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นางสาวสิวลี สุระประสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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IDENTIFICATION OF BACTERIAL AND VIRAL PROTEINS INTERACTING WITH *Penaeus monodon* ANTILIPOPOLYSACCHARIDE FACTOR ISOFORM 3

Miss Sivalee Suraprasit

A Thesis Submitted in Partial Fulfillment of the Requirements
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โปรตีนแอนติลิโพพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 (ALF*Pm*3) เป็นโปรตีนที่มีฤทธิ์การยับยั้งการเจริญของแบคทีเรียแกรมลบ แบคทีเรียแกรมบวก เชื้อรา และไวรัสตัวแดงดวงขาวในกุ้งได้ดี งานวิจัยนี้จึงสนใจศึกษาหน้าที่ของโปรตีน ALF*Pm*3 ในระบบภูมิคุ้มกันของกุ้งกุลาดำและการเกิดอันตรกิริยาของโปรตีน ALF*Pm*3 กับโปรตีนของเชื้อก่อโรค ด้วยการค้นหาโปรตีนทั้งจากเซลล์เม็ดเลือดของกุ้งที่ติดเชื้อแบคทีเรียวิบริโอฮาวิอาย (*Vibrio harveyi*) และไวรัสตัวแดงดวงขาว (WSSV) และโปรตีนจากเชื้อแบคทีเรีย *V. harveyi* และเชื้อไวรัส WSSV โดยใช้เทคนิค Yeast two-hybrid screening (Y2H) ผลการค้นหาจากห้องสมุดของเซลล์เม็ดเลือดของกุ้งที่ติดเชื้อแบคทีเรียและไวรัสไม่พบโคลนที่เกิดอันตรกิริยากับ ALF*Pm*3 แสดงว่าโปรตีน ALF*Pm*3 ทำงานได้โดยไม่ได้เกิดอันตรกิริยากับโปรตีนอื่นๆของกุ้งระหว่างการติดเชื้อ แต่ ALF*Pm*3 จะเกิดอันตรกิริยากับเชื้อก่อโรคได้โดยตรง จากนั้นทำการค้นหาโปรตีนของเชื้อก่อโรคที่โปรตีน ALF*Pm*3 ไปจับได้ จากห้องสมุดของเชื้อแบคทีเรียวิบริโอฮาวิอาย (VH library) และเชื้อไวรัสตัวแดงดวงขาว (WSSV library) จากการคัดเลือกใน VH library ไม่พบโคลนที่เกิดอันตรกิริยากับโปรตีน ALF*Pm*3 แสดงให้เห็นว่ากลไกการทำงานของโปรตีน ALF*Pm*3 ในการต่อต้านเชื้อแบคทีเรีย *V. harveyi* อาจไม่เกี่ยวข้องกับการจับกันของโปรตีนของเชื้อแบคทีเรีย สำหรับการคัดเลือกใน WSSV library พบโปรตีนจากไวรัส WSSV ที่เกิดอันตรกิริยากับโปรตีน ALF*Pm*3 ได้ทั้งหมด 5 โคลนได้แก่ WSSV186 WSSV189 WSSV395 WSSV458 และ WSSV471 จากการตรวจสอบการแสดงออกของยีน WSSV เหล่านี้เมื่อกุ้งติดเชื้อไวรัส WSSV ในเวลาต่างๆด้วยวิธี RT-PCR พบว่ายีน WSSV ทั้ง 5 ยีนเป็นยีนที่มีการแสดงออกในระยะท้ายของการติดเชื้อ (24 ถึง 48 ชั่วโมงหลังจากการติดเชื้อ) ต่อมาได้เลือกยีน WSSV189 และ WSSV471 ซึ่งเป็นยีนที่ไม่ทราบหน้าที่ มาศึกษาสมบัติและพิสูจน์การเกิดอันตรกิริยากับโปรตีน ALF*Pm*3 โดยโคลนยีนที่ครบสมบูรณ์ของ WSSV189 และ WSSV471 เข้าสู่เวกเตอร์ pET-19b แล้วนำเข้าสู่เซลล์เจ้าบ้าน *Escherichia coli* สายพันธุ์ต่างๆ ได้แก่ BL-21(DE3) Rosetta(DE3)pLysS และ BL21-CodonPlus(DE3)-RIL โดยสามารถผลิตโปรตีนรีคอมบิแนนท์ WSSV189 ขนาด 26 กิโลดาลตันและ WSSV471 ขนาด 20 กิโลดาลตันที่มีกรดอะมิโนฮิสทิดีนบริเวณปลายคาร์บอกซิลได้สำเร็จใน *E. coli* สายพันธุ์ BL21-CodonPlus(DE3)-RIL จากนั้นนำมาทำให้บริสุทธิ์โดยใช้นิกเกิลคอลัมน์แบบสัมพรรคภาพ และได้พิสูจน์การเกิดอันตรกิริยาระหว่างโปรตีน ALF*Pm*3 กับโปรตีนรีคอมบิแนนท์ WSSV189 และ WSSV471 ด้วยเทคนิค *in vitro* pull-down assay จากผลการทดลองแสดงให้เห็นว่าการที่ ALF*Pm*3 สามารถจับ WSSV189 และ WSSV471 ได้อย่างจำเพาะจึงน่าจะเกี่ยวข้องกับแอกทิวิตีของ ALF*Pm*3 ในการต่อต้านเชื้อไวรัส WSSV

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SIVALEE SURAPRASIT: IDENTIFICATION OF BACTERIAL AND VIRAL PROTEINS INTERACTING WITH *Penaeus monodon* ANTILIPOPOLYSACCHARIDE FACTOR ISOFORM 3. ADVISOR: ASST. PROF. KUNLAYA SOMBOONWIWAT, Ph.D., CO-ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., 126 pp.

A broad-spectrum antimicrobial peptide from *Penaeus monodon*, namely anti-lipopolysaccharide factor isoform 3 (ALF*Pm*3) is active against bacteria, fungi and a shrimp pathogenic virus, white spot syndrome virus (WSSV). To study in depth on ALF*Pm*3 function in shrimp immune responses and on ALF*Pm*3-pathogen interaction, we screened the *Vibrio harveyi* (VHH)- and WSSV (WH)-infected shrimp hemocytes, *V. harveyi* (VH) and WSSV libraries for ALF*Pm*3 interacting protein using yeast two-hybrid technique (Y2H). Screening of the bacterial- and viral-infected shrimp hemocyte libraries could not identify any ALF*Pm*3-interacting partners. These suggested that the ALF*Pm*3 did not interact with other shrimp proteins during pathogenic infection, implying that the ALF*Pm*3 solely acted on the pathogens. Accordingly, no positive clone from VH library was found to interact with ALF*Pm*3 suggesting that the antibacterial mechanism of ALF*Pm*3 against *V. harveyi* might not involve its binding to bacterial proteins. For WSSV library, five true positive clones including WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471 were found to be the ALF*Pm*3-interaction proteins. Temporal transcriptional analysis in WSSV-infected *P. monodon* hemocytes revealed that all WSSV genes were expressed in the late phase of infection (24 h and 48 h post infection). Then WSSV189 and WSSV471, an unknown protein, were selected for further analysis. The open reading frame (ORF) of each WSSV189 and WSSV471was cloned into pET-19b vector and subsequently transformed into different strains of *Escherichia coli* including BL-21(DE3), Rosetta(DE3)pLysS, and BL21-CodonPlus(DE3)-RIL. The recombinant proteins containing a His-tag at C-terminus of WSSV189 (rWSSV189) and WSSV471 (rWSSV471) were successfully expressed in *E. coli* strain BL21-CodonPlus(DE3)-RIL. The rWSSV189 (26 kDa) and rWSSV471 (20 kDa) were purified through Ni mediated affinity chromatography and checked by SDS-PAGE and Western blot analysis. *In vitro* pull-down assay using rWSSV189 and rWSSV471 as baits confirmed the true interaction between ALF*Pm*3 with both WSSV proteins. To our knowledge, the specific binding of ALF*Pm*3 to WSSV189 and WSSV471 might involve in anti-WSSV activity of ALF*Pm*3.

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LIST OF ABBREVIATIONS

A	absorbance
AMP	antimicrobial peptide
ALF	anti-lipopolysaccharide factor
bp	base pair
CFU	colony forming units
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EST	expressed sequence taq
EtBr	ethidium bromide
h	hour
kb	kilobase
kDa	kilodalton
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
ml	millilitre

mM	millimolar
ng	nanogram
nm	nanometer
O.D.	optical density
^{o}C	degree celcius
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
sec	second
WSSV	white spot syndrome virus
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I

INTRODUCTION

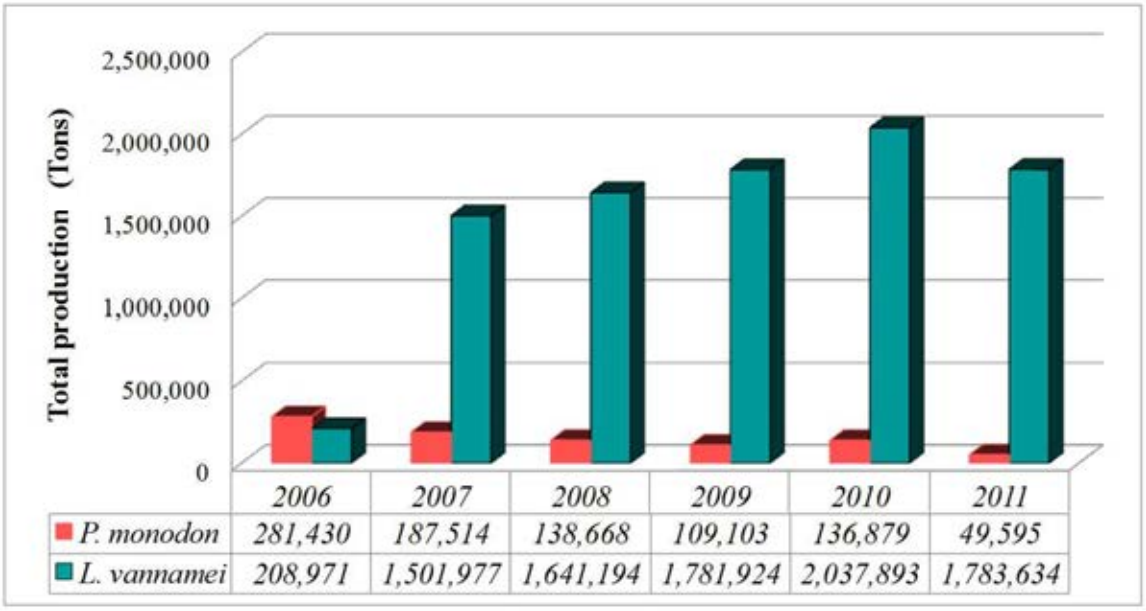
1.1 General introduction

Shrimp farming is a very important agro-industry in many countries. Since 1970 it has developed into a multi-billion-dollar industry. Shrimp farming provides approximately 30% of the shrimp supplied to the world market. Many species of shrimp are cultured in several parts of the world. The eight important shrimp species are the giant tiger shrimp, *Penaeus monodon*, the western white shrimp, *Litopenaeus vannamei*, the Chinese white shrimp, *Fenneropenaeus chinensis*, the northern brown shrimp, *Farfantepenaeus aztecus*, the yellow leg shrimp, *Farfantepenaeus californiensis*, the Indian white shrimp, *Fenneropenaeus indicus*, the western blue shrimp, *Litopenaeus stylirostris*, and the Japanese kuruma prawn, *Marsupenaeus japonicas* (Conklin, 2003). In Thailand, the shrimp species mainly produced are the black tiger shrimp (*P. monodon*) and the white shrimp (*L. vannamei*).

The shrimp culture in Thailand started in early 1980s and really began to spread in the mid 1980´s. In 1992, Thailand has been the world's leading producer export earning more than 1 billion US dollar per year (Flegal, 2006). The industry has been valued approximately 300,000-400,000 mectric 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 black tiger shrimp, *P. monodon* is farmed everywhere in Asia such as India, Thailand, Indonesia, Philipines, Vietnam and Malaysia. It has been proceeded for more than two decades. Until these days, the black tiger shrimp production in Thailand has rapidly decrease since 2006 (Fig. 1.1) because it seriously affected by the disease outbreaks mostly caused by viruses (Flegel, 2006) and *Vibrio* bacteria (Bachère, 2000). Hence, the western white shrimp, *L. vannamei* has become more popular than black tiger

shrimp. This is because the white shrimp species is a genetically improved species. It contains several advantages over the black tiger shrimp such as rapid growth, high stocking density tolerance, low salinities, temperatures tolerance and disease resistance. However, the *L. vannamei* may have certain disadvantages because it is an alien 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 alien species should be considered as necessary for Thailand. Therefore, *P. monodon* immunity and development of agriculture technology should be intensively studied.



	2006	2007	2008	2009	2010	2011
P. monodon	281,430	187,514	138,668	109,103	136,879	49,595
L. vannamei	208,971	1,501,977	1,641,194	1,781,924	2,037,893	1,783,634

(Source: Office of Agriculture Economics)

Figure 1.1 The shrimp *P. monodon* and *L. vannamei* production in Thailand between 2006-2011

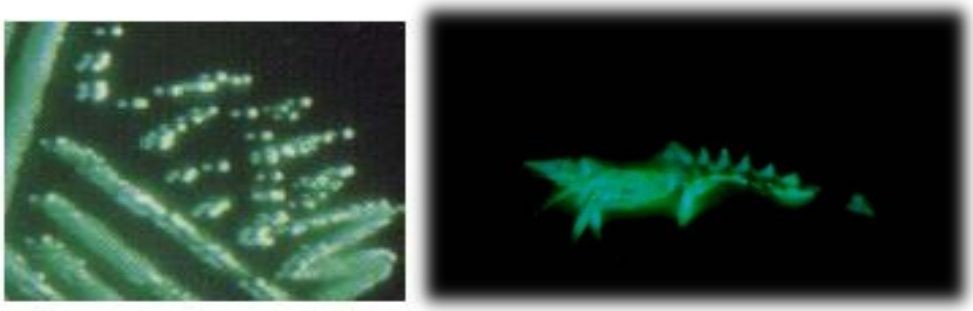
1.2 Shrimp disease

Viruses and bacteria are the major cause of the infectious diseases in *P. monodon*. Because of the artificial conditions in the shrimp farms where water quality, microbiological flora and nutrition are immensely difference from those in the natural habitat. The bacterial diseases of shrimp are due mainly to *Vibrio* species (Saulnier et al., 2000). On the other hand, the major shrimp viral pathogens are white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), taura syndrome virus (TSV), and hematopoietic virus (IHHNV) (Flegal, 2006).

