein. Moreover, the antibody specific to rWSSV471 was also produced using the purified rWSSV471 obtained.

3.1.1 Expression of recombinant WSSV proteins

The recombinant WSSV proteins which are rWSSV189, rWSSV458 and rWSSV471 proteins were produced using the pET-19b *E. coli* expression system. The rWSSV189 and rWSSV471 proteins were expressed in *E. coli* strain BL21-CodonPlus(DE3)-RIL and the rWSSV458 protein was over-produced in *E. coli* strain BL21(DE3). All rWSSV proteins were 6× His-tag fusion protein that can be induced the expression by IPTG for 2 h for thr rWSSV189 and 4 h for rWSSV458 and rWSSV471. The crude rWSSV proteins were analyzed by 15% SDS-PAGE and Coomassie brilliant blue staining. The WSSV proteins were successfully produced. The rWSSV189 (Fig.3.1) and rWSSV458 proteins (Fig.3.2) with the expected size of about 26 kDa were detected. The rWSSV471 protein (Fig.3.3) whose size of about 20 kDa was also over-produced.

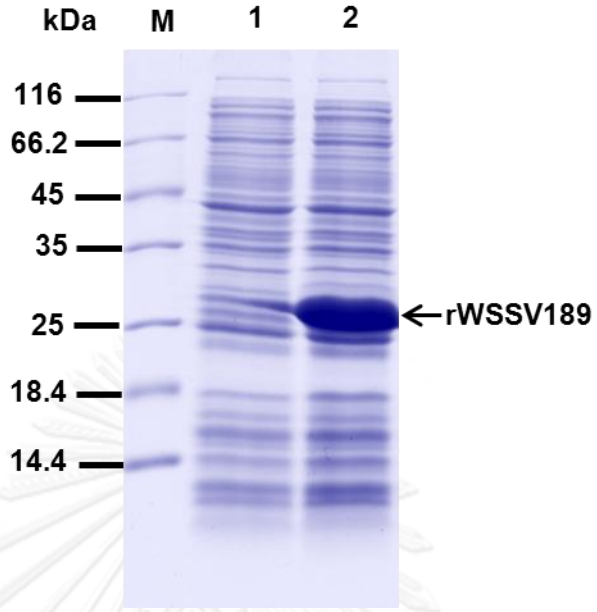


Figure. 3.1 Production of the rWSSV189 protein in *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL. The recombinant *E. coli* strain BL21-CodonPlus(DE3)-RIL expressing rWSSV189 was cultured in LB medium until OD_{600} reached 0.4. The uninduced cells were collected (Lane 1). The expression of rWSSV189 was then induced with 1 mM IPTG for 2 h (Lane 2). The whole cells were analyzed for the protein expression by 15% SDS-PAGE staining with Coomassie blue staining. The arrow indicates the expected size (~ 26 kDa) of the rWSSV189 protein. Lane M is the Page Ruler™ unstained protein Ladder (Thermo Scientific).

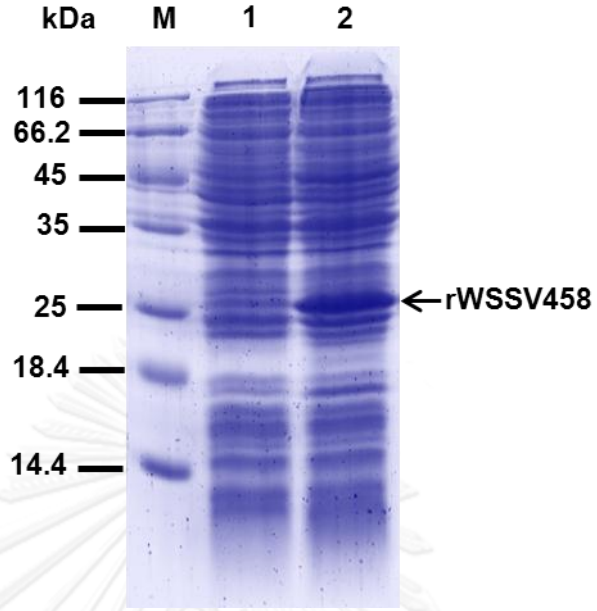


Figure. 3.2 Production of the rWSSV458 protein in *Escherichia coli* strain BL21(DE3). The recombinant *E. coli* strain BL21(DE3) expressing rWSSV458 was cultured in LB medium until OD_{600} reached 0.4. The uninduced cells were collected (Lane 1). The expression of rWSSV458 was then induced with 1 mM IPTG for 4 h (Lane 2). The whole cells were analyzed for the protein expression by 15% SDS-PAGE staining with Coomassie blue staining. The arrow indicates the expected size (~ 26 kDa) of the rWSSV458 protein. Lane M is the Page Ruler™ unstained protein Ladder (Thermo Scientific).

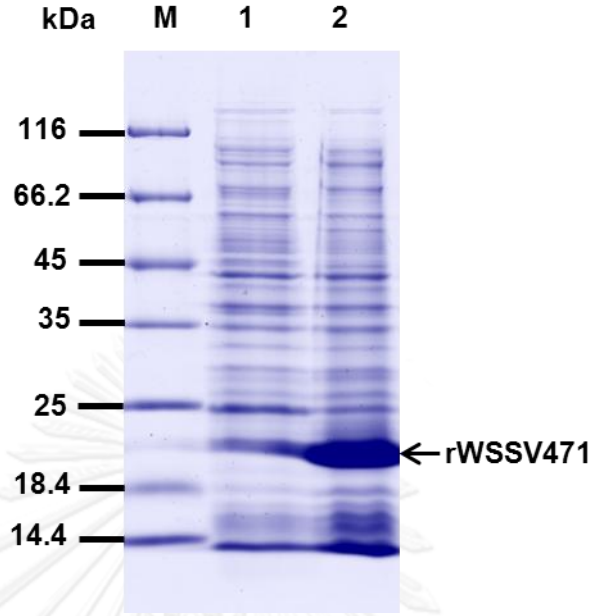


Figure. 3.3 Production of the rWSSV471 protein in *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL. The recombinant *E. coli* strain BL21-CodonPlus(DE3)-RIL expressing rWSSV471 was cultured in LB medium until OD_{600} reached 0.4. The uninduced cells were collected (Lane 1). The expression of rWSSV471 was then induced with 1 mM IPTG for 4 h (Lane 2). The whole cells were analyzed for the protein expression by 15% SDS-PAGE staining with Coomassie blue staining. The arrow indicates the expected size (~ 20 kDa) of the rWSSV471 protein. Lane M is the Page Ruler™ unstained protein Ladder (Thermo Scientific).

3.1.2 Purification of recombinant WSSV proteins

As reported previously, the rWSSV189, rWSSV458 and rWSSV471 proteins containing the His_6-tag were expressed as the inclusion bodies (Suraprasit, 2012). Denaturing buffer (Tris-HCl buffer, pH 7.4 and 6M urea) and non-denaturing buffer (Tris-HCl buffer, pH 7.4) were used for solubilizing the proteins from the inclusion bodies. The rWSSV189, rWSSV458 and rWSSV471 proteins were further purified by Ni Sepharose 6 Fast Flow bead. The rWSSV189 protein was purified under non-denaturing condition as described previously (Suraprasit, 2012). The purified

WSSV189 was dialyzed against 20 mM sodium carbonate buffer, pH 10 and the purity of the protein was verified by 15% SDS-PAGE and Western blot analysis (Fig. 3.4). The expected size of rWSSV189 (26 kDa) was detected. Using Bradford assay, the concentration of the purified rWSSV189 protein was 77 μM.

On the other hand, the rWSSV458 and rWSSV471 proteins were purified under the denaturing condition. For rWSSV458 protein purification, the elution buffers; 20 mM Tris-HCl, 0.3 M NaCl, pH 7.4 buffer supplement with 6 M urea and 50, 100, 150, 250 and 500 mM imidazole, respectively, were used. The purified fractions were subsequently analyzed by 15% SDS-PAGE (Fig. 3.5). To purify the rWSSV471 protein, the elution buffers; 20 mM Tris-HCl, pH 7.4 buffer supplement with 6 M urea and 50, 100, 150, 250 and 500 mM imidazole, respectively, were used and the purified fractions were analyzed by 15% SDS-PAGE (Fig. 3.6). The bound rWSSV458 protein eluted with 20 mM Tris-HCl, 0.3 M NaCl, pH 7.4 buffer supplement with 6 M urea and 100 and 500 mM imidazole showing the high purity rWSSV458 protein was then pooled. The fractions containing the high purity rWSSV471 protein eluted with 20 mM Tris-HCl, pH 7.4 buffer supplement with 6 M urea and 250 and 500 mM imidazole was pooled. Then, the purified rWSSV458 and rWSSV471 proteins were dialyzed against 0.1X PBS, pH 7.4. The purified proteins were concentrated by aquacide I. Next, the purified proteins of rWSSV458 (Fig 3.7) and rWSSV471 (Fig. 3.8) were analyzed by 15% SDS-PAGE and Western blot analysis. The expected bands of rWSSV458 protein (26 kDa) and rWSSV471 protein (20 kDa) were observed. Then, these proteins were measured the concentration by Bradford assay. The concentration of the rWSSV458 and rWSSV471 proteins obtained was 55 μM and 70 μM, respectively.

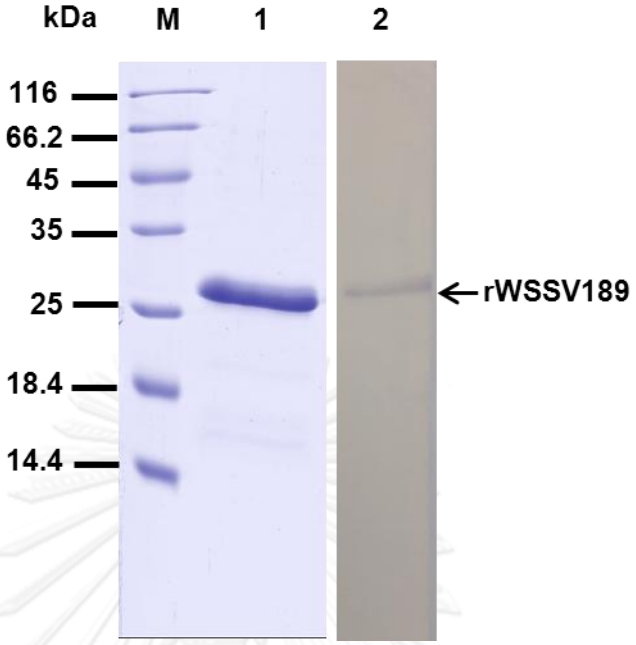
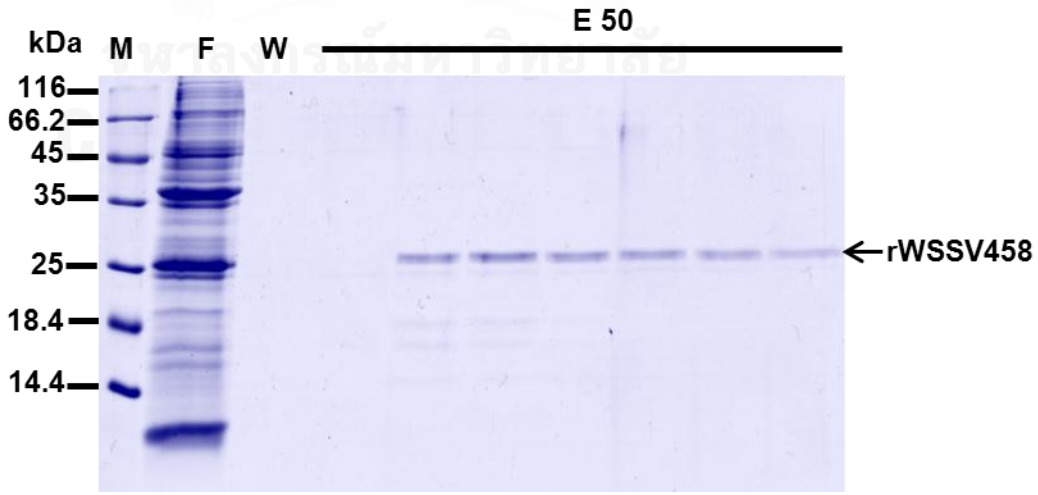
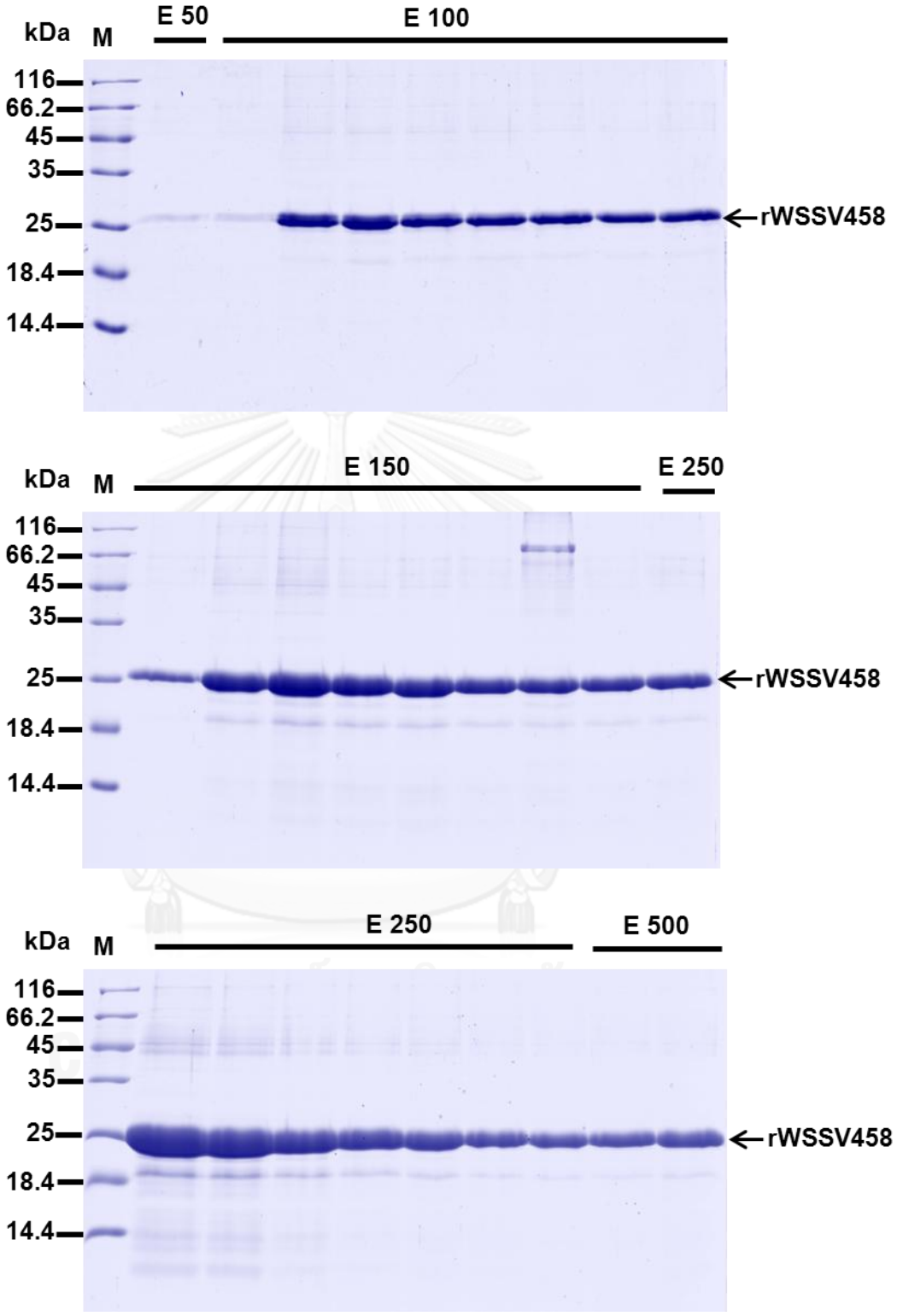


Figure. 3.4 Analysis of the purified rWSSV189 protein. The purified protein of rWSSV189 was analyzed by 15% SDS-PAGE (Lane 1) and Western blot analysis using the mouse anti-rWSSV189 antiserum as a primary antibody and the alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody (Lane 2). Lane M is unstained protein marker (Page Ruler™ unstained Protein Ladder, Thermo Scientific). The arrow indicates the expected band of the purified rWSSV189 protein.

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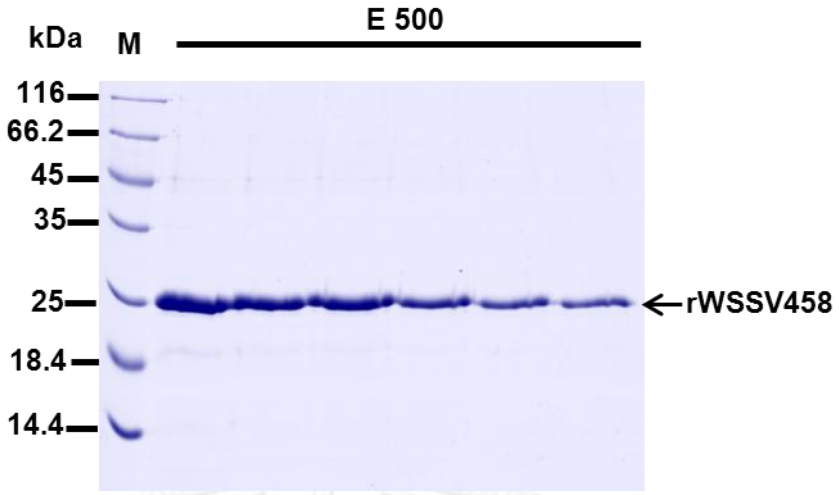

Figure. 3.5 Purification of the rWSSV458 protein by Ni affinity chromatography. The crude soluble fractions of rWSSV458 were passed through Ni Sepharose 6 Fast Flow column under the denaturing condition. After washing, the bound protein was eluted with 20 mM Tris-HCl, 0.3 M NaCl, pH 7.4 buffer supplement with 6 M urea and 50, 100, 150, 200 and 500 mM imidazole, respectively. The fractions were then run on 15% SDS-PAGE. M: the Page Ruler™ unstained protein Ladder (Thermo scientific); F: flowthrough fraction; W: wash fraction; E 50: elute fractions with elution buffer containing 50 mM imidazole; E 100: elute fractions with elution buffer containing 100 mM imidazole; E 150: elute fractions with elution buffer containing 150 mM imidazole; E 250: elute fractions with elution buffer containing 250 mM imidazole; E 500: elute fractions with elution buffer containing 500 mM imidazole. The arrow indicates the expected band of the purified rWSSV458 protein.

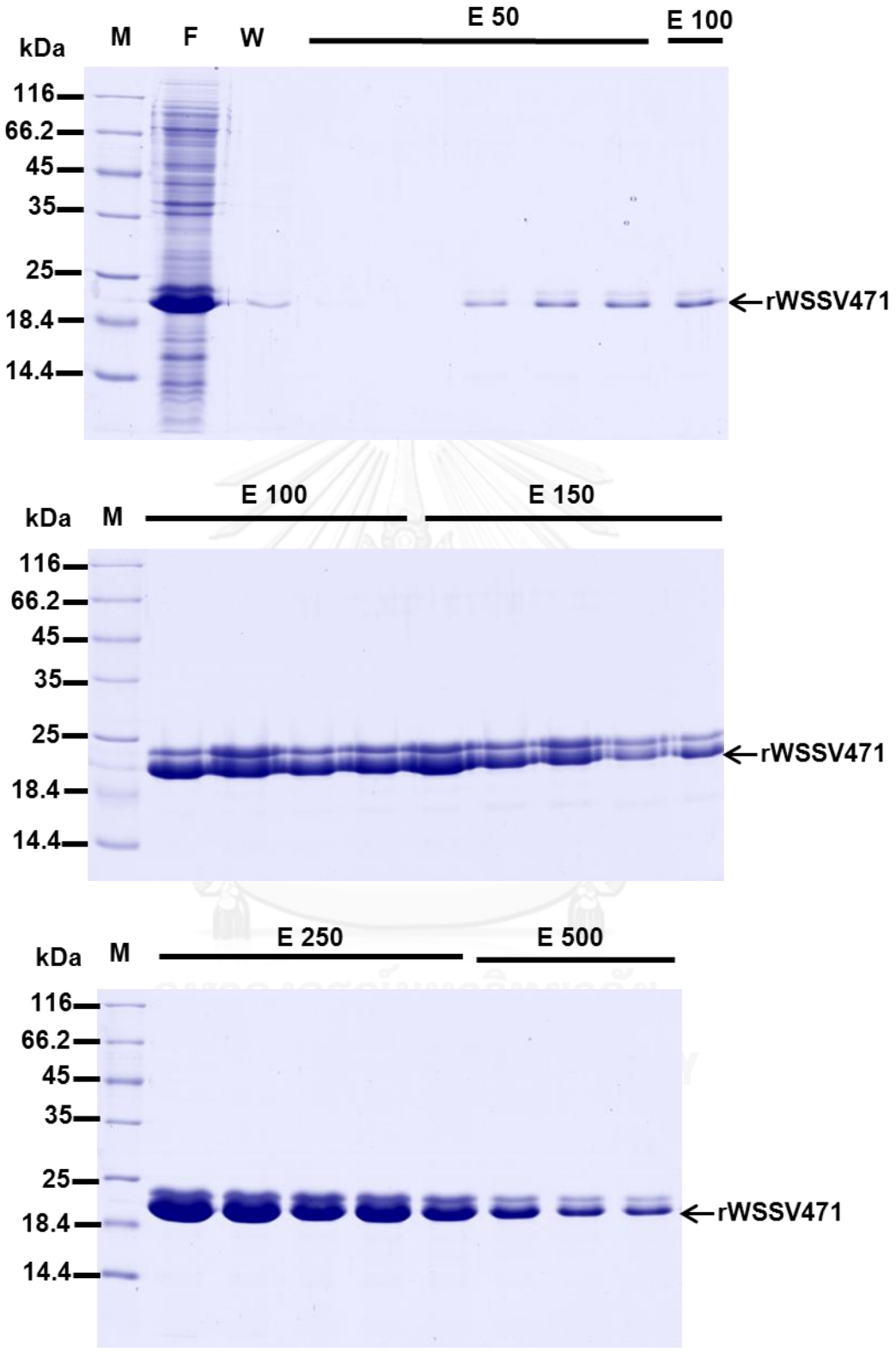

Figure. 3.6 Purification of the rWSSV471 protein by Ni affinity chromatography. The crude soluble fractions of the rWSSV471 protein were purified through Ni Sepharose

6 Fast Flow column under denaturing condition. The crude protein was incubated with Ni bead pre-incubated with the equilibration buffer. The unbound protein was washed and the bound protein was eluted with 20 mM Tris-HCl, pH 7.4 buffer supplement with 6 M urea and 50, 100, 150, 200 and 500 mM imidazole, respectively. The protein fractions were then run on 15% SDS-PAGE. M: the Page Ruler™ unstained protein Ladder (Thermo scientific); F: flowthrough fraction; W: wash fraction; E 50: elute fractions with elution buffer containing 50 mM imidazole; E 100: elute fractions with elution buffer containing 100 mM imidazole; E 150: elute fractions with elution buffer containing 150 mM imidazole; E 250: elute fractions with elution buffer containing 250 mM imidazole; E 500: elute fractions with elution buffer containing 500 mM imidazole. The arrow indicates the expected band of the purified rWSSV471 protein.

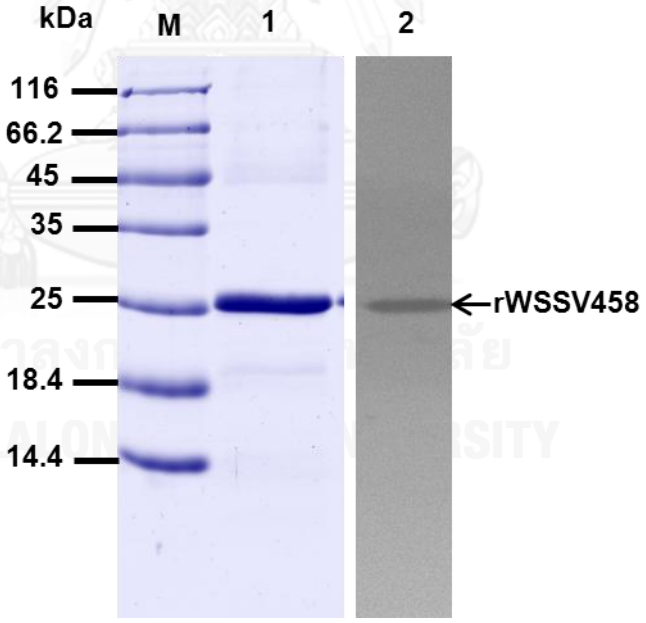

Figure. 3.7 Analysis of the purified rWSSV458 protein. The purified of rWSSV458 protein was analyzed by 15% SDS-PAGE (Lane 1) and Western blot analysis using the mouse anti-rWSSV458 antiserum as a primary antibody and the alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody (Lane 2). Lane M is

unstained protein marker (Page Ruler™ unstained Protein Ladder, Thermo Scientific). The arrow indicates the expected band of the purified rWSSV458 protein.