1.2.1 Bacteria disease

Vibriosis is the major bacterial infectious disease problems in the farmed Penaeid shrimp causing the mass mortality of cultured shrimp worldwide (Delves-Broughton and Poupard, 1976; Sinderman, 1977; Lightner et al., 1992; Chen et al., 2000). *Vibrio*-related infections frequently occur in hatcheries, but epizootics also commonly occur in pond reared shrimp species. Vibriosis is caused by Gram-negative bacteria in the family Vibrionaceae. Outbreaks may occur when environmental factors trigger the rapid multiplication of bacteria already tolerated at low levels within shrimp blood (Sizemore & Davis, 1985), or by bacterial penetration of host barriers. The exoskeleton provides an effective physical barrier to pathogens trying to penetrate the external surface of crustaceans, as well as the foregut and hindgut. Vibriosis is caused high mortality and a severe economic loss in all producing countries (Mohney et al.,1990). Disease outbreaks caused by *Vibrio harveyi, Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio alginolyticus,* and *Vibrio penaeicida* (Brock and Lightner, 1990; Ishimaru et al., 1995) have been reported in nursery or growout ponds of *P. monodon*, *L. vannamei*, *P. japonicas* and *P. stylirostris* (Saulnier et al., 2000).

Vibrio harveyi, a Gram-negative, luminous bacterium, is one of the important etiologic agents of mass mortalities of shrimp larval rearing systems. It is a rod-shaped, 0.5-0.8 μm in width and 1.4-2.6 μm in length. It is capable to emit light of a blue-green color. This bacterial outbreak causes mortality of the affected shrimp up to 100% (Lightner, 1993). Presumptive diagnosis is made on the basis of clinical sign and culture of the suspensions of hepatopancreas or blood on tryptic plate supplemented with 2% (w/v) NaCl. After incubation at 30 °C overnight, colonies of *V. harveyi* show strong luminescence in a dim light (Fig. 1.2). Other features of the infected shrimps are the milky white body and appendages, weakness, disoriented swimming, lethargy and loss of appetite. Eventually, these lead to death.



(Source: www.thailandshrimp.com)

Figure 1.2 The colonies of luminescent bacteria, *V. harveyi* are grown on selective media (left) and *V. harveyi* infected shrimp (right)

Antibiotics have been used to control of luminous *Vibrio* but it might have led to the problems of drug resistance. The development and use of a probiotics, a marine bacterial strain *Pseudomonas* I-2 and *Bacillus subtilis* BT23 can relieve the infection for it can produced a compound with inhibitory property against shrimp pathogen (Chythanya and Karunasagar, 2002; Vaseeharan and Ramasamy, 2003).

1.2.2 Viral Disease

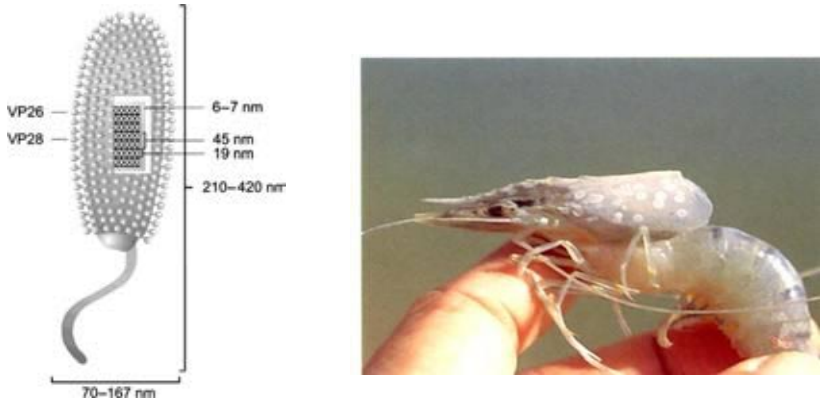
The problem of penaeid shrimp farming is mostly caused by viral pathogens. The prevention and control of diseases afflicting shrimp is now a high priority research topic in Thailand. There are 7 families of viral pathogens including Parvoviridae, Bacuroviridae, Iridoviridae, Picornaviridae, Rhabdoviridae, and Togaviridae identified in penaeid shrimp (Jittivadhna, 2000). Two major of viral pathogens of *P. monodon* are white spot syndrome virus (WSSV) and yellow head virus (YHV) (Boonyatatpalin et al., 1993; Wongteerasupaya et al., 1995). In this study, we focused only on WSSV.

1.2.3 White spot syndrome virus (WSSV)

White spot syndrome virus is a pathogen of the major economic importance. It has appeared globally as one of the most prevalent and widespread, which was first discovered in Taiwan in 1992 (Chou et al. 1995). In 1994, WSSV was reported in Thailand (Lo et al., 1996).

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 (van Hulten et al., 2001; Huang et al., 2005; 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 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. A tail-like appendage at one end of the WSSV virion (Wongteerasupaya et al., 1995; Durand et al., 1996) (Fig. 1.3).More than 40 proteins have been characterized from this virus. 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). Others are structural related and were located in virion or nucleocapsid (Escobedo-Bonilla et al., 2007).

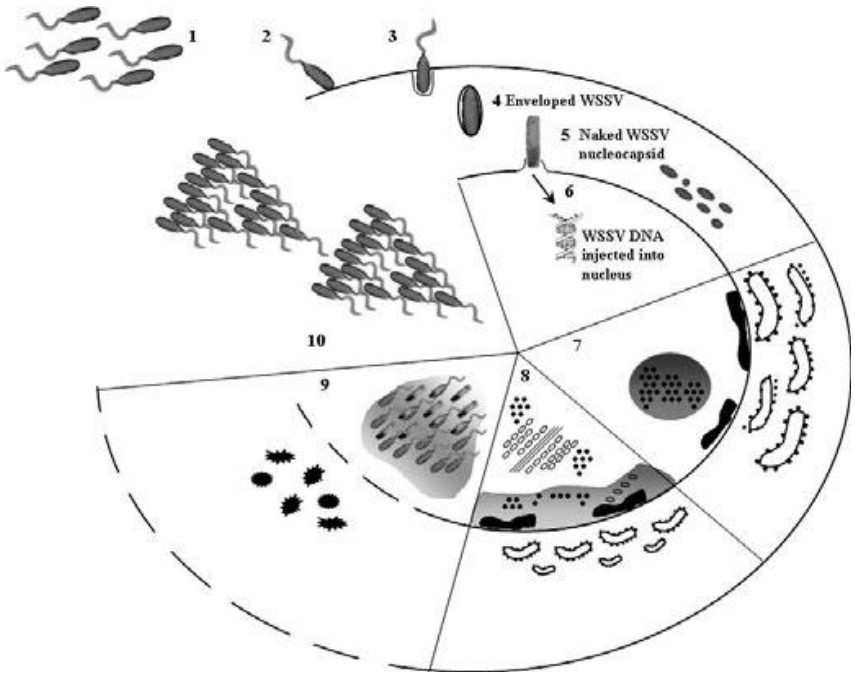
WSSV has spread rapidly to shrimp-farming areas all over the world and become one of the major pathogens in shrimp farms. After infection, the shrimp exhibits very high mortality rate up to 100% within 3 to 10 days, resulting in large economic losses to the shrimp farm industry (Lightner, 1996). It has a broad host range including shrimp, crab and crayfish (Wang et al, 1998; Zhang et al., 2004). WSSV infects and causes disease in many shrimp species worldwide including *P. monodon*, *L. vannamei*, *M. japonicas*, *F. chinensis* and *L. stylirostris.* 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, 1996). The virus severely damages the stomach, gills, subcuticular epithelial cells, lymphoid organs, antennal gland, and hemocyte (Chang et al., 1996; Lightner, 1996). Nevertheless, the mechanism of infection and spreading of WSSV in crustacean host is not clear. It is believed that the envelop proteins of virus might be important roles in viral infections (Zhang et al., 2004; Wu et al., 2005).



(Source: www.vetres.org) (Source: shrimpdiseases.worldpress.com)

Figure 1.3 The morphology of the WSSV viral particle and WSSV infection on shrimp carapace

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; Wang et al., 1999; Tsai et al., 2006; Escobedo-Bonilla et al., 2008). They have several stages of development (Fig. 1.4). At the early stage of cell infection the WSSV particles infect host cells and show gently hypertrophied nuclei. A viral nucleosome appears before the formation of viral particles. It composes of viral proteins organized in fibrillar fragments. In the nucleus, the fibrillar material induces the formation of circular membranes that are soon filled with viral core material starting viral assembly. The nucleocapsids appear with low electron density and grow from one end towards the other. The presence of most abundant viral particles. Afterwards, the nucleocapsid is completed of globular protein and become completely enclosed by the envelope. At the late stage of viral development, the viral particles become egg-shape and a long tail-like projection derived from the envelope is observed. Subsequently, the nucleocapsids become shorter, thicker and more electron-dense because of the packing of the viral DNA-VP15 complex. During the final phase of morphogenesis, the virions are elliptical with complete envelopes enclosing an electron-dense nucleocapsid and with a tail-like projection at the last enclosed end. The infected host cells are severely damaged and disrupted.



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

Figure 1.4 A model of the morphogenesis of white spot syndrome virus (WSSV)

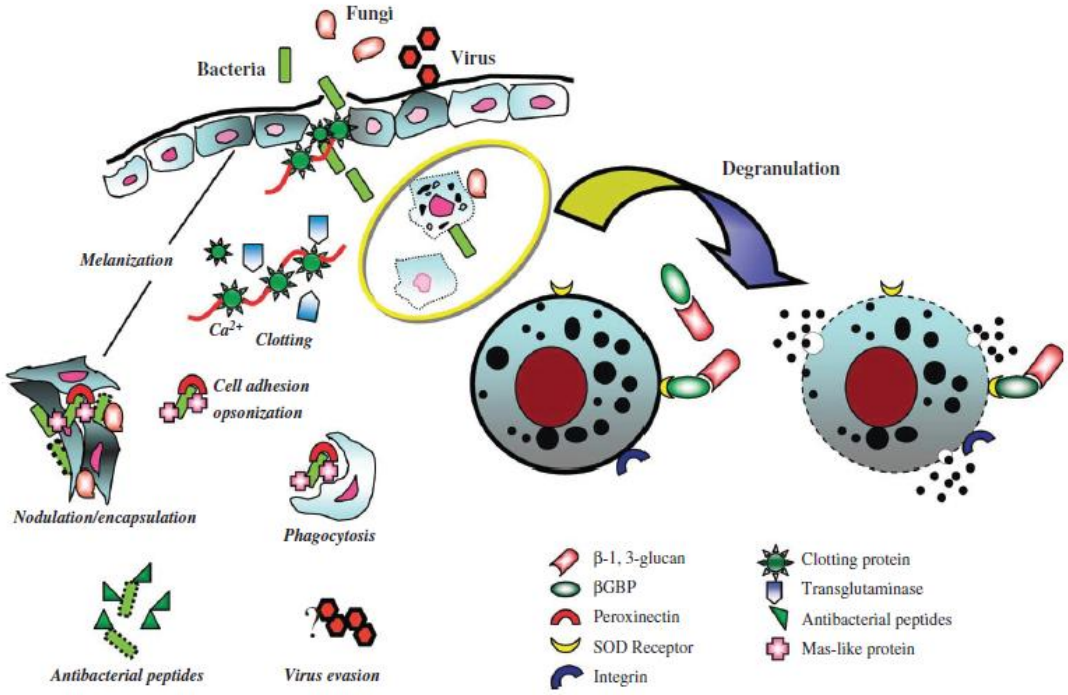
Because of the devastation effects on shrimp aquacultures by WSSV, various diagnostic methods with high efficiency, simplicity of use, and accuracy have been developed in many different laboratories to control the spread WSSV. Several diagnostic methods have been described such as polymerase chain reaction (PCR) (Kimura et al., 1996; Lo et al., 1996; Nunan and Lightner, 1997; Kim et al., 1998), *in situ* hybridization (Wang et al., 1998), miniarray (Quere et al., 2002), observation of tissues subjected to fixation or negative staining (Inouye et al., 1993), gastic epithelium by dark field microscopy (Momoyama et al., 1995), immunological methods using monoclonal and polyclonal antibodies to WSSV or their component proteins (Huang et al., 1998; Nadala et al., 1997; Poulos et al., 2001) and reverse passive latex agglutination (RPLA) method (Okumura et al., 2005).

1.3 The crustacean immune response

Crustaceans have the innate immune response based on cellular and humoral defense components of the open circulatory system to fight against pathogens. The cuticle that covers the external surfaces of the crustacean works as the first physical barrier, and it contains antimicrobial substances, coagulation factors, proteinases and proteinase inhibitor (Söderhäll and Cerenius, 1992). However, It responses rapidly if microorganisms can invade the animals. The major defense responses are carried in the hemolymph, which contains cells called hemocytes. Cellular defense components include all those reactions performed directly by hemocytes; for example phagocytosis, encapsulation, nodule formation, blood coagulation and cytotoxicity system. On the other hands, the humoral components related to production of soluble component playing roles in the defense system such as anticoagulant proteins, agglutinin, prophenoloxidase (ProPO), antimicrobial peptides and clotting system (Jiravanichpaisal et al., 2006; Holmblad and Söderhäll, 1999).

The cellular response is the cell-mediated reaction against pathogen invasion including phagocytosis, encapsulation, and nodule formation. Crustacean hemocytes have different functions depending on hemocyte types, which can be classified based on the presence and size of cytoplasmic granules. The hemocyte can be divided by light or electron microscopic observation into hyaline (the smallest hemocytes with minute cytoplasmic granule), semi-granulars (cell with small granules in cytoplasm), and granulars (the biggest hemocytes with large cytoplasmic granules) (Tsing et al., 1989; Rodrigues et al., 1995; Van de Braak et al., 2000). Even though the proportion and function of hemocytes can vary among species, it is generally considered that granular and semi-granular hemocytes have the ability of producing melanin by the pro-phenoloxidase system (Johansson et al., 2000). Hyaline cells involved in encapsulation, degranulation, and phagocytosis process (Giulianini et al., 2007). Hemocyte activation results in the release of various types of the immune effectors which function to

elimination the attacking pathogens. The current knowledge of crustacean immunity is summarized in Fig. 1.5.



(Source: Jiravanichpaisal et al., 2006)

Figure 1.5 The model of innate defense mechanism

1.4 Antimicrobial peptides

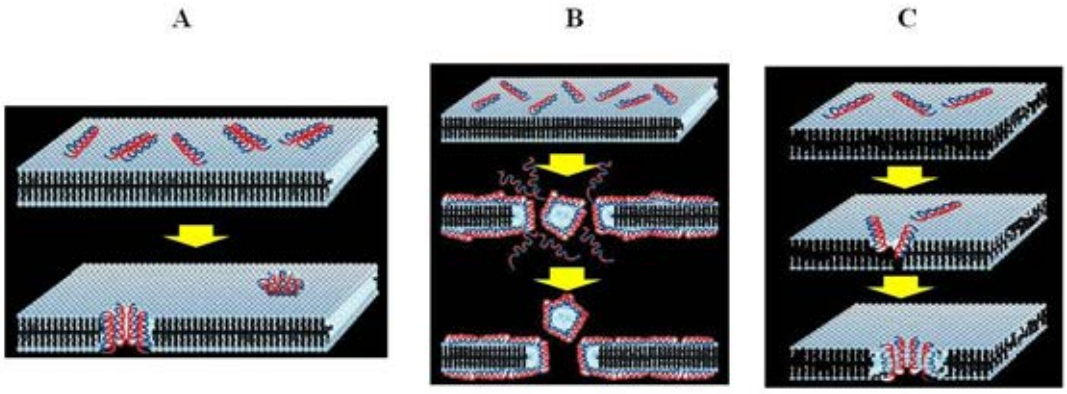
Antimicrobial peptides (AMPs) are components of the innate immune system and function as a first line of defense against many microorganisms (Hancock et al., 2006). They found in multicellular organisms. The biochemical features of AMPs are typically small size, generally less than 150-200 amino acid residues, and have amphipathic structure and cationic property. Nevertheless, the anionic peptides also exist. These peptides are active against a broad spectrum of microorganisms such as bacteria, virus,

yeast, parasite, and fungi and may also exhibit an anti-tumor activity (Hancock and Diamond et al., 2000; Cruciani et al., 1991; Krepstakies et al., 2012). AMPs have been founded in mammalians, and arthropods such as insects, horseshoe crabs, and shrimp (Tassanakajon et al., 2011; Bachère et al., 2004; Bulet et al., 1999, Vizioli and Salzets, 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.