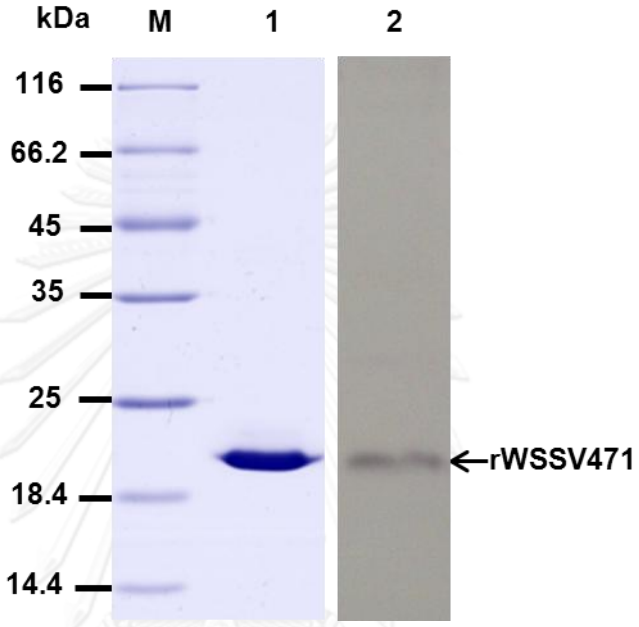

Figure. 3.8 Analysis of the purified rWSSV471 protein. The purified of rWSSV471 protein was analyzed by 15% SDS-PAGE (Lane 1) and Western blot analysis using the mouse anti-rWSSV471 antiserum as a primary antibody and the alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody (Lane 2). Lane M is unstained protein marker (Page Ruler™ unstained Protein Ladder, Thermo Scientific). The arrow indicates the expected band of the purified rWSSV458 protein.

3.1.3 Expression and purification of the recombinant ALF*Pm*3 protein

The rALF*Pm*3 protein was produced from yeast *Pichia pastoris* system. The expression culture was collected at day 2 after methanol induction and analyzed by 15% SDS-PAGE and Coomassie brilliant blue staining (Fig.3.9). The result showed the expected size of rALF*Pm*3 protein (about 11.3 kDa) indicating the successful expression of rALF*Pm*3 protein in yeast *P. pastoris* system.

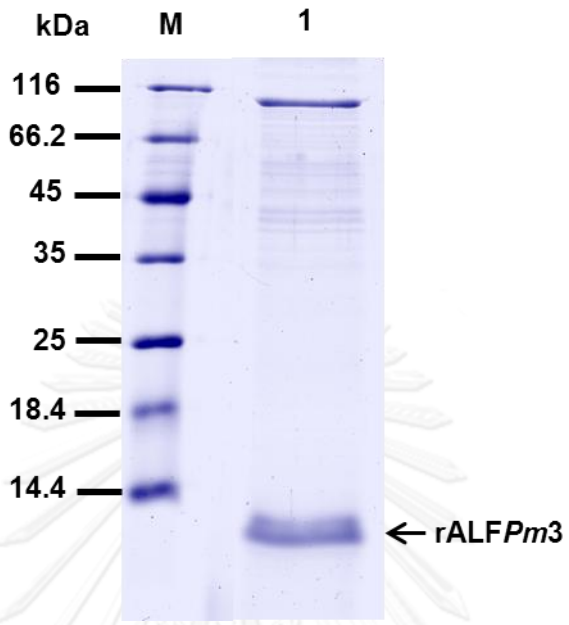

Figure. 3.9 Analysis of the rALF*Pm*3 protein expressed by the yeast *Pichia pastoris*. Lane M: unstained protein marker (Page Ruler™ unstained Protein Ladder, Thermo Scientific); Lane 1: crude supernatant at day 2 after methanol induction. The arrow indicates the expected band of the rALF*Pm*3 protein.

Then, the crude supernatant was purified by a strong cation exchange chromatography as described in section 2.5.4 and the elution fractions containing rALF*Pm*3 were analyzed by 15% SDS-PAGE. The result showed the purified rALF*Pm*3 with expected size of about 11.3 kDa (Fig.3.10). The purified rALF*Pm*3 fractions were then pooled and dialyzed against the sterile distilled water at 4 ℃ to eliminate salt. Next, the purified of rALF*Pm*3 protein was concentrated and determined for the antibacterial activity against *Escherichia coli* 363 and *Bacillus megaterium.* The concentration of rALF*Pm*3 protein is 280 μM. The minimum inhibitory concentration (MIC) values were 0.095-0.19 and 0.19-0.39 μM, respectively, showing the existance of rALF*Pm*3 antimicrobial activity. The purified protein was then used for the *in vitro*

pull-down assay and study the effect of rWSSV protein binding on WSSV-neutralizing activity of ALF*Pm*3. Prior to use, the purified rALF*Pm*3 protein was usually analyzed by 15% SDS-PAGE.

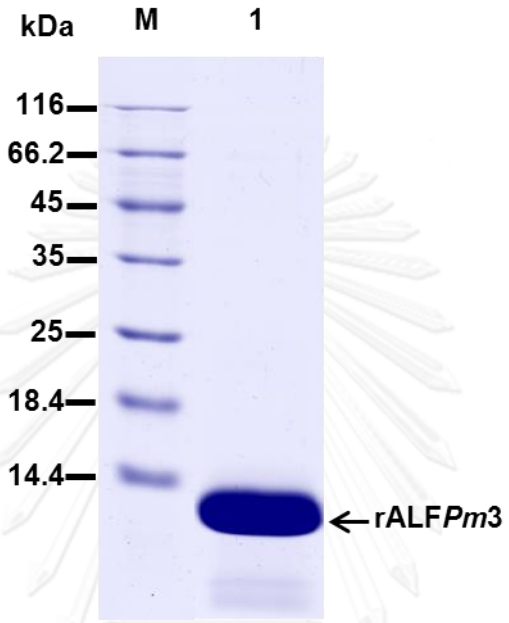

Figure. 3.10 Analysis of the purified rALF*Pm*3 protein. The purified rALF*Pm*3 protein with antimicrobial activity was analyzed by the 15% SDS-PAGE staining with Coomassie brilliant blue. Lane M: unstained protein marker (Page Ruler™ unstained Protein Ladder, Thermo Scientific); Lane 1: the purified rALF*Pm*3. The arrow indicates the expected band of purified rALF*Pm*3 protein.

3.2 Protein-protein interaction assay between WSSV458 and rALF*Pm*3 by *in vitro* pull-down assay

Out of ALF*Pm*3-interacting proteins identified from WSSV by the yeast two-hybrid screening, the WSSV189 and WSSV471 were previously confirmed by *in vitro* pull-down assay (Suraprasit, 2012). Therefore, in this study the interaction between WSSV458 and ALF*Pm*3 was further confirmed. The purified protein of rWSSV458 and

rALF*Pm*3 were used in the *in vitro* pull-down assay to confirm the genuine interaction between rWSSV458 and rALF*Pm*3. In this experiment, rWSSV458 protein containing the 6× His-tag was used as a bait. It was incubated with a nickel chelating resin and the resin was washed to remove the excess protein. The final wash fraction was collected and checked on SDS-PAGE to ensure that there was no excess rWSSV458 protein left in the column. Then, the prey protein (rALF*Pm*3) was added and allowed to bind to the rWSSV458 protein. The resin was again washed to remove excess rALF*Pm*3 protein. The wash fraction was collected and checked on SDS-PAGE to ensure that there was no excess rWSSV458 protein and rALF*Pm*3 protein left in the column. Then, the protein complex was eluted and resolved by SDS-PAGE followed by Western blot analysis. As shown in Fig. 3.11, the nickel chelating bead effectively pulled-down the protein complex of rWSSV458 (~26 kDa) and rALF*Pm*3 (~11 kDa). The results indicated that the rWSSV458 protein could specifically bind to rALF*Pm*3 *in vitro*.

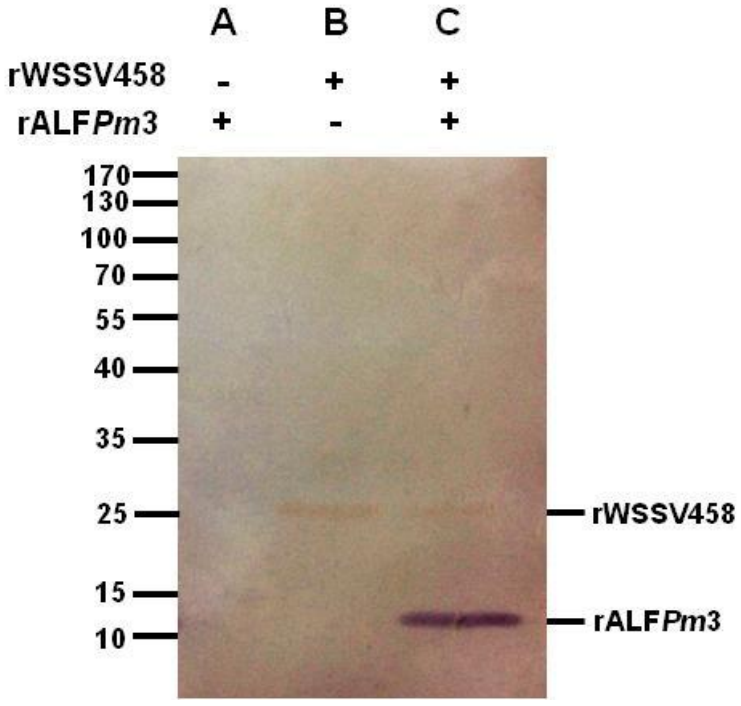

Figure. 3.11 Interaction between rWSSV458 and rALF*Pm*3 proteins by *In vitro* pull-assay. The rWSSV458 protein was incubated with the Ni bead for 1 h. After extensive washing, the rALF*Pm*3 protein was added and incubated for 1 h. Then, the excess protein was removed and the bound protein complex was eluted. The elution fraction was analyzed by 15% SDS-PAGE and Western blot analysis using antibody specific to rWSSV458 and rALF*Pm*3, respectively. Lane A: prey control (rALF*Pm*3 protein only was incubated with the bead.), Lane B: bait control (rWSSV458 protein only was incubated with the bead.) and Lane C: elution of bait-prey protein complex.

3.3 WSSV purification

In order to prepare the intact WSSV virion for WSSV protein localization, the WSSV was purified from gills of moribund WSSV-infected shrimp, *Litopenaeus vannamei*. The purified WSSV was adsorbed to Formvar-supported, nickel grids and negatively stained with uranyl acetate. The quality of the purified WSSV was examined with a transmission electron microscope. Base on the morphology observation, it was found that the fully intact WSSV was hardly detect, most of WSSV particles observed were nucleocapsid (Fig.3.12) which have the unique stacked ring structure. Our result indicated that WSSV purified from shrimp might not be able to use for WSSV protein localization. However, injection of this purified WSSV into shrimp still caused the shrimp death revealing its infectivity.