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 from sheep, cattle and human. 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).

Several AMPs have been studied for their antibacterial mechanisms. There are 2 kinds of antibacterial mechanism; one is transmembrane pore-forming and another is intracellular antimicrobial peptide activity several studies have been carried out to seek a better understanding of two kinds mode of action these peptide (For review, Brogden, 2005 and Salditt et al., 2006). For the transmembrane pore-forming mechanism, the peptide insertion based on 3 proposed models and membrane permeability can be

described (Fig. 1.6). The first model is the barrel-stave model where the attached peptides aggregate and insert into the membrane bilayer in order that the hydrophobic peptide regions align with the lipid core regions and the hydrophilic peptide regions from the interior region of the pore (Fig. 1.6A). The second model is carpet-like model as the peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive layer or carpet (Fig. 1.6B). The third model is toroidal models where the attached peptides aggregate and induce the lipid monolayers to bend continuously trough the pore so that the water core is in line by both the inserted peptides and the lipid head groups (Fig. 1.6C). The microorganisms are then destroyed upon membrane permeabilization. An examples of peptides exhibiting their activity via carpet model pore formation is dermaseptins (Pouny et al., 1992).



(Source: Brogden, 2005)

Figure 1.6 **Transmembrane pore-forming mechanisms of the AMPs.** Hydrophilic and hydrophobic regions of the peptide are shown in red, and blue. (A) The barrel-stave model, (B) The carpet model, and (C) The toroidal model.

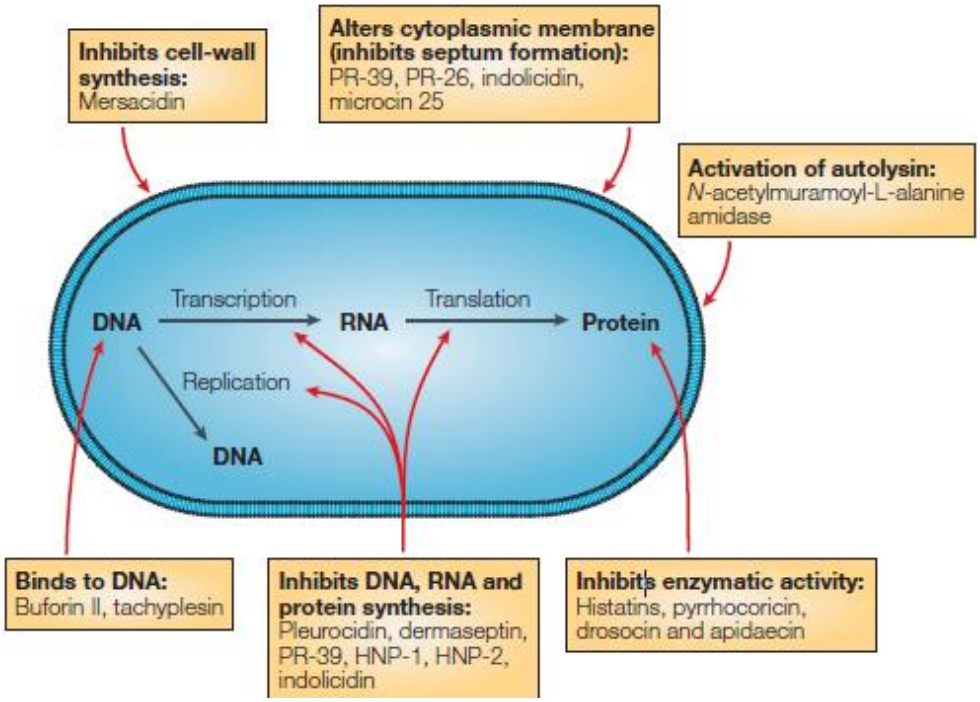
On the other hand, intracellular killing is the mechanism where antimicrobial peptides penetrate into the cells and bind to intracellular molecules that are crucial to cell living. Translocated peptides can alter cytoplasmic membrane septum formation, inhibit cell-wall synthesis, inhibit nucleic-acid synthesis, inhibit protein synthesis or inhibit

enzymatic activity (Fig. 1.7). There are many evidences showing the concurrence of intracellular killing and the ultrastructural damage. In that case, cellular damage lags substantially behind the time required for antimicrobial killing. In other instances, cellular damage occurs at the same rate as that of killing. Lehrer et al. (1989) noted that membranous blebs on HNP1-treated *E. coli* continued to accumulate as viable counts decreased and concluded that the appearance of blebs followed, rather than caused, the loss of bacterial viability (Lehrer et al., 1989). In similar studies, *E. coli* incubated with DEFB118 were killed in 15 minutes but cellular damage continued 30–120 minutes after exposure to the peptide (Yenugu et al., 2004). Furthermore, *P. aeruginosa* cells incubated with SMAP29 or CAP18 were killed in 15 minutes, whereas cellular damage continued for up to 8 hours (Kalfa et al., 2001).

In many cases, the mechanism of killing is unknown. In contrast to many conventional antibiotics, these peptide appear to be bacteriocidal or bacteria killer instead of bacteriostatic or bacteria growth inhibitior. In general, the antimicrobial activity of these peptides is determined by measuring the minimal inhibitory concentration (MIC), which is the lowest concentration of antimicrobial peptide that inhibits bacterial growth.

There are many reports on antimicrobial peptides in crustaceans. In 1997, the hemolymph of blue crab, *Callinectes sapidus*, have been founded to have the antimicrobial activity. The small antimicrobial peptide named calliectin has been identified (Khoo et al., 1999). Like most invertebrates, shrimps lack a true adaptive immune system and depend on innate immunity. Therefore, the AMPs are critical for them to fight the pathogenic invasion. Various antimicrobial peptides have been identified in shrimps such as penaeidins, lysozymes, crustins, anti-lipopolysaccharide factors (ALFs), and stylicin (Rolland et al., 2010; Tassanakajon et al., 2011). They are produced by and stored in the hemocyte, a prime cell in the immune system (Burge et al., 2007; Destoumieux et al., 2000; Somboonwiwat et al., 2005). Penaeidins, first isolated from the hemolymph of the pacific white shrimp, *L. vannamei*, which exhibited antifungal and anti-Gram-positive bacterial properties (Destoumieux et al., 1997).

Penaeidins contain a proline rich domain at the N-terminal and a six cysteine residues at C-terminal domain that form three disulfide linkages. cDNA clones of penaeidin isoform were also isolated from the hemocytes of *L. vannamei*, *P. setiferus* (Gross et al., 2001), and *P. monodon* (Supungul et al., 2004; Woramongkolchai et al., 2011). Crustins, an antimicrobial peptide homologue of an 11.5 kDa antibacterial peptide were identified from *L. vannamei*, *L. setiferus* and *P. monodon* (Bartlett et al., 2002; Vargas-Albores et al., 2004; Supungul et al., 2004). Peptide fragment derived from hemocyanin of *L. vannamei*, *L. stylirostris* and *P. monodon* possessing antiviral activity has also been identified (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004) and it can inhibit growth of Gram-positive bacteria.



(Source: Brogden, 2005)

Figure 1.7 Mode of action for intracellular antimicrobial peptide activity

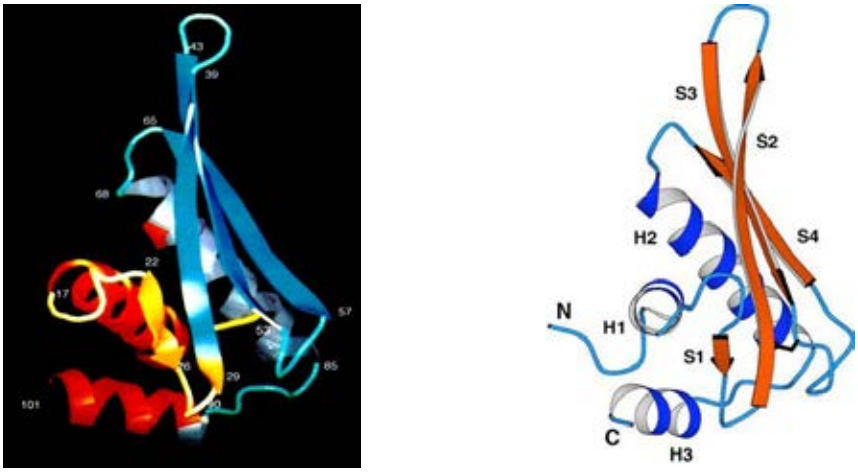
1.5 Anti-lipopolysaccharide factor

Anti-lipopolysaccharide factor (ALF) is an antimicrobial peptide previously found in the hemocytes of horseshoe crabs, *Limulus polyphemus* (LALF) and *Tachypleus tridentatus* (TALF) (Morita et al., 1985, Tanaka et al., 1982). So far, several ALFs have been identified and characterized in many crustacean species including *P. monodon* (Supungul et al., 2002), *L. setiferus* (Gross et al., 2001), *F. chinensis* (Liu et al, 2005), *M. japonicas* (Nagoshi et al., 2006), *P. leniculus* (Liu et al., 2006), *L. vannamei* (Jiménez-Vega and Vargas-Albores 2007; de-la-Re-Vega et al., 2008), *F. paulensis, L. schmitti* (Rosa *et al*., 2008). Recently, it has been shown that ALFs from crabs, *Scylla paramanosian*, was characterized exhibited to be phylogenetically related to the ALFs from the horseshoe crabs, shrimps and crabs (Imjongjirak et al., 2007, 2011).

In general, ALFs are small basic proteins that contain two-highly conserved-cysteine residues and highly conserved cluster of positively charged residues within the disulfide loop. All ALFs have a molecular weight of about 11 kDa with a isoelectric point usually in the range of 6.10-10.29. The activity of ALF depends on the positively charged cluster within a disulfide loop (Yang et al., 2009). From the three-dimensional structure of ALF (Fig. 1.8), the amphipathic disulfide loop binds to lipid A and neutralizes the biological effect of LPS (Hoess et al., 1993; Pristvsek et al., 2005; Yang et al., 2009). The synthetic disulfide loops from ALF*Pm*3 and ALF*Sp* has been shown unequivocally to be antimicrobial activity, though the actual mechanism is not known (Imjongjirak et al., 2007; Somboonwiwat et al., 2005).

The Limulus ALF has previously been reported to inhibit lipopolysaccharide-mediated coagulation system or endotoxin, and inhibit the growth of some Gram-negative bacteria (Morita et al., 1985). Many studies on the antimicrobial activity of shrimp ALFs (ALF*Pm*3, *Lv*ALF) and mud crab (ALF*Sp*) have also been reported a broad anti-microbial activity against bacterial and fungal (de la Vega et al., 2008; Imjongjirak et al., 2007; Somboonwiwat et al., 2005). *M. japonicus* ALF-like peptide (M-ALF) exhibits LPS neutralizing activity in the *Limulus* ameabocyte lysate and nitric oxide production

from the murine macrophage cell line RAW264.7 (Nagoshi *et al*., 2006). *Lv*ALF1 from *Litopenaeus vannamei* can protect shrimp from *Vibrio penaeicida* and *Fusarium oxysporum* as revealed by gene knockdown experiment (de-la-Re-Vega *et al*., 2008). In freshwater crayfish, *Pacifastacus leniusculus*, ALF has been shown to interfere with WSSV replication in cell culture and animals (Liu *et al*., 2006). However the mechanism of antiviral activity against WSSV of ALF is still unknown.



(Source: Hoess et al., 1993) (Source: Yang et al., 2009)

Figure 1.8 The structure of an anti-lipopolysaccharide factor from horseshoe crabs and shrimps. (left) X-ray structure of *Limulus* ALF; (right) NMR structure of rALF*Pm*3.

In *P. monodon*, several isoforms of ALF have been identified from the express sequence taq (EST) database (for review, see 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) (Supungul et al., 2004; Prapavorarat et al., 2010; Ponprateep et al., 2012) have been identified. They can divided into 3 groups: ALF*Pm*1 and ALF*Pm*2 formed one groups (group A). ALF*Pm*3-ALF*Pm*5 formed another group (group B) and ALF*Pm*6 belongs to a new ALF*Pm* group (group

C). Among them, the LPS binding site is different which 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* and WSSV infection revealing its roles in shrimp immune response (Somboonwiwat et al., 2008; Ponprateep et al., 2012). 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. In addition, the rALF*Pm*3 can efficiently protect *P. monodon* from WSSV infection (Tharntada et al., 2009).

1.6 Shrimp antiviral immunity

Viral components like genomic DNA and RNA or dsRNA generated in virally infected cells can be sensed by host pattern recognition receptors (PRRs) after which the appropriate antiviral response is triggered (Liu et al., 2009). Such antiviral responses have been review in shrimp, crayfish and other crustaceans. 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).

In shrimp, many studies have reported that virus-inhibiting proteins such as ALF, Beta-integrin, *Pm*AV, Syntenin etc. could interact with WSSV (Liu et al., 2009). 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* in *P. monodon*. 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., 2007). 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).

The proteins or genes involved in anti-WSSV response have been briefed by Liu et al. (2009). 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., 2007). 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 JAKSTAT (Janus kinase/signal transducer and activator of transcription) pathway but benefits from STAT activation in shrimp (Chen et al., 2008). 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 (Robalino et al., 2005; Kim et al., 2007; Xu et al., 2007; Liu et al., 2009). Effect of injection of WSSV specific dsRNA/siRNA can block viral disease progression. This effect has been confirmed with different unrelated viruses.

1.7 Protein-protein interaction technologies

Protein-protein interactions have attracted much attention because they form the basis of a wide variety of biochemical reactions. The identification of proteins that interact with the known protein is an essential aspect of the protein-functional characterization. The study of interaction between two or more proteins interacting with a specific functional objective can be elucidated in several different ways. The measurable effects of protein interactions have been outlines by Phizicky and Fields, 1995.

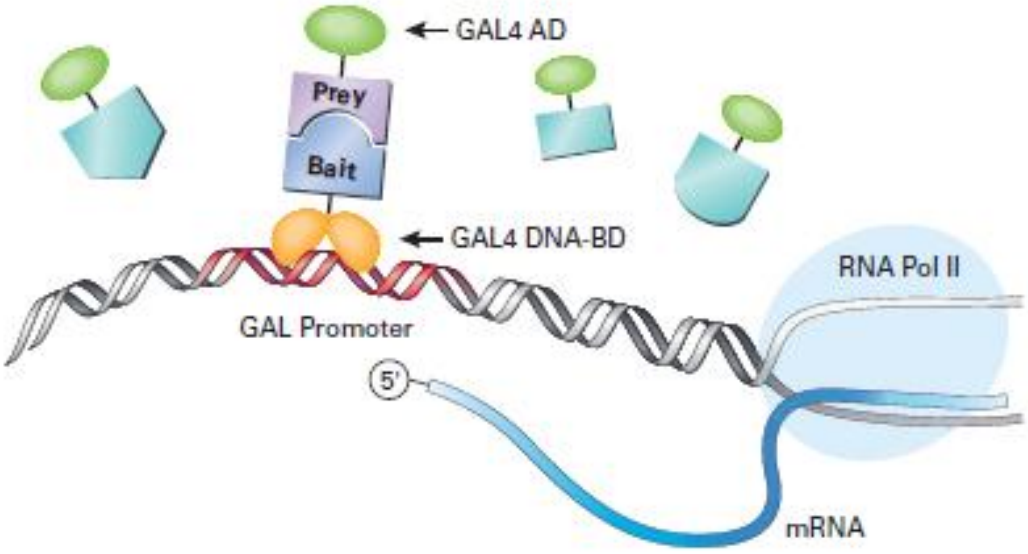
Interest has stimulated the development of the number of biochemical and genetics approach to identify and clone genes encoding interacting proteins. Yeast two-hybrid system and *in vitro* pull-down assay appear to have the greatest impact on the interaction methodology.

1.7.1 Yeast two-hybrid system

The yeast two-hybrid system has been devised to identify gene encoding protein that physically associate with a given protein *in vivo*. This is a versatile and powerful method that is applicable to most, if not all, proteins once their genes have been isolated. In contrast to biochemical methods detecting protein-protein interactions, this system is based on yeast genetic assay in which the interaction of two proteins are measured by reconstitution of functional transcription activator in yeast (Field and Song, 1989; Chien et al., 1991). This method not only allows identification of proteins that interact, but also can be used to define and/or test the domain/residues necessary for the interaction of two proteins (Li and Field, 1993). Since its development, a large number of genes from a variety of studies have been identified using this method, including many cell cycle regulators that have contributed significantly to our understanding of the eukaryotic cell cycle (Harper et al., 1993; Toyoshima and Hunter, 1994; Bai et al., 1996)

The basis of the yeast two-hybrid system relies on the structure of particular transcription factors that have two physically separable domains: a DNA-binding domain

and transcription activator domain. The DNA binding domain serves to target the transcription factor to specific promoter sequence whereas the activating domain serves to facilitate assembly of the transcription complex allowing the initiation of transcription. The fact that a functional transcription factor can be reconstituted through non-covalent interaction of two independent hybrid proteins containing either a DNA-binding domain or an activation domain constitutes the basis of the two-hybrid approach (Fields and Song, 1989). The hybrid proteins are normally transcriptionally inactive alone or when co-expressed with a non-interaction hybrid protein. However, when co-expressed they associate via interaction between the two fusion protein partners. Their association results in activation and thus expression of the reporter gene driven by the specific UAS for the DNA-binding domain (Fig. 1.9) (Fields and Song, 1989).

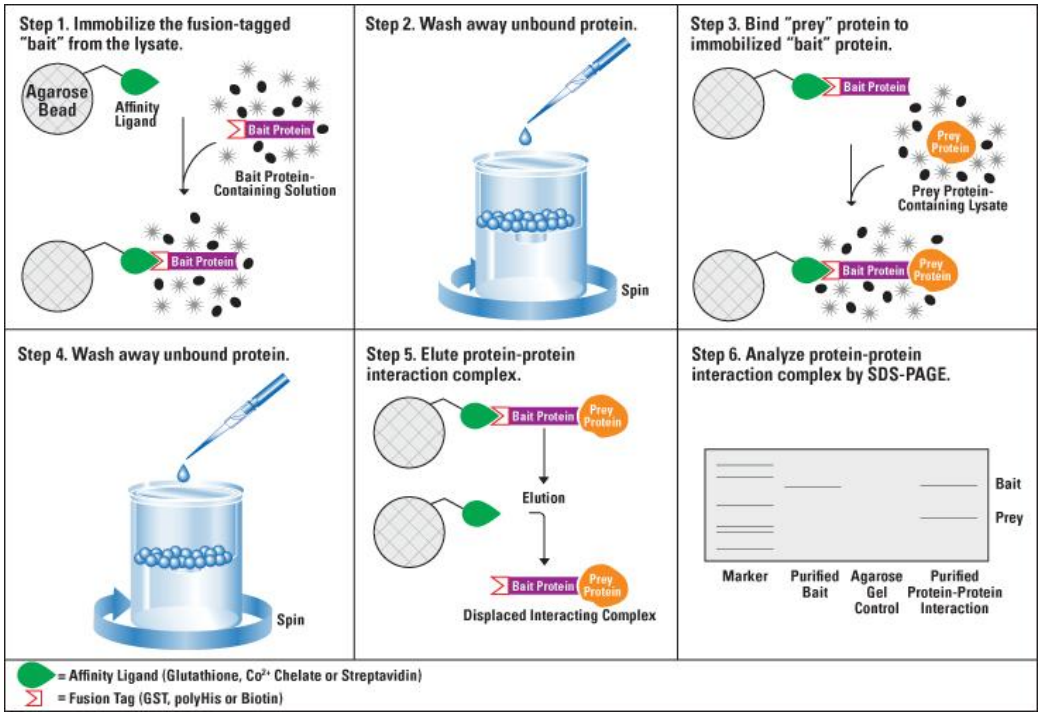


(Source: Mathmaker^{TM}Gold Yeast Two-Hybrid System User Manual)

Figure 1.9 A schematic representation of the basis of yeast two-hybrid assay

1.7.2 *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 et al., 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. 1.10). 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 1.10 A schematic of *in vitro* pull-down assay

1.8 Objectives of the dissertation

In this current research we aim to study the shrimp immune response especially that involves the ALF*Pm*3 functions upon bacterial and viral infection. We then apply the yeast two-hybrid screening to screen *V. harveyi*- and WSSV-infected shrimp hemocyte libraries for shrimp proteins that can interact with the ALF*Pm*3 protein. Moreover, to fulfill the understanding in pathogen-host interaction, the yeast two-hybrid screening again is used to screen for ALF*Pm*3-interacting proteins from the *V. harveyi* and WSSV libraries. The interaction between ALF*Pm*3 and its interacting partners is further confirmed by *in vitro* pull-down technique.

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^{TM} 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)

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

Minicentrifuge (Costar, USA)

Minipulser electroporation system (Bio-RAD)

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)

PD-10 column (GE Healthcare)

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)

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)

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

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

Anti-His antiserum (GE Healthcare)

Benchmark™ Pre-stained Protein Ladder (Invitrogen)

Benchmark™ Unstained Protein Marker (Invitrogen)

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)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

D-Glucose anhydrous (Ajax)

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

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

Hydrochloric acid (HCl) (Merck)

HiTrap SP Sepharose^{TM} Fast Flow 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)

Paraformaldehyde (Sigma)

Phenol:chloroform:isoamyl alcohol (Sigma)

Phosphoric acid (Labscan)

Prestained protein molecular weight marker (Fermentas)

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)

Unstained protein molecular weight marker (Fermentas)

Urea (Affy Metrix USB)

2.1.3 Kits

High-speed plasmid mini kit (Geneaid)

Matchmaker™ Gold Yeast Two-Hybrid System (Clontech)

Nucleospin® Extract II kit (Macherey-Nagel)

ProFound™ Pull-down PolyHis Protein (Thermo scientific)

RevertAID™ first strand cDNA synthesis kit (Fermentas)

T&A cloning vector kit (RBC Bioscience)

Yeastmaker™ Yeast Transformation System 2 (Clontech)

2.1.4 Enzymes

Advantage® 2 Polymerase Mix (Clontech)

*Hind*III (Biolabs)

*Nde*I (Biolabs)

*Bam*HI (Biolabs)

Phusion® Hot Start High-Fidelity DNA polymerase (Finnzymes)

Taq DNA polymerase (RBC Bioscience)

T4 DNA ligase (Biolabs)

2.1.5 Antibiotics

Aureobasidin A (Clontech)

Ampicillin (BioBasic)

Chloramphenicol (Sigma)

Kanamycin

Tetracycline

2.1.6 Bacterial, yeast and virus strains

Escherichia coli strain XL-1-Blue

E. coli strain 363

E. coli strain BL21(DE3)

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

E. coli strain Rosetta(DE3)pLysS

Pichia pastoris strain KM71

Saccaromyces cereviseae strain Y2H Gold (Clontech)

White spot syndrome virus (WSSV)

2.1.7 Softwares

BlastX (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)

ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

ExPASy ProtParam (http://au.expasy.org/tools/protparam.html)

GENETYX version 7.0 program (Software Development Inc.)

SECentral (Scientific & Educational Software)

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

2.1.8 Vectors

pET-19b (Novagen®, Germany)

pGBKT7 (Clontech, USA)

T&A cloning vector (RBC Bioscience)

2.2 General protocol purpose

2.2.1 Quantitative method for DNA determination

The concentration of DNA fragment was determined by measuring the A_{260} and estimated in μg/ml using an equation: [DNA] (μg/ml) = A_{260} × dilution factor × 50 for one A_{260} corresponds to 50 μg/ml of DNA (Sambrook et al., 1989).

2.2.2 Primers design

PCR primer pairs were designed based on nucleotide sequences of the template DNA using the SECentral (Scientific & Educational Software). Each primer in the pair should have about the same T_m values. They were checked for minimal self-priming and primer dimer formation.

2.2.3 Competent cells preparation and transformation

There are two types of *E. coli* competent cells used in this study, $CaCl_2$-treated cells and electrocompetent cells.

A single colony of *E. coli* strain XL-1-Blue, BL21(DE3), BL21-CodonPlus(DE3)-RIL, or Rosetta(DE3)pLysS were inoculated into fresh LB media (1% (w/v) tryptone

type-I, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl) containing an appropriate antibiotic (34 μg/ml of chloramphenicol for Rosetta(DE3)pLysS, and 34 or 50 μg/ml of chloramphenicol for BL21-CodonPlus(DE3)-RIL). The culture was grown overnight at 37 °C with 250 rpm shaking as a starter. The starter was diluted 1:100 in LB broth and incubated until OD_{600} reached 0.5-0.6.

2.2.3.1 Competent cells for electro-transformation

The cell suspension was cooled down by chilling on ice for 30 min, before harvested by centrifugation at 4,000 × g for 15 min at 4 °C. The cell pellet was washed twice using 1 and 0.5 volume, respectively, of sterile pre-cooled distilled water. Then, cell pellet was resuspended in an appropriate volume of iced-cooled 10% (v/v) glycerol. Forty microliters of cell suspension were aliquoted and immediately frozen at -80 ^{o}C until used.

2.2.3.2 Competent cells for $CaCl_2$-transformation

The 10 min pre-chilled culture was centrifuged at 4,000 × g for 5 min. Then, the cells were washed once with 0.5 volume of 10 mM $CaCl_2$ solution. The final cell pellet was resuspended in an appropriate volume of 100 mM $CaCl_2$ solution supplemented with 10% (v/v) glycerol and chilled on ice for about 30 min. One hundred microliters of competent cells were taken into an aliquot and immediately frozen at -80 ^{o}C until used.

2.2.3.3 Transformation

For electro-transformation, plasmid solution at the maximum volume of 2 μl per 40 μl cells was transformed into electro-competent cells. The plasmid was incubated with competent cells on ice for 1 min, and then the mixture was transferred into cooled and cleaned 0.2 cm electrode gap cuvette for electroporation. The mixture was pulsed using Minipulser electroporation system (Bio-Rad) at constant 2.5 kV. The mixture was immediately transferred into 1 ml fresh LB media.

For $CaCl_2$ transformation, up to 20 µl plasmid was mixed with 100 µl of competent cells. The plasmid and competent cells were mixed and chilled on ice for at least 30 min. After that, the reaction was incubated at 42 ^{o}C for 1 min and optional on ice for 3 min. One milliliter of fresh LB media was subsequently added to the mixture.

Afterward, 1 ml culture containing recombinant cell was incubated at 37 °C with shaking for 1 h. The cell suspension was spread onto LB agar plate supplemented with appropriate selective substances.

2.2.4 Plasmid DNA preparation using High-speed plasmid mini kit (Geneaid)

A recombinant bacterial clone was inoculated into 5 ml of Luria-Bertani (LB) medium containing appropriate antibiotic and grew overnight at 37 °C with shaking. The cells were collected by centrifugation for 10 min at 8,000 × g. The cell pellet was resuspended in 200 µl of PD1 buffer containing RNase A. The 200 µl of PD2 buffer was added and mixed gently by inverting the tube 10 times to lyse the cells. The cell lysate was neutralized by adding 300 µl of PD3 buffer and mixed immediately by inverting the tube 10 times. After centrifugation at 15,000 × g for 3 min, the supernatant containing the plasmid was applied to a LP column by pipetting. The column was centrifuged at 15,000 × g for 1 min and the flow-through was discarded. The column was washed twice with 400 µl of W1 buffer and 600 µl of wash buffer containing ethanol, respectively, and then centrifuged to remove residual ethanol from wash buffer. Finally, the plasmid DNA was eluted by adding 50 µl of pre-heated elution buffer to the center of each column, incubating at room temperature for 2 min and centrifugation at 15,000 × g for 2 min. The eluent containing the plasmid was then stored at -20 °C until use.

2.2.5 Purification of PCR product from agarose gel

The PCR product was purified from agarose gel by Nucleospin® Extract II kit (Macherey-Nagel). The PCR band was excised from agarose gel with a clean sharp scalpel. Extra agarose was removed to minimize the size of the gel slice. The gel slice was weighted and transferred to a clean microcentrifuge tube. For each 100 mg of agarose gel, 200 µl of NT buffer was added into the tube. The sample was incubated at 55 °C for about 10 min or until gel was completely melted. A NucleoSpin extract column was placed into a 2 ml collecting tube. The sample was loaded into the column and centrifuged at 11,000 × g for 1 min. The flow-through was discarded, and the column was washed with 600 µl of NT3 buffer and centrifuged at 11,000 × g for 1 min. After centrifugation, the silica membrane in column was dried by centrifugation at 11,000 × g for another 2 min to completely remove NT3 buffer. The column was placed into a clean 1.5 ml microcentrifuge tube. The DNA was eluted by adding 50 µl of NE buffer into the center of silica membrane, leaving at room temperature for 1 min to increase elution yield of eluted DNA and centrifugation for 1 min. The flow-through containing the DNA fragment was measured at A_{260} for its concentration and stored at -20 °C until used.

2.2.6 Agarose gel electrophoresis

The 1-1.5% agarose gel was prepared using 1× TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). After melting the agarose gel, the solution was cooled down to 60 °C before pouring into a tray with a well-forming comb. The gel was placed onto the running chamber. DNA samples with 1/10 volumes of the 10X loading dye (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. A DNA ladder (100 bp or 1 kb marker, Fermentas) was used as standard DNA markers. The electrophoresis was performed in 1× TBE buffer at 100 volts for 30-40 min. The gels were stained with

ethidium bromide solution for a while and de-stained in water for about 10-15 min. The DNA bands were visualized under the UV transilluminator.