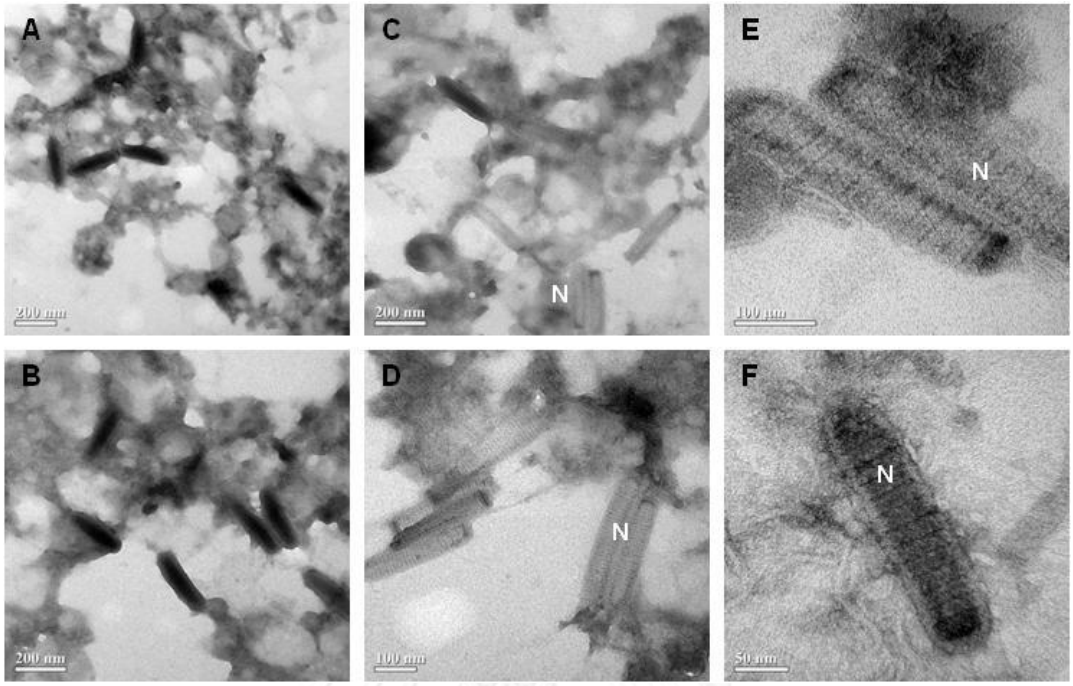

Figure. 3.12 Transmission Electron micrograph of the negative stained-WSSV purified from gill of *Litopenaeus vannamei*. The negative staining of the purified WSSV visualized by TEM is shown in the different fields and magnification (A-F). N indicate nucleocapsid.

3.4 Effect of WSSV proteins binding on the WSSV-neutralizing activity of the rALF*Pm*3 protein

3.4.1 Determination of the dosage of WSSV used for cumulative mortality assay

To investigate the appropriate dosage of WSSV for cumulative mortality assay, WSSV stock was serially diluted to 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} and injected into shrimp. Here, we selected 10^{-9} fold of diluted WSSV for cumulative mortality assay. The cumulative mortality of shrimp reached 100% within 3 days for 10^{-3} dilution, 3.5 days for 10^{-5} dilution, 4.5 days for 10^{-7} dilution and 5 days for 10^{-9} dilution (Fig. 3.13).

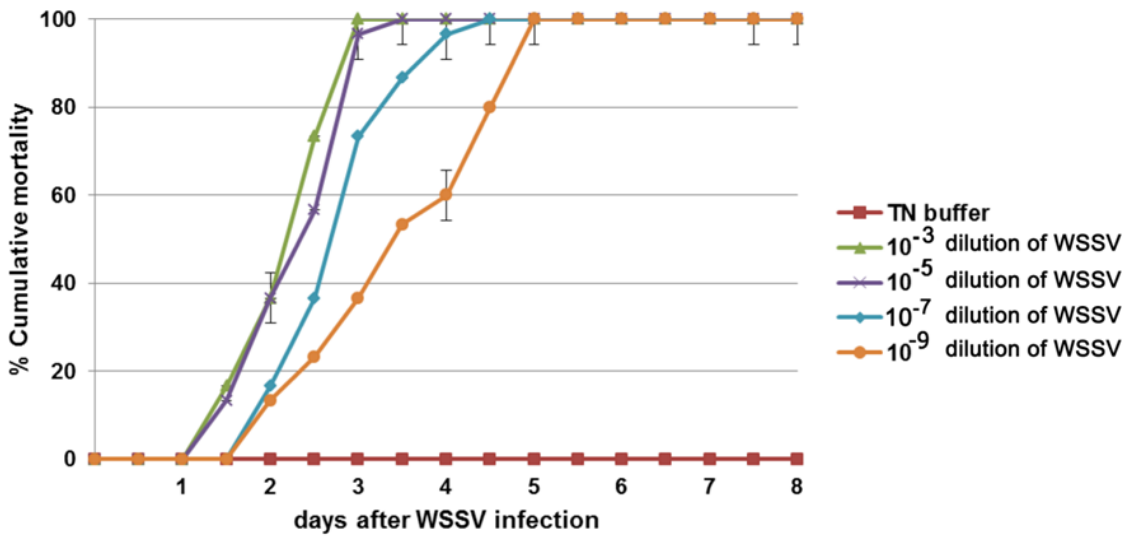

Figure. 3.13 Determination of the dosage of WSSV used for cumulative mortality assay. The purified WSSV was serially diluted in TN buffer to 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} fold. Fifty microliters of diluted purified WSSV were injected into the shrimp at second abdominal segment. Five groups of 10 shrimps were challenged with TN buffer (—■—), 10^{-3} dilution of WSSV (—▲—), 10^{-5} dilution of WSSV (—×—), 10^{-7} dilution of WSSV (—◆—), and 10^{-9} dilution of WSSV (—●—). The cumulative mortality of WSSV-infected shrimp was observed for 8 days. The experiment was performed in triplicate. The values shown in the graph are means of % cumulative mortality ± SD.

3.4.2 WSSV-neutralizing activity of the rALF*Pm*3 protein

In order to find an appropriate amount of rALF*Pm*3 that are effectively neutralized WSSV for the WSSV-neutralizing activity. Various concentration (100, 200 and 280 μM) of the purified rALF*Pm*3 protein was incubated with 10^{-9} fold-diluted WSSV for 30 min then injected into shrimp. The cumulative mortality assay was observed. The result showed that no significant difference of WSSV-neutralizing activity of the rALF*Pm*3 was observed (Fig 3.14). To ensure that the rWSSV proteins of interest can effectively bind to ALF*Pm*3 and reduce the WSSV-neutralizing activity.

The excess amount of the rALF*Pm*3 protein (280 μM) was chosen for testing the effect of WSSV protein binding on ALF*Pm*3-neutralizing activity.

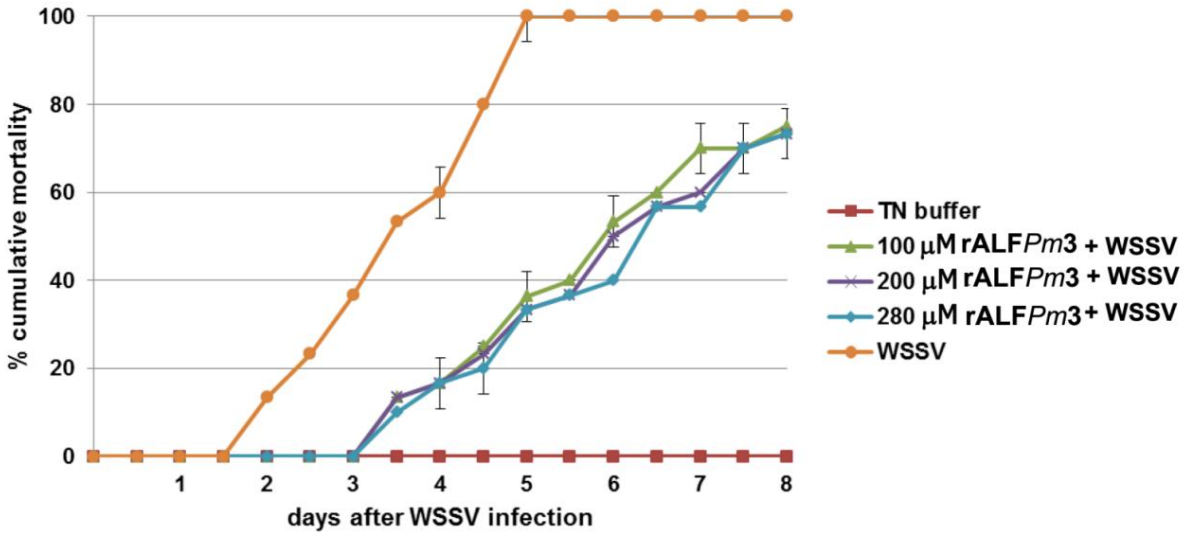

Figure. 3.14 WSSV-neutralizing activity of the rALF*Pm*3 protein. Shrimp were challenged with TN buffer (—■—), 100 μM of rALF*Pm*3 and WSSV (—▲—), 200 μM of rALF*Pm*3 and WSSV (—✕—), 280 μM of rALF*Pm*3 and WSSV (—◆—), and WSSV (—●—). The percentage of cumulative mortality of WSSV-infected shrimp was observed for 8 days. The experiment was performed in triplicate. The values shown here in the graph are means of % cumulative mortality ± SD.

3.4.3 Effect of the rWSSV189, rWSSV458 and rWSSV471 binding on WSSV-neutralizing activity of the rALF*Pm*3 protein

Previously, the rALF*Pm*3 protein has been shown to inhibit the replication of WSSV in shrimp. Also, the neutralization effect of the rALF*Pm*3 protein on WSSV in *P. monodon* lowering the percentage of cumulative mortality has been reported (Tharntada et al., 2009). If WSSV189, WSSV458 and WSSV471 proteins are the target proteins of ALF*Pm*3 neutralization, they should be able to intervene with and protect

the virus from the action of ALF*Pm*3. In this experiment, we tested whether three recombinant proteins could interfere with the WSSV neutralizing activity of rALF*Pm*3 if each rWSSV protein and rALF*Pm*3 were pre-incubated together before being injected along with WSSV into the shrimp. Then, the percentage of survival of shrimp was observed. The shrimp injected with only TN buffer as the control did not die during the observation period of 10 days. In contrast, the percentage of survival of the shrimp injected with only WSSV decreased to 0% within 5 days. The WSSV pre-incubated with rWSSV189, rWSSV458 and rWSSV471 proteins also killed the shrimp to the same extent as WSSV alone, indicating that the exogenous rWSSV proteins had no effect on WSSV infectivity. Also, injection of an exogenous protein, GST, together with either WSSV alone or WSSV and rALF*Pm*3, did not affect the WSSV infectivity and rALF*Pm*3 activity (Fig. 3.18).

As expected, rALF*Pm*3 was able to neutralize the WSSV causing a higher in the percentage of survival of shrimp as compared to shrimp injected with WSSV alone. However, the preincubation of rWSSV189, rWSSV458 and rWSSV471 proteins with the rALF*Pm*3 protein reduced the WSSV-neutralizing activity of the rALF*Pm*3 protein; a 0% survival was observed in 7-8 days. The results are shown in Figs. 3.15, 3.16, and 3.17, respectively. The decrease in the WSSV-neutralizing activity of the rALF*Pm*3 protein was presumably due to the binding of rWSSV189, rWSSV458 and rWSSV471 proteins to the rALF*Pm*3 and blocking the access to the WSSV.

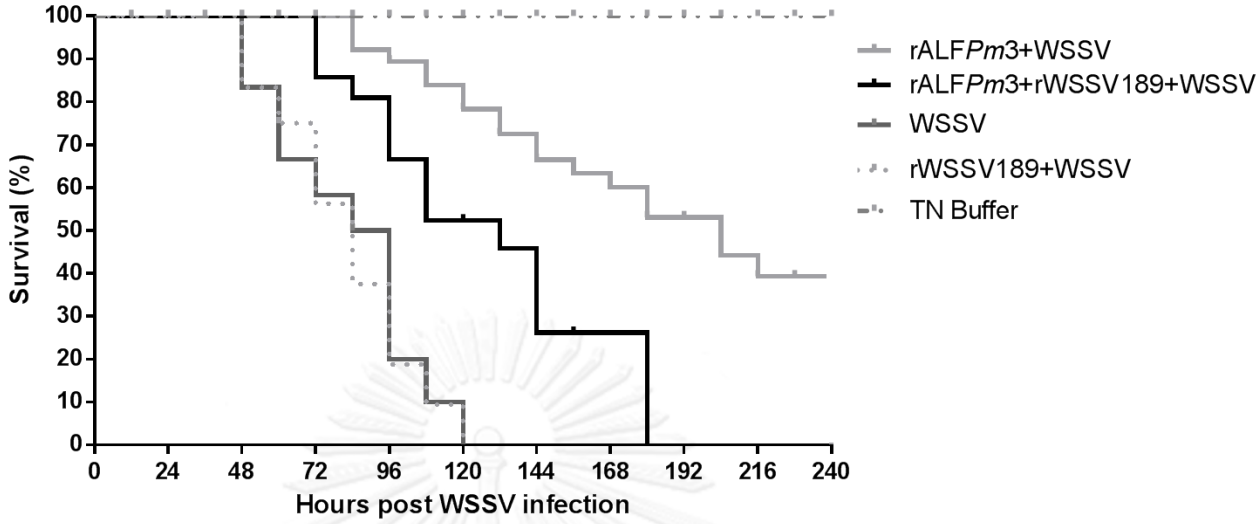

Figure. 3.15 Effect of the rWSSV189 protein binding on the WSSV-neutralizing activity of the rALF*Pm*3 protein. Five groups of shrimp were challenged with the TN buffer (), WSSV pre-incubated with the rWSSV189 protein (), WSSV only (), WSSV pre-incubated with a mixture of rALF*Pm*3 and rWSSV189 proteins (), and WSSV pre-incubated with the rALF*Pm*3 protein (). The survival of WSSV-infected shrimp was observed for 10 days. The experiment was performed in triplicate. The values shown here in the graph are means of % survival ± SD.