2.2.7 Protein analysis

2.2.7.1 Analysis of recombinant protein by SDS-PAGE

A discontinuous system of SDS-PAGE was used. The gel solutions were prepared as shown in the Appendix. The glass plates and spacers were assembled. Then, the separation gel solution was pipetted into the gel plate set and layered on top with distilled water was ensure a flat surface of gel. When the polymerization was complete, water of poured off. The stacking gel solution was prepared and poured on top of the separating gel. Then, a comb was placed immediately in position with excess gel solution overflowing the front glass plate. After the stacking gel was polymerized, the comb was removed and the wells were rinsed with distilled water to remove excess 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 10 min and spun down. The samples were either held at room temperature or kept on ice until loaded into gel.

Electrophoresis was performed in 1× SDS running buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 0.1% (w/v) SDS at a constant current of 25 mA per gel. The protein samples and the pre-stained protein marker were loaded into the wells. After electrophoresis, the gels were stained with 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 overnight or 1 h. After that, the gels were dipped into the destaining solution (10% (v/v) acetic acid, 10% (v/v)

methanol) and shaken at room temperature with agitation for 1-3 h or overnight. The solution of destaining was replaced regularly to assist the removal stain.

2.2.7.2 Protein detection by Western Blot analysis

After electrophoresis through the SDS-PAGE, the protein gel was removed from the glass plates. The nitrocellulose membrane, gel, and filter papers were soaked in transfer buffer (25 mM Tris base, 150 mM glycine and 20% methanol) for 15-30 min together with nitrocellulose membrane which were cut to the size of gel. The pre-soaked filter paper was placed onto the Trans-Blot® SD (Bio-Rad). A pipette was rolled over the surface of filter paper to exclude all air bubbles, followed by the nitrocellulose membrane, the gel, and the filter papers, respectively.

The transfer of protein was performed at constant 160 mA for 100 min. After protein was transferred, 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 for overnight with gentle shaking. The membrane was washed 3 times for 10 min with PBS/Tween™-20. The membrane was then incubated with anti-His antiserum (GE Healthcare) that was diluted 1:3,000 in PBS/Tween™-20 containing 1% (w/v) skim milk for about 3 h at 37 °C with gentle shaking. Subsequently, the membrane was washed 3 times for 10 min each with PBS/Tween20 and incubated with a alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) that was diluted 1:10,000 in PBS/Tween™-20 containing 1% (w/v) skim milk at room temperature for an hour. The membrane was washed 3 times for 10 min with PBS/Tween™-20 at room temperature. The color development was performed by adding 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 until the bands were detected. Finally, the membrane was washed with distilled water to stop the reaction.

2.2.7.3 Determination of protein concentration

The protein concentration was measured according to the method of Bradford (Bradford, 1976) using bovine serum albumin (Fluka) as a standard protein. This method is based on the binding of Coomassie brilliant blue G250 dye to proteins in sample converting the red dye color to blue. A sample solution of 100 µl was mixed with 1 ml of Bradford working buffer and incubated for 10 min at room temperature before A_{595} was measured. The Bradford working buffer (100 ml) was a mixture of 6 ml Bradford stock solution (350 g of Coomassie blue G250, 100 ml of 95% ethanol and 200 ml of 85% phosphoric acid), 3 ml of 95% ethanol, 6 ml of 85% of phosphoric acid and 85 ml of distilled water.

2.3 Identification of ALF*Pm*3 binding protein by yeast two-hybrid screening

2.3.1 Construction of ALF*Pm*3 bait vector in pGBKT7 plasmid

2.3.1.1 Amplification of ALF*Pm*3 gene

The DNA sequences corresponding to mature peptide of ALF*Pm*3 was amplified by PCR from a pBluescript SK plasmid containing ALF*Pm*3 gene pBS-ALF*Pm*3 using a specific primer pair; ALF*Pm*3F (5′-ATA CTA GAA TTC CAA GGG TGG GAG GCT GTG GCA-3′) and ALF*Pm*3R (5′-TAT TAT GGA TCC CTA TGA GCT GAG CCA CTG GTT GGC CT-3′). The *Eco*RI and *Bam*HI recognition sites for ALF*Pm*3F and ALF*Pm*3R primers, respectively, are underlined. The ALF*Pm*3 gene fragment was PCR amplified in a final reaction volumn of 50 µl containing 5 µl of 10× Advantage 2 PCR buffer, 1 µl of 50× dNTP mix, 1 µl of 10 µM primer each, 1 µl of pBS-ALF*Pm*3 plasmid, 40 µl of deionized water and 1 µl 50× Advantage 2 Polymerase Mix (Clontech). The PCR amplification was as followed by 30 cycle of 95 °C for 1 min; 95 °C for 30 s and 68 °C for 1 min. The amplified products were analyzed using 1%

agarose gel electrophoresis preparing with 1× TBE buffer, excised and purified using Nucleospin® Extract II kit (Macherey-Nagel) as described in section 2.2.5. The concentration of the purified PCR products was examined.

2.3.1.2 Ligation and transformation

The *Eco*RI/*Bam*HI digested ALF*Pm*3 fragment ligated into a pGBKT7 vector (Clontech) (Fig. 2.1) cut with the same enzymes. The total 20 µl of ligation mixture contained 2 µl of 10× T4 ligation buffer, 50 ng of *Eco*RI/*Bam*HI digested pGBKT7 vector, 40 ng of *Eco*RI/*Bam*HI digested ALF*Pm*3, and 1 µl of 400U/µl T4 DNA ligase (New England, Biolabs). The reaction was incubated at 22°C for 3-5 h.

Afterwards, 2 µl of the ligation mixture was electro-transformed into an *E. coli* XL1-blue. The transformants were selected on LB agar plate with 30 µg/ml kanamycin. The selected clones were sequenced using T7 promoter primer with an automated sequencer by a commercial service (Macrogen Inc., Korea). The bait vector that encoded the Gal4-DNA binding domain fused with ALF*Pm*3 was named as pGBKT7-ALF*Pm*3. The pGBKT7-ALF*Pm*3 was tested for autoactivation of reporter genes, toxicity to yeast cells and subsequently used for two-hybrid screening.

2.3.2 Transformation bait vector (pGBKT7-ALF*Pm*3) into *Saccaromyces cereviseae* strain Y2H Gold

2.3.2.1 Preparation of competent yeast cells

A single colony of *S. cereviseae* strain Y2H Gold was cultured and used as starter in 3 ml of YPDA medium at 30 °C with shaking 250 rpm for 8-12 h. Then, transfer 5 µl of the culture to 50 ml of YPDA medium and grown overnight to an OD_{600} 0.15-0.3. The cells were pelleted by centrifugation at 700 × g for 5 min at room temperature. The cell pellet was resuspended to a final volume of 100 ml in fresh YPDA

medium and incubated at 30 °C until OD_{600} reached 0.4-0.5. The culture was centrifuged at 700 × g for 5 min at room temperature. The pellet was washed by resuspending in a total of 60 ml of deionized water, gently mixing and centrifugation. The pellet was resuspended in 1.5 ml of 1.1xTE/LiAc. This cell suspension was equally transferred to two respective 3 ml microcentrifuge tubes and centrifuged at 15,000 × g for 15 sec. Finally, each cell pellet was resuspended in 600 µl of 1.1xTE/LiAc. The cells are now ready to be transformed with plasmid DNA.

2.3.2.2 Transformation of competent yeast cells

The bait vector, pGBKT7-ALF*Pm*3, was transformed into *S. cereviseae* Y2H Gold using Yeastmaker™ Yeast Transformation System 2 (Clontech). A 50 µl aliquot of *S. cereviseae* Y2H Gold competent cells was thawed on ice and gently mixed with 100 ng of pGBKT7-ALF*Pm*3 plasmid, 50 µg of yeast maker carrier DNA, and 500 µl of PEG/LiAc. The mixture of cell was incubated 30 °C for 30 min. DMSO (20 µl) was added, mixed gently and incubated at 42 °C for 15 min. The yeast cells were pelleted by centrifugation at 15,000 × g for 15 sec. The cell pellet was resuspended in YPD plus medium, incubated at 30 °C with shaking 250 rpm for 90 min and centrifuged at 15,000 × g for 15 sec.The pellet yeast cells were resuspended in 0.9% (w/v) NaCl solution.

2.3.2.3 Plating and determination of transformation efficiency

The cell suspension diluted at 1/10 and 1/100 folds in one hundred microliters of the diluted cells suspension were plated onto a selective medium which was SD medium lacking Tryptophan (SD/-Trp) and incubating at 30 °C until colonies appear. Then, the transformation efficiency was calculated using the following equation:

$$\text{Transformation Efficiency} = \frac{\text{cfu} \times \text{Suspension Volume (ml)}}{\text{Volume plated (ml)} \times \text{amount of DNA (µg)}}$$

2.3.2.4 Testing bait for autoactivation and toxicity

The yeast transformant containing pGBKT7-ALF*Pm*3 plasmid was tested for autoactivation. To confirm that ALF*Pm*3 do not autonomously activate the reporter genes in *S. cereviseae* Y2H Gold, in the absence of the prey protein. 100 ng of pGBKT7-ALF*Pm*3 plasmid was transformed using Yeastmaker™ Yeast Transformation System 2 (Clontech) as described in section 2.3.2.2. One hundred microliters of 1/10 and 1/100 fold diluted cell suspension were plated onto separate plates, as followed, SD/-Trp plates, SD/-Trp containing X-alpha-Gal (SDO/X) plates and SD/-Trp containing X-alpha-Gal and Aureobasidin A (SDO/X/A) plates and incubating at 30 °C until colonies appear. If bait vector autoactivates the *AUR1-C* reporter gene of *S. cereviseae* Y2H Gold such as blue colonies appear on SDO/X/A.

For toxicity test, pGBKT7-ALF*Pm*3 plasmid transformed into the yeast cells, both solid and liquid cultures will grow more slowly. 100 ng of pGBKT7-ALF*Pm*3 plasmid and 100 ng pGBKT7 plasmid (control) were transformed into *S. cereviseae* Y2H Gold using Yeastmaker™ Yeast Transformation System 2 (Clontech) as described in section 2.3.2.2. The cell suspension was spreaded 100 µl of 1/10 and 1/100 fold diluted onto SD/-Trp plates and incubating at 30 °C until colonies appear. If bait vector is toxic, the colonies containing bait vector are significantly smaller than colonies containing the empty pGBKT7 vector.

2.3.3 Control experiments

To perform a control mating before screening a two-hybrid library. The plasmids were transformed into competent yeast cells using Yeastmaker™ Yeast Transformation System 2 (Clontech) as described in section 2.3.2.2. The transformants were spreaded onto selective media plates and incubating at 30 °C for 3 days (Table 2.1).

Table 2.1 Transformation of control plasmids to competent yeast cells

Strain	Transformation plasmid	Plating Medium
Y2H Gold	pGBKT7-53	SD/-Trp with agar
Y2H Gold	pGBKT7-Lam	SD/-Trp with agar
Y187	pGADT7-T	SD/-Leu with agar

After that, a single colony of pGBKT7-53 in Y2H Gold and pGADT7-T in Y187 were mated in 500 µl of 2× YPDA broth as positive control mating. A negative control were yeast mated between pGBKT7-Lam in Y2H Gold and pGADT7-T in Y187. The solutions were mixed in microcentrifuge tubes and incubated at 30 °C with shaking 200 rpm for overnight. The 100 µl of mated culture (100 µl of 1/10, 1/100 and 1/1,000 dilution) was spreaded onto separate plates, as followed: SD/-Trp plates, SD/-Leu plates, SD/-Leu/-Trp (DDO) plates and SD/-Leu/-Trp containing X-alpha-Gal and Aureobasidin A (DDO/X/A) plates and incubating at 30 °C until colonies appear. The expected results of positive interactions, the number of colonies on DDO should be the same on DDO/X/A agar plates and colonies on DDO/X/A are blue. For negative interactions, the colonies appear on DDO but no colonies on DDO/X/A agar plates to determine if there was a problem with the bait or prey cultures.

2.3.4 Yeast two-hybrid screening

2.3.4.1 Yeast mating

To perform the Yeast Two-Hybrid screening, the Y2H Gold contained pGBKT7-ALF*Pm*3 pre-cultured in 5 ml SD/-trp medium was mated with 1 ml aliquot of *V. harveyi* (VHH)- or WSSV (WH)-infected shrimp hemocyte prey library and *V. harveyi* (VH) in *S. cerevisiae* strain Y187 constructed by Somboonwiwat., et al (unpublished

data) as described in the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech). The mating solution were mixed in 45 ml of 2× YPDA broth containing 50 μg/ml of kanamycin. The mating culture was grown for 20-24 hours at 30 °C with gentle swirling (50 rpm). The mating mixture was centrifuged at 1,000 × g for 10 minutes to collect the cell pellet that was resuspended in 10 ml of 0.5× YPDA containing 50 μg/ml of kanamycin.

The mating cultures are subsequently plated on the double dropout media lacking leucine and tryptophan (SD/-Leu/-Trp) containing X-alpha-Gal and Aureobasidin A (DDO/X/A). Positive clones were the blue colonies from *MEL1* reporter gene, *MEL-1* encodes α-galactosidase, an enzyme occurring naturally in many yeast strains. As a result of two-hybrid interactions, α-galactosidase was expressed and secreted by the yeast cells. Yeast colonies that express *MEL1* turn blue in the presence of chromagenic substrate X-α-Gal. After that positive clones were subsequently grown on the higher stringency quadruple dropout media in the absence of leucine, tryptophan, adenine and histidine (SD/-Leu/-Trp/-Ade/-His) containing X-alpha-Gal and Aureobasidin A (QDO/X/A).

2.3.4.2 Determination of the mating efficiency

One hundred microliters of mating cultures (1/10, 1/100, 1/1,000 and 1/10,000 dilution) were spreaded onto separate plates described above, as followed: SD/-Trp plates, SD/-Leu plates and SD/-Leu/-Trp containing X-alpha-Gal (DDO/X) plates and incubating at 30 °C until colonies appear. Then, the colonies were calculated the number of screened clones (diploids) from DDO/X and mating efficiency using the following equation:

$$\text{Number of screened clones} = \text{cfu/ml of diploids} \times \text{resuspension volumes (ml)}$$

$$\%\ \text{Mating efficiency} = \frac{\text{Number of cfu/ml of diploids}}{\text{Number of cfu/ml of limiting partner}} \times 100$$

2.3.4.3 Rescue plasmid

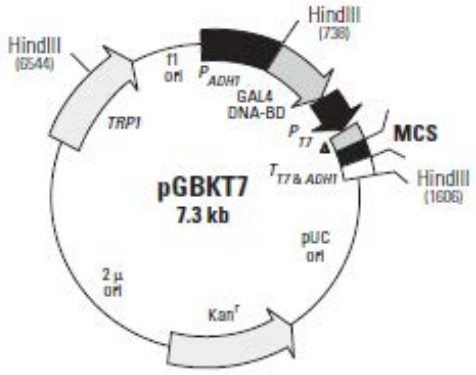
In order to screen the real positive clones containing the interacting proteins, prey plasmid DNA (pGADT7 plasmid containing hemocyte gene) was isolated from those clones that all four yeast reporter genes [*ADE2, HIS3, MEL1* (encode α-galactosidase) and *AUR1-C*] were activated. The rescued plasmid was transformed into *E. coli* XL-1-Blue to recover the plasmid for sequencing. The obtained sequences were searched against the GenBank database to identify the clones.

2.3.4.4 Confirmation of positive interaction by co-transformation

To confirm the screening results, each pGADT7 plasmid containing hemocyte gene or recovered plasmid was co-transformed with pGBKT7-ALF*Pm*3 or, parental pGBKT7 plasmid into a Y2H Gold strain as described in section 2.3.2.2 and plated on DDO/X and QDO/X/A according to the manufacturer's instruction (Clontech).

pGBKT7 Vector Information PT3248-5

Cat. No. 630489
630443



▲ c-Myc epitope tag

Restriction Map and Multiple Cloning Site (MCS) of pGBKT7. Unique restriction sites are in bold.

Figure 2.1 The pGBKT7 vector map (Clontech, USA)

2.4 Shrimp

The black tiger shrimp, *P. monodon*, each weighing of about 5-7 grams were kindly provided from a local shrimp farm at Suratthani province, Thailand. Shrimps were acclimatized in the laboratory aquaria at a temperature of 28±4 °C and at the salinity of about 15 ppt for at least 1 week before used in each experiment.

2.5 Preparation of WSSV stock

WSSV stock for experimental infections was firstly prepared according to the method described by Xie et al., 2005. *P. monodon* were infected with WSSV until they are moribund. Gills were dissected from the moribund shrimp and then homogenized in TNE buffer (50 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.5). After centrifugation at 5,000 × g for 5 min 4 °C, the supernatant was filtered through 0.45 μM filter membrane and centrifuged at 30,000 × g for 30 min. After the supernatant was discarded, the pellet was resuspended in TM buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5). Finally, the purified virus was kept at -80 °C until used for WSSV injection. The WSSV solution was diluted in 0.9% NaCl at 1:10,000 dilutions. One hundred microliters of diluted WSSV was used for injection into each shrimp.

2.6 RNA preparation and first-stranded cDNA synthesis

2.6.1 Total RNA extraction

At 0, 3, 6, 12, 24 and 48 h post-injection, gills were individually collected. The gill samples were briefly homogenized by a pestle in 1 ml of ice-cold TriReagent® (Molecular Research Center). The homogenate was stored at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. Then, 200 μl of chloroform was added. The sample were vigorously shaken for 15 min and incubated at room

temperature for 2-5 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 isopropanol. The mixture was left at room temperature for 5-10 min and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was removed. The pellet of total RNA was washed in 1 ml of 75% ethanol and stored at -80 °C until used.

The total RNA pellet in 75 % ethanol was centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was removed. The RNA pellet was briefly air-dried for 5-10 min. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC)-treated water and leaving it on ice until it was completely dissolved.

2.6.2 Determination of the quantity of RNA samples

The quantity and quality of total RNA was spectrophotometrically measured at 260 nm based on the specific property of UV adsorption.

The concentration of total RNA could be determined using the following formular: [RNA] = A_{260} × dilution factor × 40. One A_{260} corresponds to 40 µg/ml of RNA (Sambrook et al., 1989).

The relative purity of RNA samples was examined by measuring the ratio of $A_{260/280}$ and $A_{260/230}$. The maximum absorption of organic solvent, nucleic acid, and protein is at 230, 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 an agarose gel electrophoresis. The gel was stained with EtBr and visualized under UV light, respectively.

2.6.3 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 °C for 30 min to remove the contaminating chromosomal DNA. Then, the RNA was purified by TriReagent® and isopropanol precipitation. The reactions of DNase-treated RNA were adjusted the volume to 30 μl with DEPC-treated water and 200 μl of TriReagent® were added. The reaction was vortexed for 15 sec. 40 μl of chloroform were added and vigorously shaken for 20 sec. The mixture was kept at room temperature for 8-10 min and centrifuged at 12,000 × g for 15 min at 4 °C. After centrifugation, the aqueous upper phase was precipitated with 1 volume of isopropanol. The RNA pellet collected by centrifugation was washed with 1 ml of 75% ethanol. The RNA pellet was briefly air-dried and dissolved with an appropriate volume of DEPC-treated water. The quantity and quality of total RNA was examined as described in the section 2.6.2.

2.6.4 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 (Fermentas). According to the kit's instruction, the reaction was performed in a final volume 12 μl containing 1 μg of the total RNA, 0.5 μg of the oligo$(dT)_{18}$ primer and adjusted the volume by DEPC-treated water. The mixture of RNA was incubated at 65 °C for 5 min and chilled on ice for 5 min to anneal the primer. 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 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.

2.6.5 Semi-quantification of WSSV gene expression by RT-PCR

Semi-quantitative RT-PCR was used to examine the expression of interesting WSSV gene in WSSV-infected shrimp at various time points. Total RNA gill from 3 individual shrimp was extracted and then subjected to cDNA synthesis as described in the section 2.6.4. The WSSV genes were amplified using gene specific primers (Table 2.2). The elongation factor-1α (EF-1α) gene was generally used as an internal control.

The fragment of WSSV transcript was amplified by RT-PCR. One microliters of 2-fold diluted cDNA was used as a template for PCR amplification. The PCR reaction in a 25 µl total volume was carried out. The reaction contained 1.25 µl of 10× PCR buffer, 0.125 µl of 10 mM each dNTP, 0.125 µl of 10 µM primer each, 8.8125 µl of ultrapure water, and 0.0625 µl of 5 U/µl RBC *Taq* polymerase (Bioscience). The reaction was pre-denatured at 94 °C for 1 min and followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 58 or 60 °C for 30 sec and extension at 72 °C for 30 sec, and final extension at 72 °C for 7-10 min.

Five microliters of PCR product were mixed with one-five volume of 10× DNA loading dye (50 mM Tris-HCl, 2.5 mg/ml bromophenol blue, 2.5 mg/ml xylene cyanol, 60% glycerol at pH 7.6) and analyzed on 1.5% agarose gel electrophoresis as described in section 2.2.6.

2.6.6 Amplification of elongation factor-1α (EF-1α) gene

For internal control of RT-PCR, elongation factor-1α gene (GenBank accession no. NM_001958.2) was used. The specific primers of shrimp elongation amplifying 145 bp product are EF-F: 5' GGTGCTGGACAAGCTGAAGGC 3' and EF-R: 5′ CGTTCCGGTGATCATGTTCTTGATG 3′. The PCR reaction mixture of 25 µl total volume consisted of 2.5 µl of 10× PCR buffer, 0.5 µl of 10 mM each dNTP, 0.25 µl of 10 µM primer each, 1 µl of 10-fold diluted cDNA of interest, 20.25 µl of ultrapure water,

and 0.25 µl of 5 U/µl *Taq* polymerase (Bioscience). The PCR profile was same as Semi-quantitative RT-PCR of WSSV gene expression (section 2.6.5) but optimized to only 25 cycles. The five microliter of PCR products was mixed with 10× DNA loading dye and analyzed on 1.5% agarose gel electrophoresis as described above (section 2.2.6).

Table 2.2 Primer pairs used for the amplification

Gene	Primer	Sequence*	PCR product	Task
WSSV186	WSSV186F	5'-GGACCCCATGGGCAGTAGCTACC TAGATCTTTTA-3'	1,455 bp	RT-PCR
	WSSV186R	5'-GGTCCAGGATCCCTAATGATGATGA TGATGATGTAGAACAGTGTTATTTCTTC-3'		
WSSV189	WSSV189F	5'-GGACCCCATGGGCGAATGGATAAACC AACGGACA-3'	699 bp	Cloning and RT-PCR
	WSSV189R	5'-GGTCCAGGATCCTTAATGATGATGATGAT GATGTTGGATAAAGTAGTTTAAAT-3'		
WSSV395	WSSV395F	5'-GGACTCCATGGGCTCGTCTAACGGA GATGAG-3'	852 bp	RT-PCR
	WSSV395R	5'-GGTCTAGGATCCCTAATGATGATGATG ATGATGAAAAACAAACAGATTGAAA-3'		
WSSV458	WSSV458F	5'-GGACCCCATGGGCTTCCAGAAATGGT TTGAATC-3'	552 bp	RT-PCR
	WSSV458R	5'-GGTCCAGGATCCTTAATGATGATGAT GATGATGTTTGTTTGATAATACAATTTT-3'		
WSSV471	WSSV471F	5'-GGACCCCATGGGCGAGGACCTAAAA TCCACTATC-3'	480 bp	Cloning and RT-PCR
	WSSV471R	5'-GGTCCAGGATCCTTAATGATGATGATG ATGATGTGCATTGTTTGTATACA-3'		
EF-1α	EF-F	5'-GGTGCTGGACAAGCTGAAGGC-3'	145 bp	Internal control for RT-PCR
	EF-R	5'-CGTTCCGGTGATCATGTTCTTGATG-3'		

* The restriction sites are underlined.

2.7 Expression of recombinant WSSV189 and WSSV471 proteins

2.7.1 Preparation of the pET-19b expression vector

Stock solution of pET-19b vector (Novagen) (Fig. 2.2) was transformed into *E. coli* strain XL-1-Blue. The positive clones were selected by plating the transformants onto LB plate containing 100 µg/ml ampicillin. The vectors were extracted and purified from an overnight culture of a single colony of transformant in LB broth containing ampicillin using High-speed plasmid mini kit (Geneaid) as described in section 2.2.4. The vector was determined for quality and concentration by spectrophotometric method according to section 2.2.1.

The vector was digested with restriction enzymes *Nco*I and *Bam*HI by incubating at 37 °C overnight. The linear vector was purified by agarose gel elution using Nucleospin® Extract II kit (Macherey-Nagel) as described in section 2.2.5, determined the concentration by spectrophotometric method according to section 2.2.1, and stored at -20 °C until used.

2.7.2 Amplification of full-length WSSV gene

In this study we selected WSSV189 and WSSV471 gene for further study. Therefore, to express the recombinant WSSV, two primer pairs, WSSV189F: 5'-GGACCCCATGGGCGAATGGATAAACCAACGGACA-3' and WSSV189R: 5'-GGTCCAGGATCCTTA**ATGATGATGATGATGATG**TTGGATAAAGTAGTTTAA AT-3' for WSSV 189; WSSV471F: 5'-GGACCCCATGGGCGAGGACCTAAAA TCCACTATC-3' and WSSV471R: 5'-GGTCCAGGATCCTTA**ATGATGATGATG ATGATG**TGCATTGTTTGTATACA-3' for WSSV471, were designed. The primers were designed for the amplification of gene fragment encoding WSSV ORF with extension of *Nco*I restriction site at 5'-end of forward primer (underlined) and hexa-histidine taq (bolded) and *Bam*HI restriction site (underlined) at 5'-end of reverse primer.

The plasmid containing WSSV189 and WSSV471 ORF were used as PCR template and amplified in a final reaction volume of 50 µl containing 10 µl of 5× Phusion HF buffer, 1 µl of 10 mM each dNTP mix, 2.5 µl of 10 µM primer each, 2 µl of 5 fold diluted DNA template, 31.5 µl of ultrapure water, and 0.5 µl of 2U/µl of Phusion DNA polymerase (Finnzymes). The PCR amplification was carried out by predenaturation at 98°C for 30 sec following with 30 cycles of denaturation at 98 °C for 10 sec, annealing 60 °C for 30 sec, and extension at 72 °C for 30 sec, before final extension at 72 °C for 10 min. The PCR products were analyzed using 1% agarose gel electrophoresis, excised and purified using Nucleospin® Extract II kit (Macherey-Nagel) as described in section 2.2.5. The PCR product of WSSV189 and WSSV471 of about 699 bp and 480 bp, respectively. The concentration of the purified PCR products were examined as described in section 2.2.1.

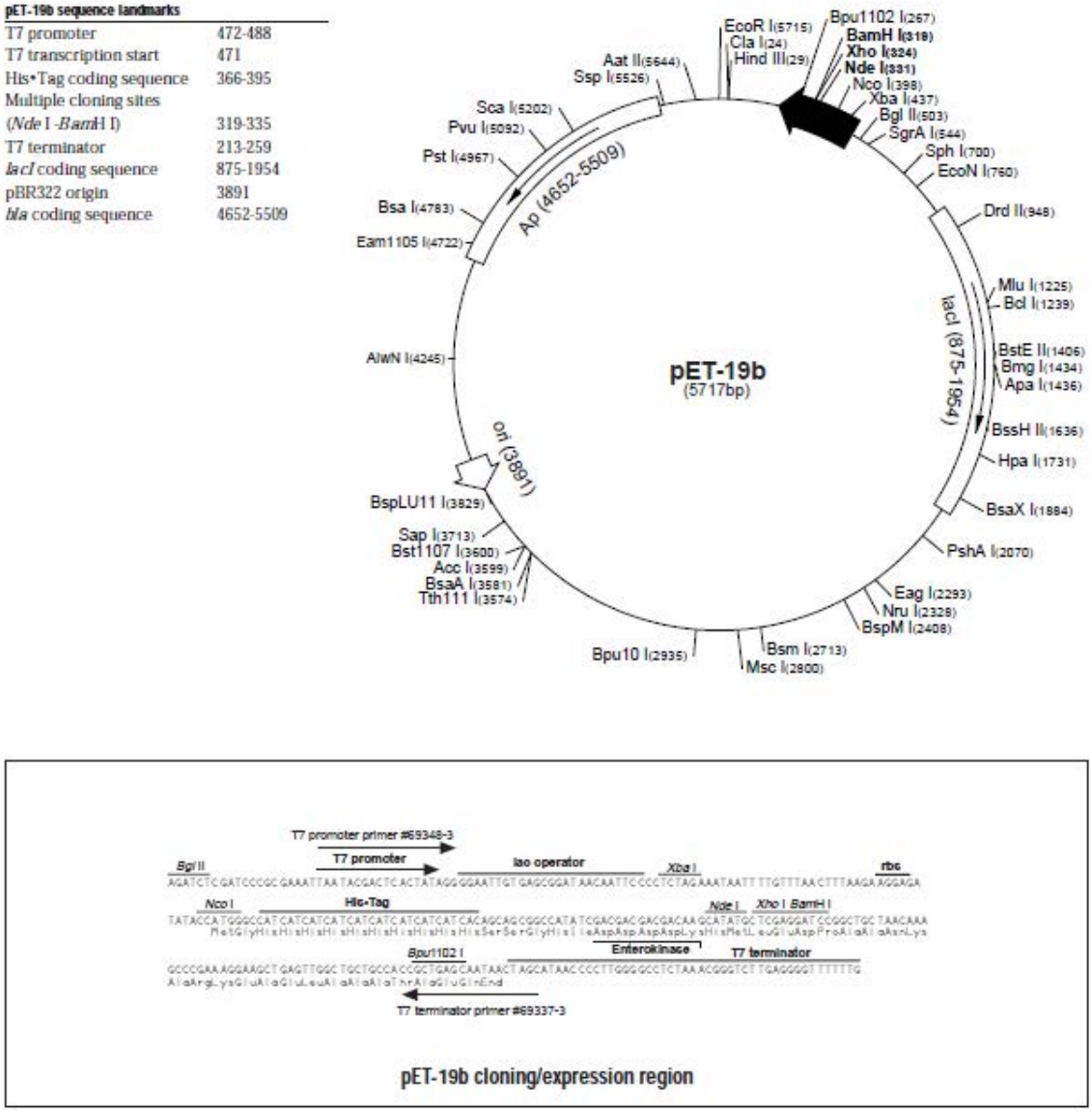


Figure 2.2 The pET-19b vector map (Novagen®, Germany)

2.7.3 Cloning the WSSV189 and WSSV471 gene into T&A cloning vector

The purified DNA fragments with *Nco*I and *Bam*HI restriction sites were cloned into T&A cloning vector. First, the poly A tail was added to the 3'-end of the insert fragment by preparing the reaction contained 5 µl of 10× *Taq* DNA polymerase buffer, 1 µl of 10 mM dATP, 30 µl of purified PCR product, 9.6 µl of ultrapure water, and 4.4 µl of 5 U/µl *Taq* DNA polymerase. The reaction was incubated at 70 °C for 30 min. The product was purified using NucleoSpin® Extract II kit. The purified DNA fragments with A-overhang were ligated into T&A cloning vector (RBC Bioscience). The insert DNA:vector molar ratio was about 5:1. DNA ligation mixture was composed of T&A cloning vector 1 µl (25 ng), Buffer A 0.5 µl, Buffer B 0.5 µl, T4 DNA ligase 0.5 µl (3 U/µl), suitable amount of the PCR product, and sterile deionized water to 5 µl. This reaction was mixed and incubated overnight at 4 °C. Then, the ligation mixtures were transformed into *E. coli* strain XL-1-blue by electro-transformation as described in section 2.2.3.3. One hundred microliters of transformants were plated onto a LB/ampicillin/IPTG/X-Gal plate.