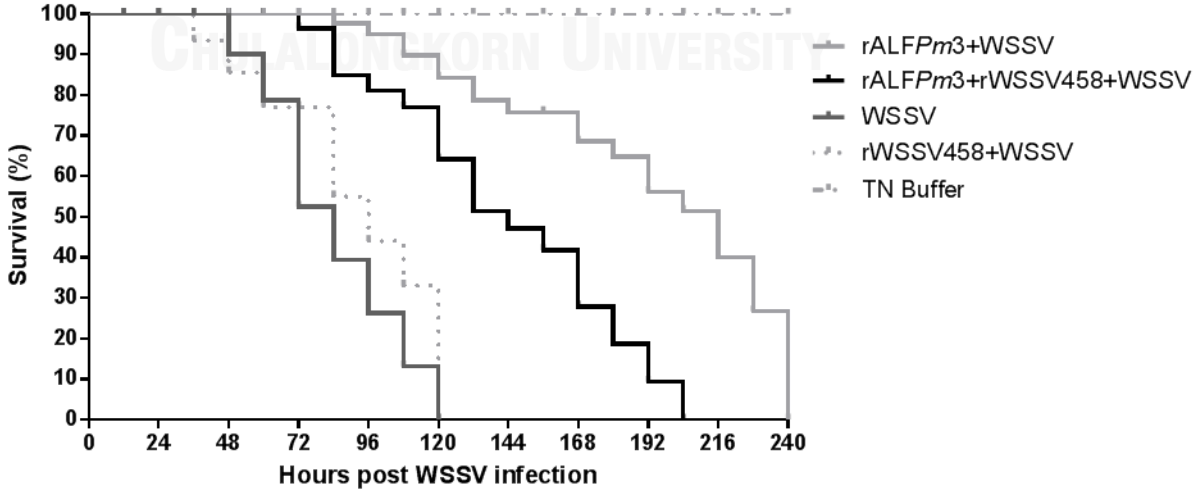

Figure. 3.16 Effect of the rWSSV458 protein binding on the WSSV-neutralizing activity of the rALF*Pm*3 protein. Five groups of shrimp were challenged with the TN buffer (-··-), WSSV pre-incubated with the rWSSV458 protein (···), WSSV only (—), WSSV pre-incubated with a mixture of rALF*Pm*3 and rWSSV458 proteins (—), and WSSV pre-incubated with the rALF*Pm*3 protein (—). The survival of WSSV-infected shrimp was observed for 10 days. The experiment was performed in triplicate. The values shown here in the graph are means of % survival ± SD.

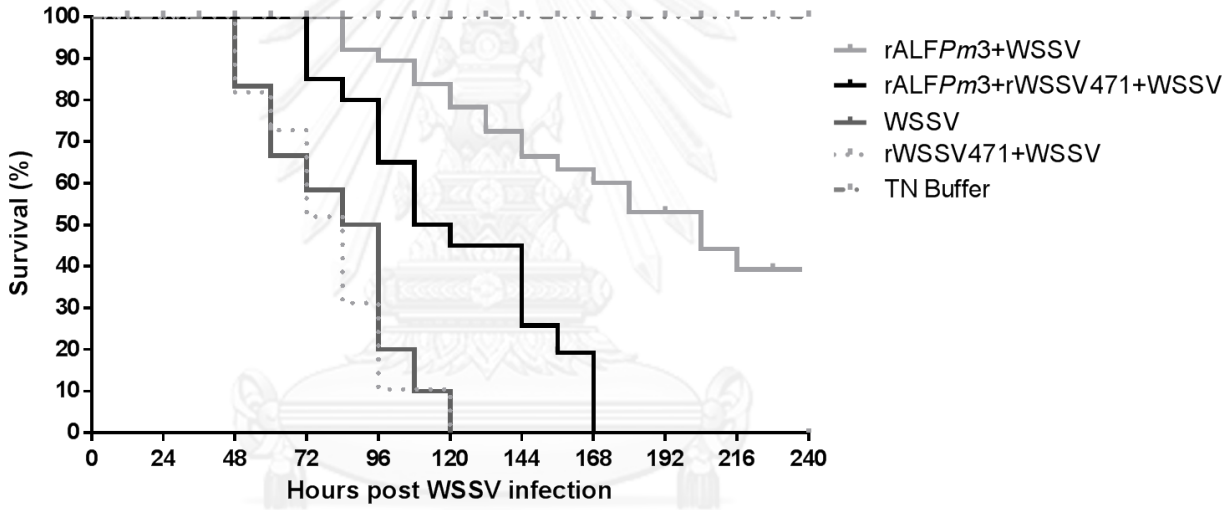

Figure. 3.17 Effect of the rWSSV471 protein binding on the WSSV-neutralizing activity of the rALF*Pm*3 protein. Five groups of shrimp were challenged with the TN buffer (-··-), WSSV pre-incubated with the rWSSV471 protein (···), WSSV only (—), WSSV pre-incubated with a mixture of rALF*Pm*3 and rWSSV471 proteins (—), and WSSV pre-incubated with the rALF*Pm*3 protein (—). The survival of WSSV-infected shrimp was observed for 10 days. The experiment was performed in triplicate. The values shown here in the graph are means of % survival ± SD.

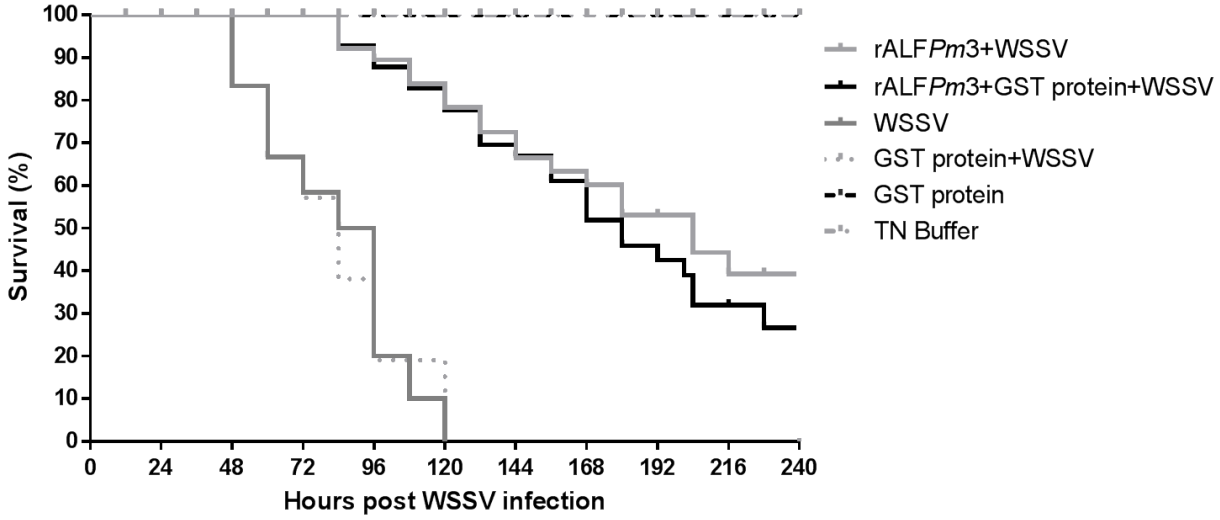

Figure. 3.18 Effect of the GST protein binding on the WSSV-neutralizing activity of the rALF*Pm*3 protein. The GST protein was used as a negative control. The WSSV-neutralizing activity of the rALF*Pm*3 protein was observed in presence of the GST protein. Shrimp were challenged with the TN buffer (), the GST protein (), WSSV pre-incubated with the GST protein (), WSSV only (), WSSV pre-incubated with a mixture of rALF*Pm*3 and GST proteins (), and WSSV pre-incubated with the rALF*Pm*3 protein (). The survival of WSSV-infected shrimp was observed for 10 days. The experiment was performed in triplicate. The values shown here in the graph are means of % survival ± SD.

3.5 Confirmation of WSSV infection in shrimp

To confirm the infection in WSSV and rWSSV proteins-treated WSSV-infection in shrimp, the expression of a WSSV late gene, VP28, and of a shrimp house keeping gene, EF-1α, was determined in hemocyte of WSSV-infected shrimp by reverse transcription-PCR (RT-PCR) at 0, 24 and 48 hours after infection. VP28 gene expression as an indicator of WSSV infection shrimp was detected at 24 and 48 h post-WSSV infection in every group of shrimp injected with WSSV only and groups of shrimp injected with WSSV pre-incubated with rWSSV proteins (rWSSV189, rWSSV471 and rWSSV458) (Fig.3.19). These results indicated that shrimp were infected with WSSV and the exogenous rWSSV proteins have no effect on WSSV infectivity.

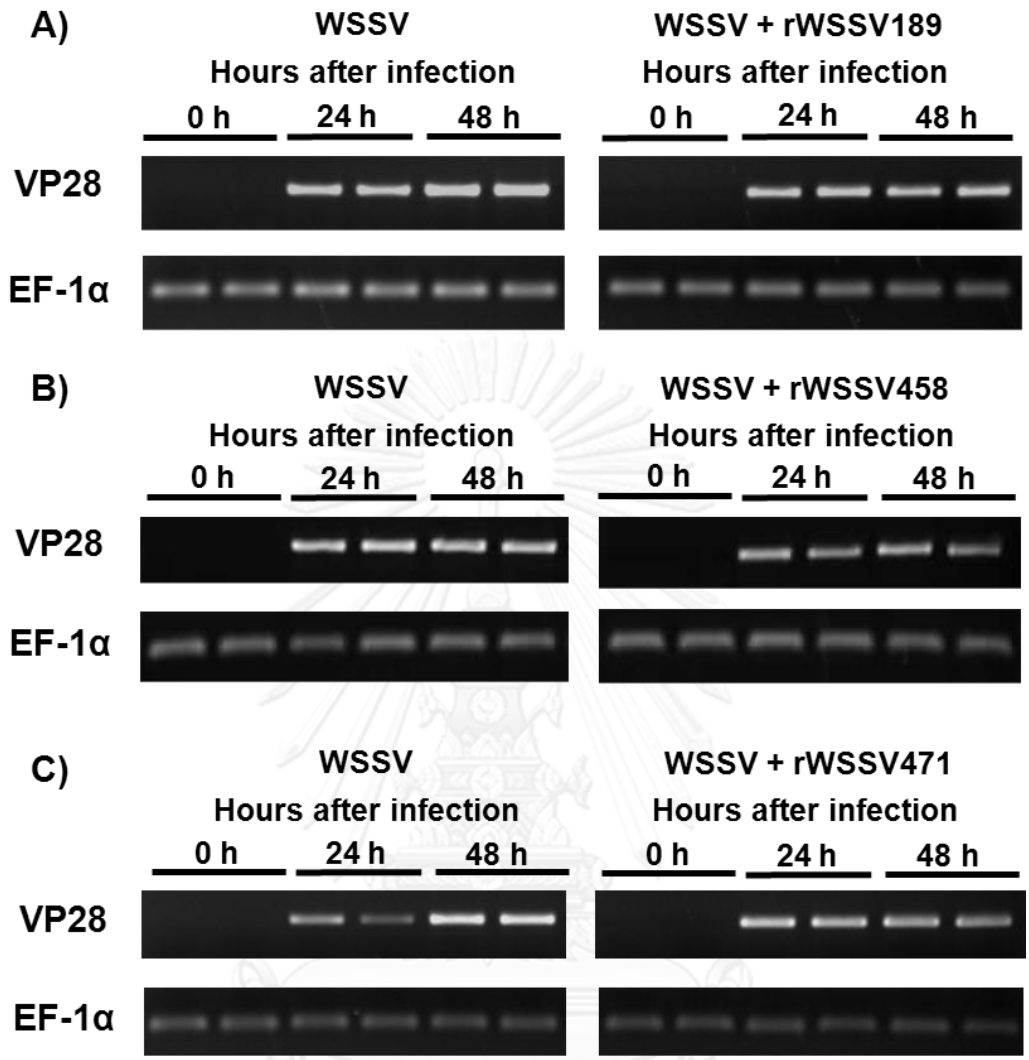

Figure. 3.19 Amplification of the VP28 and EF-1α genes from hemocyte of WSSV-infected shrimp. The total RNA from hemolymph of WSSV- and rWSSV proteins (rWSSV189, rWSSV458 and rWSSV471) treated WSSV-infected shrimp was collected from 2 individuals at 0, 24 and 48 hours after infection. The first-stranded cDNA was synthesized and used as a template for RT-PCR. The expression of VP28 and EF-1α transcript of (A) WSSV and rWSSV189-treated WSSV, (B) WSSV and rWSSV458-treated WSSV and (C) WSSV and rWSSV471-treated WSSV were analyzed on 1.5 % agarose gel electrophoresis stained with EtBr.