The blue colony will be observed if the vector is recircularized, while the colony will be white if the gene of β-galactosidase is disrupted by gene insertion. The white positive colonies were selected and detected for gene insertion by PCR using M13 forward and reverse primers. The product was analyzed by 1% agarose gel electrophoresis.

The individual colonies were picked and grown in 5 ml LB medium containing 100 µg/ml ampicillin at 37 °C overnight with shaking for plasmid preparation as described in section 2.2.4. The recombinant plasmids were screened by digestion with *Nco*I and *Bam*HI and analyzed by 1% agarose gel electrophoresis. The expected size of WSSV189 and WSSV471 were about 699 bp and 480 bp, respectively. The clones containing desired insert were subjected to DNA sequencing (Macrogen).

2.7.4 Construction of recombinant pET-19b/WSSV189 and pET-19b/WSSV471

To construct the recombinant pET-19b/WSSV189 and pET-19b/WSSV471 plasmids for recombinant protein expression in *E. coli*, the T&A plasmid containing WSSV gene were double digested with *Nco*I and *Bam*HI. The digested products were run on 1% agarose gel, then the desired band were purified by agarose gel extraction using Nucleospin® Extract II kit. The *Nco*I-*Bam*HI overhang WSSV gene fragments were ligated into pET-19b cutting with the same enzymes. The total of 20 µl of ligation mixture contained 26 ng of plasmid DNA insert, 2 µl of 10× ligation buffer, 30 ng of linear pET-19b vector, and 2 µl of 400 U/µl T4 DNA ligase. The ligation mixture was incubated at 16 °C overnight. Afterwards, 2 µl of ligation mixture was transformed into *E. coli* strain XL-1-blue by electro-transformation as described in section 2.2.3.3. The transformants were selected on LB agar plate supplemented with 100 µg/ml ampicillin. All two recombinant plasmids were digested with *Nco*I-*Bam*HI to confirm the presence of gene insert. The digestion was analyzed by 1% agarose gel electrophoresis and subjected to sequence analysis to ensure the correctness of DNA insert.

2.7.5 Transformation of pET-19b/WSSV189 and pET-19b/WSSV471 into *Escherichia coli* expression system

The pET-19b/WSSV189 and pET-19b/WSSV471 plasmid were transformed into expression host, *E. coli* strain BL21(DE3), Rosetta(DE3)pLysS and BL21-CodonPlus(DE3)-RIL by heat shock method. The positive clones were then screened on LB agar plate containing 100 µg/ml ampicillin for *E. coli* strain BL21(DE3), and LB agar plate supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol for *E. coli* strain Rosetta(DE3)pLysS and BL21-CodonPlus(DE3)-RIL.

2.7.6 Recombinant expression of pET-19b/WSSV189 and pET-19b/WSSV471 gene in the *E. coli* expression system

Each single colony of *E. coli* strain BL21(DE3), Rosetta(DE3)pLysS, and BL21-CodonPlus(DE3)-RIL containing pET-19b/WSSV189 and pET-19b/WSSV471 were cultured with shaking 250 rpm at 37 °C overnight in 5 ml of LB broth containing 100 μg/ml of ampicillin for BL21(DE3), 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol for Rosetta(DE3)pLysS, and 100 μg/ml of ampicillin and 50 μg/ml of chloramphenicol for BL21-CodonPlus(DE3)-RIL. Each starter was diluted 1:100 fold in 50 ml of LB-broth. The culture were grown at 37 °C with shaking at 250 rpm until the OD_{600} reached 0.3-0.4. The recombinant protein was induced by the addition of 1 M isopropyl-β-galactopyranoside (IPTG) to a final concentration of 1 mM.

The culture was incubated at 37 °C further with shaking for 0-6 h. One ml of cell suspension was aliquot at each time point after induction and the pellet harvested by centrifugation at 8,000 × g for 10 min at 4 °C, and the expression proteins were lysed in 100 μl of 1× SDS loading dye and analyzed by 15% SDS-PAGE and western blotting using monoclonal anti-His antibody (GE healthcare) as a primary antibody and mouse anti-rabbit IgG as a secondary antibody at the dilution of 1:3,000 and 1:10,000, respectively. The optimum induction time for over-expression of rWSSV189 and rWSSV471 were then determined and compared between the host used.

The expression host that the highest expression was observed, was selected. It was further analyzed whether the recombinant protein produced was either soluble or inclusion body form. After culturing and induction for recombinant protein expression, the cells were collected at an appropriate time point by centrifugation at 8,000 × g for 10 min at 4 °C and resuspended with 300 μl of 1× PBS. They were freeze-thawed for 3 times and the cells were broken using sonicator (Bandelin Sonoplus, Germany). After centrifugation at 16,000 × g for 20 min, the pellet and supernatant were collected and analyzed for the presence of recombinant protein by 15% SDS-PAGE and Western blotting as above.

2.8 Purification of recombinant proteins

After sonication, the inclusion bodies of rWSSV189 and rWSSV471 proteins were collected. They were tested for solubilizing conditions with either 20 mM Tris-HCl supplement with 6M urea, pH 7.4 for denaturing condition or 20 mM Tris-HCl, pH 7.4 for non-denaturing condition. The recombinant proteins were subjected to SDS-PAGE analysis. Both of them were completely soluble in denaturing condition.

For rWSSV189 protein, it was purified under non-denaturing condition using nickel affinity chromatography (GE Healthcare). The solubilized crude proteins were dialyzed in 20 mM sodium carbonate buffer, pH 10.0, purified using Ni Sepharose 6 Fast Flow bead. The Ni Sepharose 6 Fast Flow bead was packed into the PD-10 column and washed with 10 column volumns of distilled water and equilibrated with 5-10 column volumes of binding buffer (20 mM sodium carbonate buffer, pH 10.0 containing 0.5 M NaCl, and 20 mM imidazole). The soluble protein fraction was applied to the column and incubated at room temperature for 2 h. Then, the column was washed with 5-10 column volumes of binding buffer to remove unbound proteins. The bound protein was eluted with 5 column volumes of 20 mM sodium carbonate supplemented with 0.5 M NaCl, pH 10.0 buffer containing 50 mM imidazole, 100 mM imidazole, 150 mM imidazole, and 500 mM imidazole, respectively. The elution fractions were run on 15% SDS-PAGE to check the purity of the protein. The fractions containing expected recombinant protein were pooled and the imidazole was removed by dialysis for overnight at 4 °C against 0.1× PBS, pH 7.4.

For rWSSV471 protein, it was purified under denaturing condition using nickel affinity chromatography (GE Healthcare). The Ni Sepharose 6 Fast Flow bead was packed into the PD-10 column and washed with 10 column volumns of distilled water and equilibrated with 5-10 column volumes of binding buffer (20 mM Tris-HCl supplement with 6M urea, pH 7.4 containing 0.5 M NaCl, and 20 mM imidazole). The soluble protein fraction was applied to the column and incubated at room temperature for 2 h. Then, the column was washed with 5-10 column volumes of binding buffer to

remove unbound proteins. The elution was performed using 5 column volumes of 20 mM Tris-HCl supplement with 6M urea, 0.5 M NaCl, pH 7.4 buffer containing 50 mM imidazole, 100 mM imidazole, 150 mM imidazole, and 500 mM imidazole, respectively. The elution fractions were run on 15% SDS-PAGE. The fractions containing expected recombinant protein were pooled and refolded protein by dialysis for overnight at 4 °C against 0.1× PBS, pH 7.4.

2.9 Production of the recombinant ALF*Pm*3 protein (rALF*Pm*3)

2.9.1 Recombinant expression of ALF*Pm*3 in *Pichia pastoris*

The stock culture of *Pichia pastoris* strain KM71 containing ALF*Pm*3 gene (Somboonwiwat et al., 2005) was streaked into a YPD agar plate (1% yeast extract, 2% peptone, 2% dextrose and 1.5% agar) and incubated at 30 °C for 3 days. A single colony was grown in 20 ml of YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) with shaking at 250 rpm, 30 °C for overnight. The starter was inoculated 1:100 in 300 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol). The culture was grown 30 °C with shaking at 300 rpm until the OD_{600} reached 4-6. The cells were harvested by centrifugation at 8,000 × g for 10 min at room temperature. After that, the cell pellet was resuspended in 60 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol) to induce the recombinant protein expression. The pure methanol was added to the culture to a final concentration of 0.5% every 24 hours in order to maintain the induction for 3 consecutive days. The supernatant was collected by centrifugation at 8,000 × g for 10 min at room temperature. The supernatant was kept and stored at -80 °C until use. The rALF*Pm*3 was analyzed by 15% Coomassie-stained SDS-PAGE.

2.9.2 Antimicrobial activity assay

The antimicrobial activity of rALF*Pm*3 was tested against *E. coli* 363, a gram-negative bacterium using liquid broth assay. Minimum inhibitory concentration (MIC) values were then determined (Somboonwiwat et al., 2005). The overnight culture were diluted 1:100 with LB broth and incubated until an OD_{600} was about 0.1 and then diluted with poor broth (1% tryptone type-1, 0.5% NaCl, pH 7.5) to an OD_{600} of 0.001. One-hundred-microliter aliquots of bacteria were mixed with 20 µl of rALF*Pm*3 at various concentration in well of a 96-well microtiter plate. Aliquots of distilled water with poor broth were used as control. The reactions were cultured for overnight under vigorous 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 that cause 100% of inhibition bacterial growth.

2.9.3 Purification of the rALF*Pm*3

The crude supernatant containing the rALF*Pm*3 was diluted 1:1 with the start buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.0) and purified by a strong cation exchange chromatography, HiTrap SP Sepharose™ Fast Flow column (GE Healthcare) using ÄKTA Prime Plus FPLC Purification System (GE Healthcare). The column was equilibrated with start buffer. Afterwards, the diluted protein was loaded into the column, and washed with start buffer to remove unbound proteins until the A_{280} was decrease to zero. The elution was performed using the elution buffer (20 mM Tris-HCl, 1 M NaCl, pH 7.0). The flow rate was controlled at 1 ml/minute throughout the purified process. The fractions were analyzed by 15% SDS-PAGE. The antimicrobial activity of the purified protein was performed against *E. coli* 363 as described in section 2.9.2. The fractions were then pooled and dialyzed overnight against distilled water at 4 °C to

eliminate salt. The purified rALF*Pm*3 were evaporated to concentrate and then measured the protein concentration at A_{280} using an equation (Somboonwiwat et al., 2005):

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

The purified protein was kept at -80 °C until use.

2.10 *In vitro* pull-down assay

The pull-down experiments was performed according to instructions of the ProFound™ Pull-down PolyHis Protein (Thermo scientific), the interaction between 6xHis-rWSSV189 or 471 and ALF*Pm*3 was examined by incubating 6xHis-rWSSV fusion protein with a cobalt chelate resin (50 µl of 50% bed slurry) for 1.50 h at 4 °C with gentle rocking. Then, the beads were washed 12 times with 600 µl of Wash Solution (1× TBS:ProFound™ Lysis Buffer(1:1), and 40 mM imidazole, pH 7.2). The purified rALF*Pm*3 protein was added, and incubation was continued for another 3.50 h at 4 °C with gentle rocking. After incubation, the beads were washed 12 times with 600 µl of Wash Solution. The bound protein was eluted by the addition of buffer containing 290 mM imidazole (1× TBS:ProFound™ Lysis Buffer(1:1), and 290 mM imidazole, pH 7.2). The proteins were resolved on 15% SDS-PAGE and detected with silver staining.

For control, the rWSSV189 or rWSSV471 was used as a bait control and the rALF*Pm*3 was used as prey control. They were incubated with a cobalt chelate resin. After incubation, the bait and prey protein was then washed 12 times with Wash Solution and eluted at the same concentration as above.

CHAPTER III

RESULTS

3.1 Identification of ALF*Pm*3 interacting protein by yeast two-hybrid screening (Y2H)

3.1.1 Preparation of bait vector

The nucleotide sequences of ALF*Pm*3 gene from a pBluescript SK plasmid was amplified with a specific primer pair containing the restriction site *Eco*RI and *Bam*HI as described in material and methods section 2.3.1.1. The expected PCR product of 318 bp was obtained (Fig. 3.1). Then, the purified PCR product was digested with *Eco*RI and *Bam*HI and cloned into pGBKT7 vector, a bait vector, cut with the same enzymes. The ligation mixtures were transformed into *E. coli* strain XL-1 blue. The recombinant clones were screened and selected for plasmid preparation. Then, the recombinant plasmids were verified by restriction enzyme digestion with *Eco*RI and *Bam*HI as shown in Fig. 3.2 and subjected to sequencing to confirm the correctness of the sequences.

3.1.2 Preparation of haploid yeast strains expressing the ALF*Pm*3 bait protein

The pGBKT7-ALF*Pm*3 vector was transformed into *S. cereviseae* strain Y2H Gold. Yeast transformants were selected by selective medium which was SD medium lacking Tryptophan (SD/-Trp). The obtained pGBKT7-ALF*Pm*3 vector was tested for autoactivation of reporter genes and toxicity to the yeast cells before used in the subsequent yeast two-hybrid assay. The result indicated that the bait protein alone did not autonomously activate the reporter genes in Y2H Gold and was not toxic to the yeast cell.

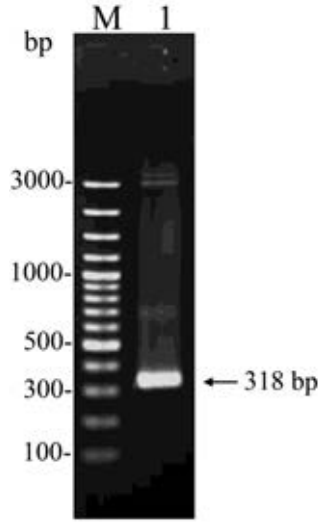


Figure 3.1 ALF*Pm*3 gene amplification. ALF*Pm*3 gene was amplified by PCR from a Bluescript SK plasmid using a ALF*Pm*3F and ALF*Pm*3R and analyzed by 1% agarose gel electrophoresis. Lane M: 100 bp DNA ladder marker; Lane 1: the amplified ALF*Pm*3 gene fragment from pBluescript SK plasmid containing ALF*Pm*3 gene.