3.6 Detection of native WSSV in shrimp tissues

Having detected the transcripts of WSSV genes that are presumably the targets of ALF*Pm*3 binding (Suraprasit, 2012). It occurred to us that the protein products should also exist in the WSSV-infected shrimp. To show that the WSSV189, WSSV458 and WSSV471 proteins were produced in WSSV-infected shrimp, hemocytes and gills were collected from WSSV-challenged shrimp at 0, 6, 24 and 48 hpi for the preparation of hemocyte lysate supernatant (HLS) and gill lysate supernatant (GLS), respectively. The HLS and GLS were separated on SDS-PAGE. The WSSV189, WSSV458 and WSSV471 proteins were detected by Western blot with anti-WSSV189, anti-WSSV458 and anti-WSSV471 polyclonal antiserum, respectively. The ALF*Pm*3 was also detected with purified anti-ALF*Pm*3 polyclonal antibody in the same HLS and GLS. The expected bands of WSSV189 (24 kDa), WSSV458 (23 kDa), WSSV471 (19 kDa) and ALF*Pm*3 (11 kDa) were observed (Fig.3.20). The WSSV189 and WSSV471 proteins were observed at 6, 24, and 48 hpi in both hemocytes and gills but the WSSV471 proteins was gradually increased at different level at 6, 24, and 48 hpi in both hemocytes and gills. When compare to others, the WSSV471 protein was highest expressed especially in the gill. As expected, the ALF*Pm*3 protein was observed mainly in hemocytes at 6 to 48 hpi and some was detected at 24 hpi in gills and this presumaly was ALF*Pm*3 produced in the infiltrating hemocyte. The WSSV458 protein was detected at all time point after WSSV infection but not at 0 hpi in both hemocytes and gills. The β-actin was generally used as a protein loading control.

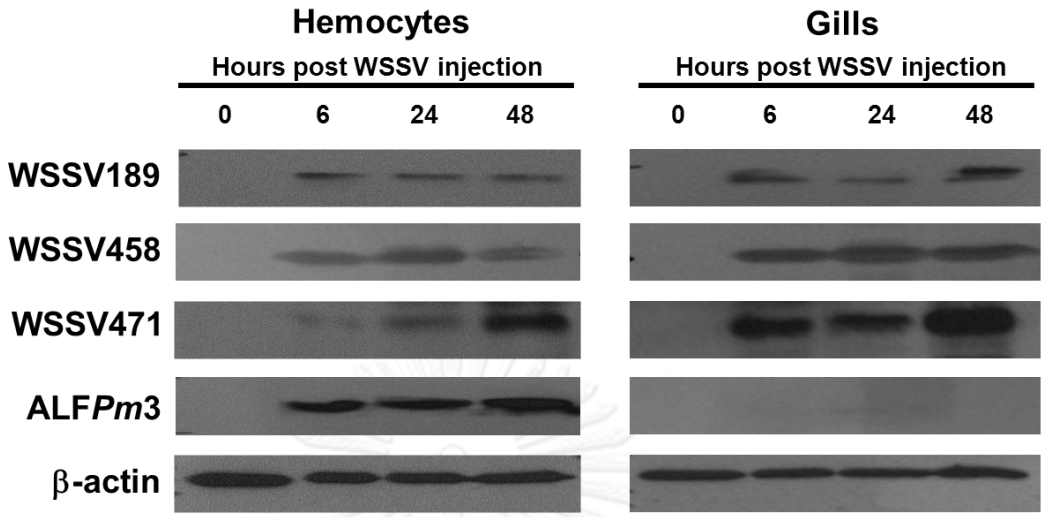

Figure. 3.20 Detection of WSSV189, WSSV458, WSSV471, and ALF*Pm*3 proteins produced in hemocytes and gills of WSSV-challenged shrimp. One hundred micrograms of HLS and GLS, respectively, of at 0, 6, 24 and 48 hpi were separated on 15% SDS-PAGE and analyzed by Western blot using polyclonal antibody specific to WSSV189, WSSV458, WSSV471, and ALF*Pm*3 proteins, as primary antibodies and the horseradish peroxidase-conjugated goat anti-mouse IgG antibodies as secondary antibodies for WSSV proteins and the horseradish peroxidase-conjugated anti-rabbit IgG antibody as a secondary antibody for ALF*Pm*3 protein. β-actin protein was detected as a loading control. The expected bands of about 24 kDa for WSSV189, 23 kDa for WSSV458, 19 kDa for WSSV471, 11 kDa for ALF*Pm*3 and 43 kDa for β-actin were detected. Chemiluminescence substrate was used for the detection of the specific protein band on the X-ray film.

3.7 Localization of WSSV189 and WSSV471

3.7.1 Localization of WSSV189 and WSSV471 proteins on WSSV by Western blot analysis

To further characterize the target WSSV proteins of ALF*Pm*3, WSSV189 and WSSV471 proteins were localized on WSSV. It has been reported previously that WSSV189 and WSSV471 were the structural proteins (Tsai et al., 2004) ; however, it is still unknown where these protein locate on WSSV virion. In this study, the WSSV virion were fractionated into envelope and nucleocapsid protein fractions. The intact WSSV and both fractions were analyzed by 12.5% SDS-PAGE with Coomassie staining (Fig. 3.21A) and the duplicated gel was subjected to Western blot analysis (Fig. 3.21B-D). The polyclonal antibodies specific to WSSV189 and WSSV471 proteins were used to detect the WSSV189 and WSSV471 protein in both fractions (Fig.3.21B and C). The VP28, the known envelope protein, was used as the envelope protein marker (Fig. 3.21D). The results showed that, the band of WSSV189 and WSSV471 proteins were found on the intact WSSV and the envelope part. These indicated that WSSV189 and WSSV471 proteins are WSSV envelope proteins.

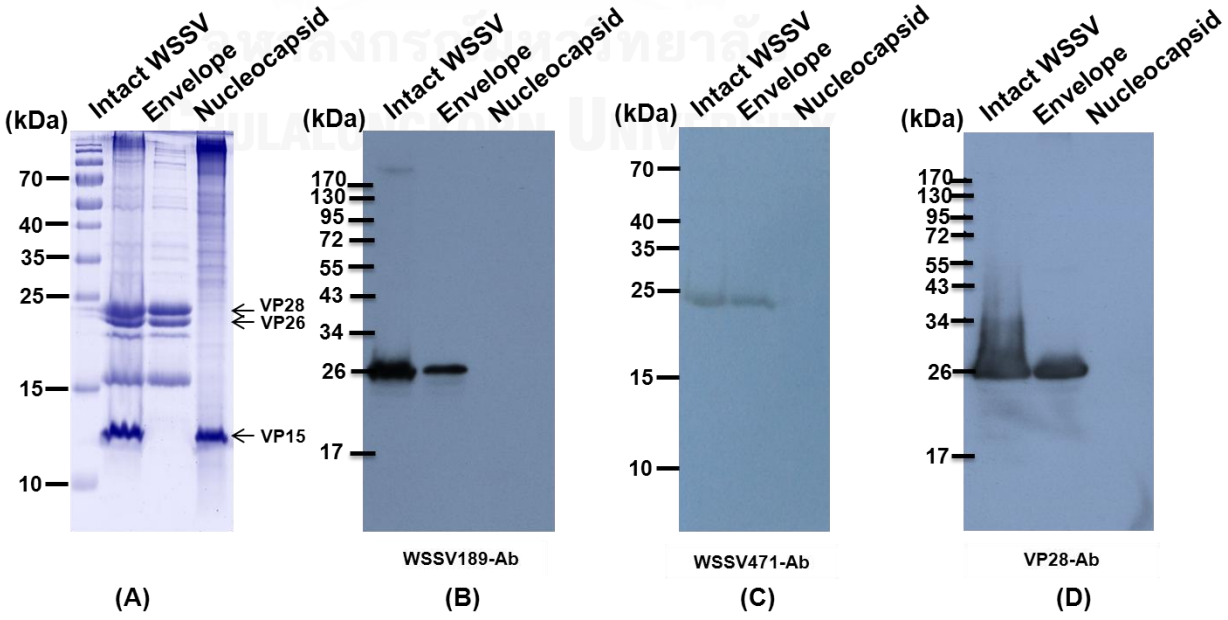

Figure. 3.21 Localization of WSSV189 and WSSV471 proteins on WSSV by Western blot. The WSSV virion was fractionated by Triton X-100 treatment into envelope and nucleocapsid protein fractions. Both fractions were separated on 12.5% SDS-PAGE and analyzed by Coomasie staining (A) and Western blot analysis using the purified polyclonal antibody specific to WSSV189 (B) and WSSV471 (C) proteins. VP28 was known envelope protein of WSSV that was used as the protein marker (D). Western Lightning® Plus-ECL, (Perkin Elmer, Inc) was applied for protein detection by chemiluminescence HRP.

3.7.2 Localization of WSSV189 and WSSV471 proteins by immunoelectron microscopy (IEM)

From the above results, both WSSV189 and WSSV471 proteins were detected in the envelope fractions but not in the nucleocapsid fraction as observed for the control VP28 envelope protein. Moreover, IEM was performed to further confirm the result. WSSV virion purified from *P. clarkiii* was used for immunogold lebeling. A gold-labeled secondary antibody together with the purified anti-WSSV189 and anti-WSSV471 polyclonal antibodies were used to detect WSSV189 and WSSV471 protein on the WSSV virion. Deposition of the gold particles was clearly observed on the envelope of the WSSV virion for the detection of WSSV189 (Fig. 3.22) and WSSV471 (Fig. 3.23) proteins. It was shown here that the gold particle was not detected in the control that is WSSV treated with the pre-immune serum. Taken together, these results indicated that WSSV189 and WSSV471 proteins are WSSV envelope proteins.

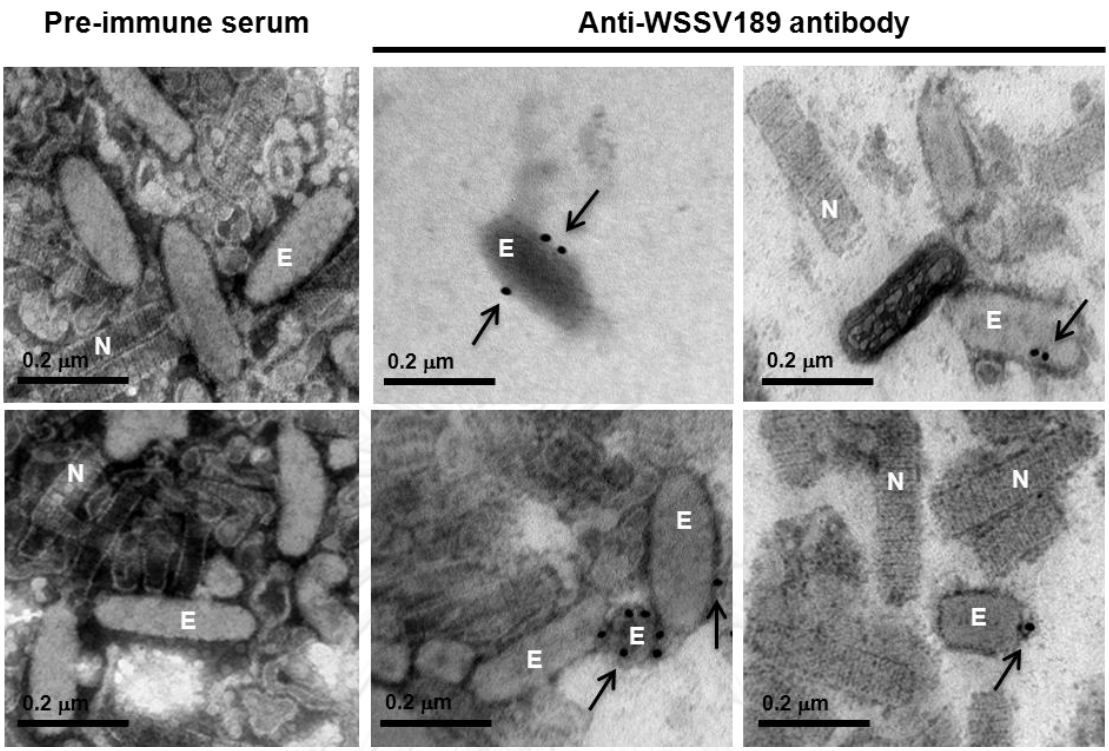

Figure. 3.22 Localization of the WSSV189 protein by immunoelectron microscopy (IEM). IEM was performed using a purified anti-WSSV189 antibody as a primary antibody and a gold-labeled secondary antibody to detect WSSV189 on the WSSV virion. As compared to the control (pre-immune serum) where the gold particle was undetectable, the positive signals were observed on the envelope of the virion as indicated by black arrows, but not on the nucleocapsid surface. E and N indicate intact virion and nucleocapsid, respectively.

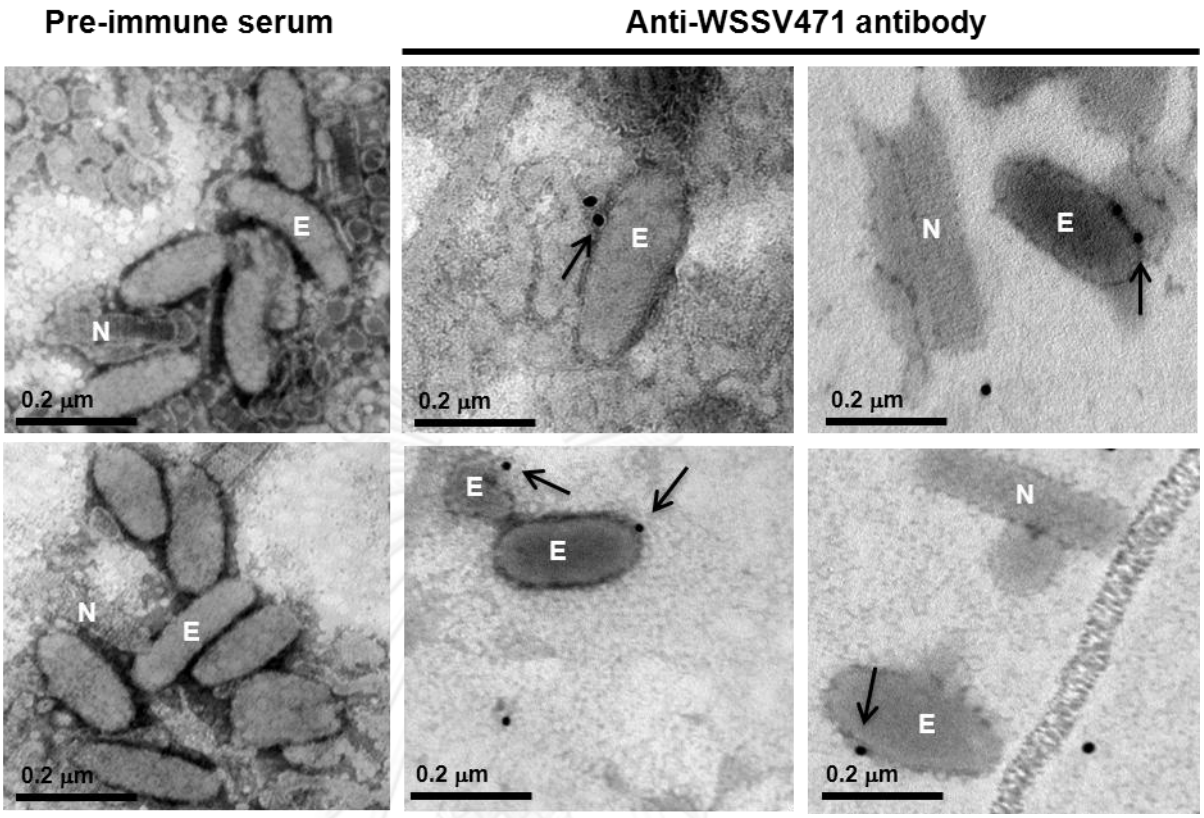

Figure. 3.23 Localization of the WSSV471 protein by immunoelectron microscopy (IEM). IEM was performed using a purified anti-WSSV471 antibody as a primary antibody and a gold-labeled secondary antibody to detect WSSV471 on the WSSV virion. As compared to the control (pre-immune serum) where the gold particle was undetectable, the positive signals were observed on the envelope of the virion as indicated by black arrows, but not on the nucleocapsid surface. E and N indicate intact virion and nucleocapsid, respectively.

CHAPTER IV

DISCUSSION

WSSV has had a massive impact on the shrimp's farm and industry worldwide because it causes the economic losses from cruel mortality. Antimicrobial peptides (AMPs) are an important effector of the innate immune system. AMPs share certain common characteristics such as small size, cationic character and similarities in structural patterns or motifs (Bachère et al., 2004; Jenssen et al., 2006b). AMPs not only possess a broad range of antibacterial activity but also antiviral activity. Antiviral properties of several AMP families against both envelope and non-envelope viruses have been characterized (Findlay et al., 2013). From previous reports, ALFs from crustaceans have been shown to exhibit a wide range of antibacterial activity, antifungal activity and antiviral activity in *Penaeus monodon* (Somboonwiwat et al., 2005), *Fenneropenaeus chinensis* (Liu et al., 2005), *Litopenaeus vannamei* (de la Vega et al., 2008), *Litopenaeus schmitti* (Rosa and Barracco, 2008), *Eriocheir sinensis* (Li et al., 2008; Wang et al., 2011; Zhang et al., 2010), *Portunus trituberculatus* (Liu et al., 2011; Liu et al., 2012b, c; Liu et al., 2013a; Liu et al., 2013b) and *Pacifastacus leniusculus* (Liu et al., 2006b). Among them ALF*Pm*3 is well characterized. Antimicrobial activity *in vitro* assays using rALF*Pm*3 have shown a strong activity against multiple Gram-positive and Gram-negative bacteria and filamentous fungi

(Somboonwiwat et al., 2005). ALF*Pm*3 is one of the antimicrobial peptide from shrimp exhibiting anti-WSSV activity (Tharntada et al., 2009).

Considering the ALF gene expression profile, a high level of ALF gene expression in WSSV-infected shrimp are generally observed (Liu et al., 2006a). The variation of the expression profile of different isoforms of ALF might be related to its different function during the WSSV progression in shrimp. In *P. leniusculus*, crayfish ALF was up-regulated by WSSV infection and involved in anti-WSSV response. After silencing of crayfish ALF, the higher rate of WSSV propagation both in the animals and HPT cell culture have been shown (Liu et al., 2006a). The transcript levels of *LvALF1* in WSSV-infected shrimp was down-regulated significantly at 24 hpi, but up-regulated significantly at 36 and 72 hpi compared to the PBS group suggesting a role of ALF in the defense against WSSV (Liu et al., 2014). The expression profile of *LvALF1* in response to WSSV infection was very similar to that of *FcALF* from *F. chinensis* (Sun et al., 2013). The expression of *PmALF* in WSSV-infected shrimp was increased and kept at a high level for 10 days (Antony et al., 2011). After WSSV infection, *MrALF5*, *MrALF6* and *MrALF7* of *Macrobrachium rosenbergii* were up-regulated and reached the peak at 24 hpi (Ren et al., 2012). The synthetic LPS-binding domain peptide of crayfish ALF was able to protect Hpt cell cultures from WSSV infection (Tharntada et al., 2009). Therefore, it was certain that ALF is one of

the important effectors of shrimp to fight against WSSV infection. However, the anti-WSSV mechanism of ALF remains elusive.

Like other shrimp proteins that play roles in antiviral responses, such as PmRab7 (Sritunyalucksana et al., 2006), β-intergrin (Ongvarrasopone et al., 2008) and C-type lectin (Zhao et al., 2009a), the antiviral activity of ALF*Pm*3 is probably due to its interaction with WSSV. It has been reported that others WSSV tegument protein such as VP26 can bind to shrimp proteins such as β-actin, WSSV-binding protein, C-type lectin from *L. vannamei* (LvCTL1) and gC1qR of *P. leniusculus* (*Plg*C1qR) (Sritunyalucksana et al., 2013; Youtong et al., 2011). From the GST pull-down assay and a far-Western overlay assay, they found that the *Plg*C1qR could bind to VP15 (nucleocapsid protein), VP26 (tegument protein) and VP28 (envelope protein) of the WSSV. Moreover, the recombinant *Plg*C1qR can reduce the WSSV replication. So far, many AMPs have been reported for their antiviral activity. The underlining mechanisms of the antiviral action of each AMP are different. Some antimicrobial peptides have a direct effect on the viral envelope protein, whereas others appear to inhibit the viral adsorption and entry process (Jenssen et al., 2006b). AMPs exhibit antiviral activity by either directly acting on the viral virion or indirectly suppressing viral replication (Mulder et al., 2013).

In order to study the mechanism of anti-WSSV activity, the previous study has identified WSSV proteins including WSSV186, WSSV189, WSSV395, WSSV458

and WSSV471 that were able to bind to ALF*Pm*3 using yeast two-hybrid assay. Among the five-ALF*Pm*3-binding proteins, the recombinant proteins, rWSSV189 and rWSSV471, were successfully produced and subjected to polyclonal antibody production. Only a specific polyclonal antibody against WSSV189 antibody was obtained but polyclonal antibody against WSSV471 antibody had low immunogenicity. Both have been confirmed for the interaction by *in vitro* pull-down assay (Suraprasit, 2012). In this study, the rWSSV189, rWSSV458 and rWSSV471 proteins were produced and only rWSSV471 protein was subjected to polyclonal antibody production. The polyclonal antibody against WSSV458 was produced by (Jaree, 2014). The *in vitro* pull-down assay was another method used to study the interaction between proteins (Li et al., 2014; Zhang et al., 2014) .The interaction between rALF*Pm*3 and rWSSV458 proteins was successfully confirmed by *in vitro* pull-down assay.

Our study was focused on interaction between ALF*Pm*3 and WSSV189, WSSV458 and WSSV471 proteins. In the antibody neutralization technique the affinity of antibody against envelope proteins of virus prevents envelope proteins from interacting with host cell receptors causing WSSV infection (Li et al., 2006; van Hulten et al., 2001; Wu et al., 2005; Xie and Yang, 2006). From previous evidence the binding of the recombinant LvCTL1 protein to WSSV increased the survival rate of shrimp after WSSV infection (Zhao et al., 2009b). Here in, we neutralized the anti-WSSV

activity of the rALF*Pm*3 protein using the rWSSV189, rWSSV458 and rWSSV471 proteins in order to test the significance of their interactions. In this assay, we used the maximum concentration of the rALF*Pm*3 protein (280 μM). We expected that at this concentration all molecules of WSSV proteins could be bound in order to confirm that the decrease in the survival rate of shrimp was the effect of rWSSV proteins binding to the rALF*Pm*3 protein.

As expected, pre-incubation of the rALF*Pm*3 protein with those rWSSV proteins decreased the *in vivo* neutralizing activity against WSSV of rALF*Pm*3. In the present study, the native WSSV proteins were detected in HLS and GLS of WSSV-infected shrimp. The expression of WSSV189, WSSV458 and WSSV471 proteins were observed at 6, 24 and 48 hpi in both HLS and GLS. It should be noted that the WSSV471 protein was highest expressed among them particularly in the gill. This implied that these rWSSV proteins are important for WSSV infection in shrimp. The ALF*Pm*3 protein was observed mainly in hemocytes at 6 to 48 hpi as reported previously. In gills, ALF*Pm*3 was slightly detected at 24 hpi. From the evidence that the ALF*Pm*3 producing hemocytes are infiltrated in gills of *V. harveyi*-infected shrimp (Somboonwiwat et al., 2008), the ALF*Pm*3 detected in the gills of 24 h WSSV-infected shrimp was from infiltrating hemocyte.

Recently, a network of WSSV-WSSV protein interaction identified WSSV395 and WSSV471 also as structural proteins acting as hub proteins interacting with over

20 WSSV proteins (Sangsuriya et al., 2014b). The knockdown of the central hubs (WSSV051 and WSSV517) in WSSV network enhances shrimp protection against WSSV by reducing the WSSV replication in shrimp and proves their significances in viral processes. Also, we found that the high up-regulation of the WSSV471 protein in WSSV-infected shrimp. Therefore, we speculated that the WSSV471 hub protein should also play a crucial role in WSSV infectivity. It was also found that, the hub proteins, WSSV051 and WSSV517, could interact with WSSV189, WSSV395 and WSSV458 (Sangsuriya et al., 2014a). Interactions between their own structural proteins are common in the envelope viruses, and they might form complexes that have specific roles in the host-viral interaction or the infectivity of viruses (Chen et al., 2007; Xie and Yang, 2006). From our results, we hypothesized that these five ALF*Pm*3-interacting proteins might interact with each other forming a complex that is the target molecule of ALF*Pm*3. From *in vitro* pull-down assay and *in vivo* neutralization activity, we know that the rALF*Pm*3 protein can interact the rWSSV proteins; WSSV189, WSSV458 and WSSV471 but the information about these WSSV proteins is limited. To further understand the roles of these WSSV proteins in the antiviral mechanism of ALF*Pm*3, the characterization of WSSV189, WSSV458 and WSSV471 proteins was performed. So far, only some WSSV proteins have been characterized. A shotgun proteomic study of WSSV (China isolate) revealed that WSSV186 (WSV131) and WSSV189 (WSV134) are WSSV structural proteins (Li et al.,

2007b; Suraprasit, 2012). WSSV395 or VP39B is determined by Western blot analysis and Immunogold electron microscopy (IEM) as an envelope protein (Tsai et al., 2004) and WSSV458 (WSV399) has been identified as a tegument protein (Jaree, 2014). The protein profiles, Western blot analysis and IEM have been used to identify VP28 as the envelope protein as well as VP15 and VP664 as the nucleocapsid proteins (Tsai et al., 2006; Zhang et al., 2002; Leu et al., 2005; van Hulten et al., 2002). It has been reported previously that WSSV189 and WSSV471 were the structural proteins (Tsai et al., 2004). Here in, Western blot analysis as well as IEM using an antibody specific to WSSV189 and WSSV471 identified WSSV189 and WSSV471 as structural proteins located at the envelope of the WSSV virion. From *in vitro* pull-down assay, the rWSSV189 and rWSSV471 proteins (Suraprasit, 2012). were shown to interact with the rALF*Pm*3 protein *in vitro* suggesting that ALF*Pm*3 directly inhibits WSSV by binding to the WSSV189 and WSSV471 envelope proteins. Accordingly, we speculated that rALF*Pm*3 directly acts on the WSSV virion as a result preventing WSSV infection in shrimp.

It has been reported that the major envelope protein VP28 plays a key role in the systemic infection of shrimp by WSSV (van Hulten et al., 2001). WSSV189 and WSSV471 envelope proteins might also contribute to WSSV infection in shrimp.

As reported previously, the antimicrobial peptides LfcinB and LfcinH (Lactoferricin) bolster the affity binding to carbohydrate molecules such as heparin

sulfate (HS) and glycosaminoglycans (GAGs), which is the viral binding site thereby blocking viral entry (Andersen et al., 2003). Interaction of antimicrobial peptide with glycoproteins in the viral envelope has been proposed to influence the viral entry process. θ-Defensin (retrocyclin 2) interacts with the HSV-2 glycoprotein B with high affinity, thus protecting the cells from HSV-2 infection. The retrocyclin-1 binds HIV gp120 with high affinity, as long as the envelope protein is glycosylated, probably resulting in an anti-HIV activity. To block the HSV entry into the cell, it has been found that the positive net charge of the peptides are critical for affinity with HS which contains many negatively-charged sulfate groups (Jenssen et al., 2004). The positive charge of Lf was found to be important for antiviral activity against human HCMV (Valenti and Antonini, 2005). When negative-charged groups were added to Lf by succinylation, the antiviral potency was mostly decreased, whereas the addition of positive charges to Lf through lamination of the protein resulted in increased anti-HCMV activity (Harmsen et al., 1995). Indolicidin causes a direct inactivation of the HIV-1 particle in a temperature-sensitive fashion, indicating a membrane-mediated antiviral mechanism (Robinson et al., 1998). The proposed anti-HIV mechanism of Lf was that the charged-modified protein binds strongly to the V3 loop of the gp120 envelope protein, increasing the net negative electric charge of viral particles, resulting in inhibition of virus-cell fusion and entry of the virus into cells (Puddu et al., 1998).

Antiviral activity of nine AMPs including PW-2, tachyplesin-1, gomesin, clavanin A, magainin, HCTF, penaeidin 3 and ALF, and mytilin A against many human viruses were reported (Carriel-Gomes et al., 2007). The peptides PW-2, ALF and penaeidin-3 exhibited higher antiviral activity against HSV-1. The peptides ALF and clavanin A showed significant antiviral activity against AdV-5 with clavanin A exhibiting a greater inhibition of viral replication (Carriel-Gomes et al., 2007). Chia reported that cyclic shrimp anti-lipopolysaccharide factor (cSALF) exhibited noticeable antiviral activity *in vitro* against nervous necrosis virus (NNV). The antiviral mechanism of this peptide was by agglutinating NNV virions into clump and preventing viral entry into the cells (Chia et al., 2010). Cecropin B and its analogue inhibit fish viral pathogens by means of direct disruption of the viral envelope and the disintegration of the viral capsids (Chiou et al., 2002).

Currently, it is not known how the shrimp ALF*Pm*3 interacts with WSSV proteins. Considering the ALF*Pm*3 structure, the LPS binding domain (LBD) is the cluster of positively charged amino acid residues (lysine and arginine). On the other side, the primary structure of WSSV189, WSSV458 and WSSV471 proteins contains a series of aspartate and glutamate negative charge residues (Asp^{103}-Ile^{104}-Glu^{105}-Asp^{106}-Asp^{107}), (Glu^{141}-Asn^{142}-Glu^{143}-Glu^{144}-Asp^{145}) and (Glu^{27}-Glu^{28}-Glu^{29}-Glu^{30}), respectively. Hence, rALF*Pm*3 probably binds directly to the WSSV by charge interaction. One of the previous studies also demonstrated that the LBD of ALFFc from *F.chinensis* had

apparent inhibition activities against Gram-negative bacteria, Gram-positive bacteria and WSSV (Guo et al., 2014). The vital function of lysine residues in LBD on its anti-WSSV activity became deficient when lysine residues were replaced. *In vivo*, FcALF-LBDc could reduce the propagation of WSSV. This mean that the lysine residues is indispensable in the anti-WSSV property (Guo et al., 2014). These data further suggested that LBD of ALF might play roles not only in the antibacterial activity, but also in antiviral activity. However, the molecular mechanism on anti-WSSV activity of LPS-binding domain still need to be further investigated. It has been reported that hydrophobicity of cecropin A-magainin 2 hybrid peptide is also important for antiviral activity (Lee et al., 2004). From H-NMR spectrum, the structure of ALF*Pm*3 contains amide, aromatic and methyl resonances and several hydrophobic amino acids involve in LPS interaction (Yang et al., 2009). It is possible that the hydrophobic amino acid residues of ALF*Pm*3 are also responsible for the interaction with WSSV proteins identified in this study.

Collectively, we demonstrated that ALF*Pm*3 exhibits WSSV neutralizing activity via direct binding to the WSSV189, WSSV458 and WSSV471 structural proteins and possibly others.

CHAPTER V

CONCLUSIONS

1. ALF*Pm*3 is an antimicrobial peptide playing an important role in *P. monodon* immunity against WSSV infection. ALF*Pm*3-interacting proteins were previously identified from WSSV by the yeast two-hybrid screening. *In vitro* pull-down assay confirmed that rWSSV189, rWSSV458, and rWSSV471 protein (from this study) could specifically bind to the rALF*Pm*3 protein.

2. Previously, the ALF*Pm*3 protein has been shown to inhibit the replication of WSSV in shrimp. Also, the neutralization effect of the ALF*Pm*3 protein on WSSV has been reported. Binding of rWSSV189, rWSSV458 and rWSSV471 proteins to the rALF*Pm*3 protein decrease the neutralizing activity against WSSV of the rALF*Pm*3 protein.

3. To characterize WSSV189, WSSV458 and WSSV471 protein, the native WSSV proteins were detected in HLS and GLS of WSSV-infected shrimp. The expression of these WSSV proteins was gradually increased at 6, 24 and 48 hpi in both HLS and GLS. WSSV471 protein, which was previously identified as a hub protein of WSSV protein network, was highest expressed. This implied that these rWSSV proteins are important for WSSV infection in shrimp.

4. To better understand how ALF*Pm*3 perform its antiviral activity, I have studied the localization of WSSV189 and WSSV471 proteins on WSSV. Western blot and IEM using an antibody specific to WSSV189 and WSSV471 identified WSSV189 and WSSV471 as structural proteins located at the envelope of the WSSV virion. We can conclude that the ALF*Pm*3 exhibits WSSV neutralizing activity via direct binding to the WSSV189 and WSSV471 structural proteins and possibly others.

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APPENDIX

Oral presentation

1. Methatham, T., Suraprasit, S., Jaree P., Phiwsaiya, K., Senapin, S., Hirono I., Lo, C. F., Tassanakajon, A., Somboonwiwat, K., Anti-lipopolysaccharide factor isoform 3 (ALF*Pm*3) from *Penaeus monodon* binds to white spot syndrome virus (WSSV) proteins for its antiviral activity. The 10th Asia-Pacific Marine Biotechnology Conference (APMBC 2014), Taipei, Taiwan (2014).

2. Methatham, T., Suraprasit, S., Jaree P., Phiwsaiya, K., Senapin, S., Hirono I., Lo, C. F., Tassanakajon, A., Somboonwiwat, K., Anti-lipopolysaccharide factor isoform 3 (ALF*Pm*3) from *Penaeus monodon* binds to white spot syndrome virus (WSSV) proteins for its antiviral activity. 2014 Symposium on Emerging Trends in Aquatic Disease and Aquaculture Biotechnology, Tainan, Taiwan (2014).

Poster Presentation

1. Methatham, T., Tassanakajon, A., Somboonwiwat, K., Anti-WSSV activity of ALF*Pm*3 possibly involved in the interactions with WSSV proteins. The 4th International Biochemistry and Molecular Biology Conference, Bangkok, Thailand (2014).

2. Methatham, T., Suraprasit, S., Tassanakajon, A., Somboonwiwat, K., Anti-white spot syndrome virus (WSSV) activity of ALF*Pm*3 possibly involved in the interactions with WSSV proteins, 18th Biology Science Graduate Congress, Kuala lumpur, Malasia (2014).

VITA

Mr. Thanachai Methatham was born in September 26, 1987 in Sukhothai. He graduated with the degree of Bachelor of Science from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2010. He has studied for the degree of Master of Science, Program in Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2011. In his third year of research work, He presented a poster entitle “Anti-white spot syndrome virus (WSSV) activity of ALFPm3 possibly involved in the interactions with WSSV proteins” at the 4th international Biochemistry and Molecular Biology Conference, Organized by Department of Biochemistry, Faculty of Science, Kasetsart University, Thailand and Biochemistry and Molecular Biology Section of the Science Society of Thailand under the Patronage of His Majesty the king at the Rama Gardens hotel & Resort, Bangkok in April 2-3, 2014. He got the outstanding poster presentation award.