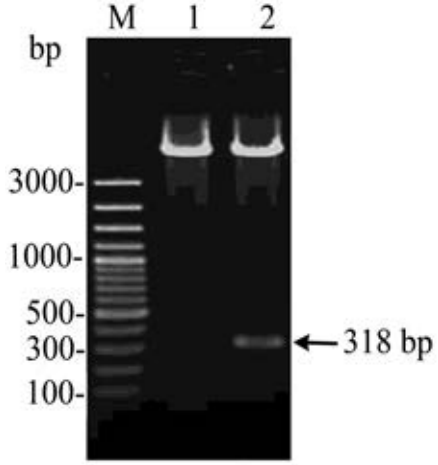


Figure 3.2 Confirmation of the recombinant plasmid, pGBKT7-ALF*Pm*3 by restriction enzyme digestion. pGBKT7-ALF*Pm*3 were digested by *Eco*RI/*Bam*HI and analyzed by 1% agarose gel electrophoresis.Lane M: 100 bp DNA ladder marker; Lanes 1 and 2: the recombinant plasmid digested with *Eco*RI and *Bam*HI. A fragment of ALF*Pm*3 gene (318 bp) is indicated by an arrow.

3.1.3 Yeast two-hybrid screening

3.1.3.1 Y2H screening on *Vibrio harveyi*- and WSSV-infected hemocyte libraries

To study the involvement of ALF*Pm*3 in shrimp immune response other than its direct pathogen killing function, yeast two-hybrid technique was used to screen for shrimp proteins that can bind to ALF*Pm*3 from *V. harveyi* (VHH)- and WSSV (WH)-infected shrimp hemocyte libraries The *S. cereviseae* strain Y2H Gold containing pGBKT7-ALF*Pm*3 vector was mated with *S. cereviseae* strain Y187 containing pGADT7-library. The result showed that, the number of positive clones obtained after screening on the DDO/X/A plates from VHH and WH libraries were 43 strong blue colonies and 13 pale blue colonies, respectively. After that, they were subsequently screened at the higher stringency on the QDO/X/A plate. Thirty-seven clones showing the positive interaction strong blue were obtained for VHH libraries (Fig. 3.3A). For the WH library, all clones were false positive (Fig. 3.3B).

Then, positive clones from VHH library were segregated by streaking yeast on DDO/X plate to obtain the pure colony. The prey plasmids were rescued and clustered to reduce the repetitive sequencing by checking restriction enzyme, *Hind*III, digestion pattern. The prey plasmids with similar banding patterns after the restriction enzyme digestion were grouped. The results showed that they could be clustered into 13 groups with different band patterns (Fig. 3.4).

3.1.3.2 Y2H screening on *Vibrio harveyi*- and WSSV-libraries

The ALF*Pm*3 was used as a bait for screening its interacting protein in the *Vibrio harveyi* (VH) and WSSV libraries in order to study its mechanism of action against each pathogen. For VH library, after mating and selection on the DDO/X/A plate the number of positive interacting clones were 14 pale blue colonies. After that, they

were subsequently screened on the QDO/X/A plate. Five positive clones were obtained from VH library (Fig. 3.3C). Afterwards, the positive clones from VH library were segregated and rescued the prey plasmids. After segregation, the results showed that 5 clones were false positive clones.

In collaboration with Dr. Sangchan from BIOTEC, the bait pGBKT7-ALF*Pm*3 vector was used to screen the library of WSSV ORF (Sangsuriya, 2010). The results showed five true positive clones that were confirmed by yeast co-transformation. The five colonies could grow and turn blue on the QDO/X plate (Fig. 3.6).

3.1.4 Confirmation of ALF*Pm*3 and positive clones by co-transformation

By Y2H screening, 13 different groups of prey plasmid from VHH library had their gene products interacting with ALF*Pm*3. However, the results should be confirmed by co-transformation each prey plasmid with either pGBKT7-ALF*Pm*3 or pGBKT7 empty vector (control). In this assay, the transformants containing a prey protein alone could not be grown on the high stringency medium (QDO/X/A) because the expression of reporter genes could occur under the activation through interaction between bait and prey protein only. The white co-transformants indicated that the examined protein could not interact with ALF*Pm*3. Co-transformation results were shown in Fig. 3.5. Therefore, all the screened clones were false positive.

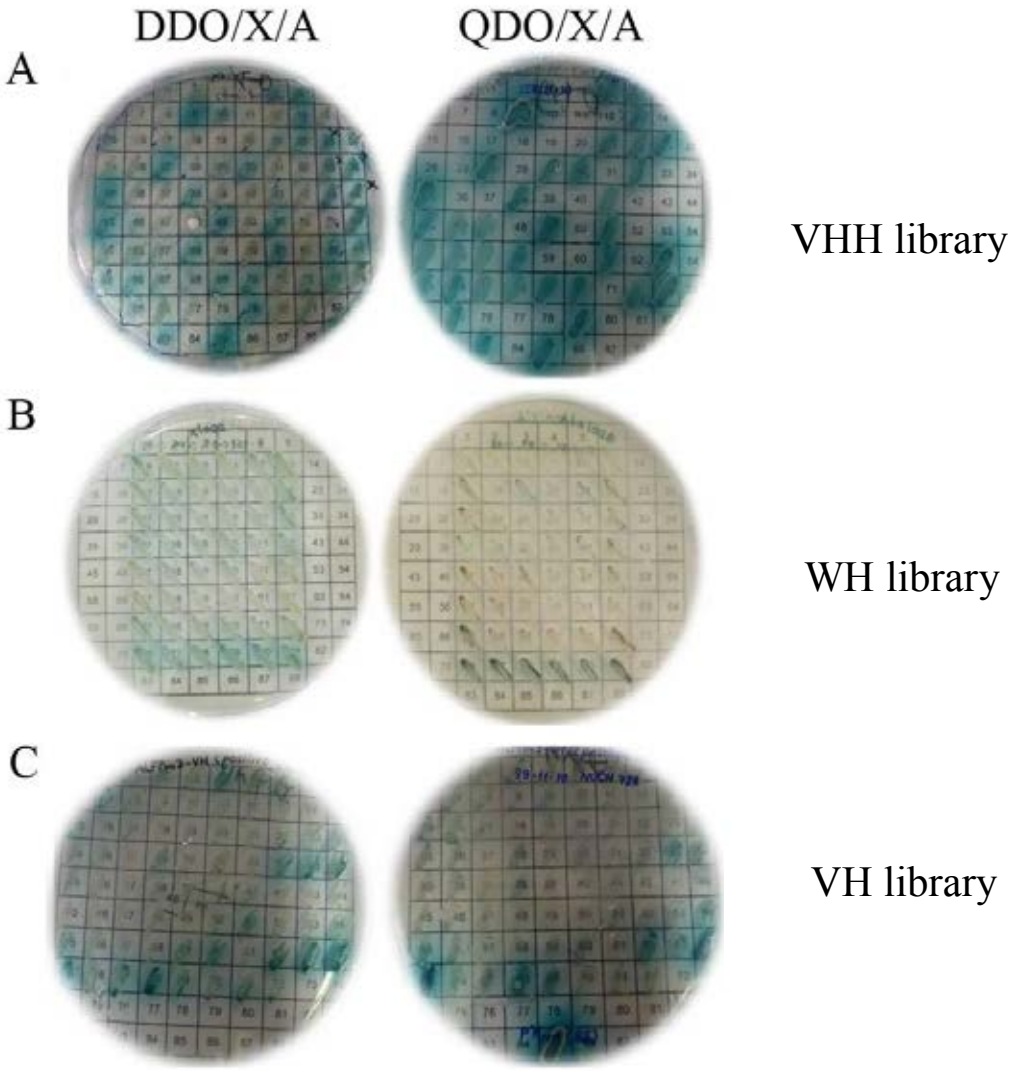


Figure 3.3 **Screening of ALF*Pm*3 interacting proteins by Y2H assay.** (A) Y2H screening on *V. harveyi*-infected shrimp hemocyte library (VHH library); (B) Y2H screening on WSSV-infected shrimp hemocyte library (WH library); and (C) Y2H screening on *V. harveyi* library (VH library). The mating yeast was selected on SD/-Leu/-Trp containing X-alpha-Gal and Aureobasidin A (DDO/X/A) and SD/-Leu/-Trp/-Ade/-His containing X-alpha-Gal and Aureobasidin A (QDO/X/A) plates. Positive interaction is indicated by normal growth and blue color of the mating yeast.

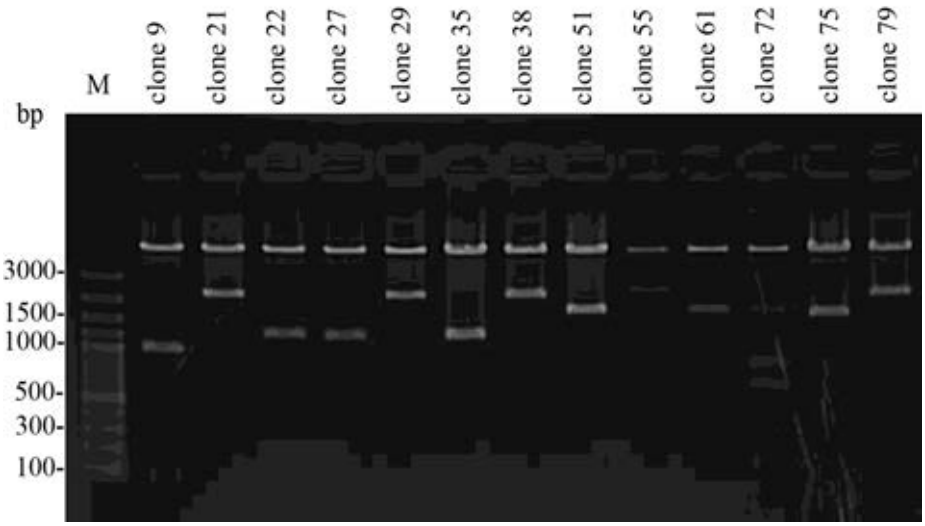


Figure 3.4 Clustering of positive clones obtained from VHH library by *Hind*III digestion. The positive clones were digested by *Hind*III and analyzed by 1% agarose gel electrophoresis. Lane M: 100 bp DNA ladder marker; Lanes 1-13: The *Hind*III-digested rescued plasmids obtained from the positive yeast clones.

3.1.5 Identification of gene encoding WSSV proteins interacting with ALF*Pm*3

The five positive clones obtained from WSSV library screening were co-transformed with ALF*Pm*3 and screened on the QDO/X plate. The positive interacting clones could grow and have blue color (Fig. 3.6). Sequence analysis and comparison of deduced amino acid sequences of these clones with the GenBank database using a BLASTX search revealed that five clones were matched 100% to mature WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471 (Fig. 3.7). To obtain the full-length sequence of WSSV186, WSSV189, WSSV395, WSSV458, and WSSV471 gene, gene specific primers were designed and then used for PCR amplification as described in material and methods section 2.6.5. The expected PCR product of 1,455, 699, 852, 552 and 480 bp, respectively and long coding for a polypeptide chain of 484, 222, 283, 183 and 158 amino acids, respectively were obtained.

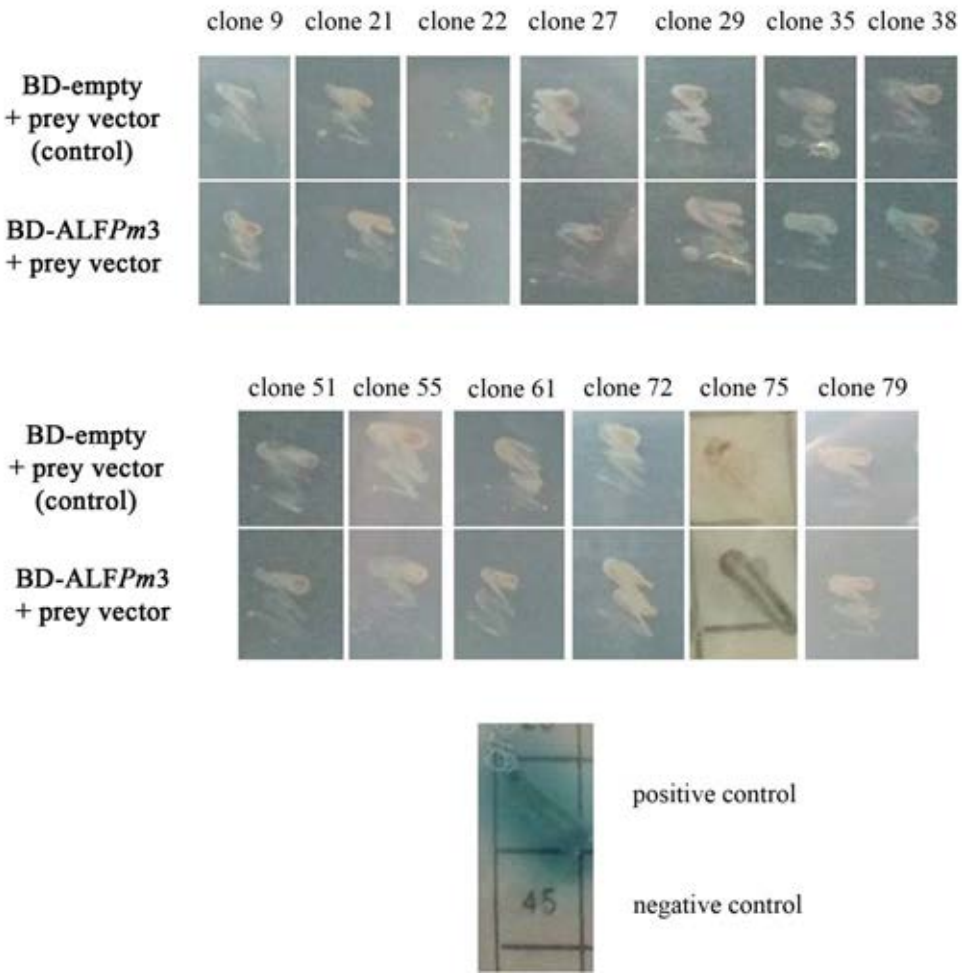


Figure 3.5 Confirmation of the interaction between ALF*Pm*3 and positive clones from VHH library by co-transformation. *S. cerevisiae* Y2H Gold cells were co-transformed with (1) pGBKT7 vector (BD-empty) and positive clone in pGADT7 (prey vector); (2) ALF*Pm*3 in the pGBKT7 vector (BD-ALF*Pm*3) and positive clone in pGADT7 (prey vector). Transformed cells were selected on SD/-Leu/-Trp/-Ade/-His containing X-alpha-Gal and Aureobasidin A (QDO/X/A) plates. The positive control is a yeast cell containing pGBKT7-53 and pGADT7-T. The negative control is a yeast cell contains pGBKT7-Lam and pGADT7-T.

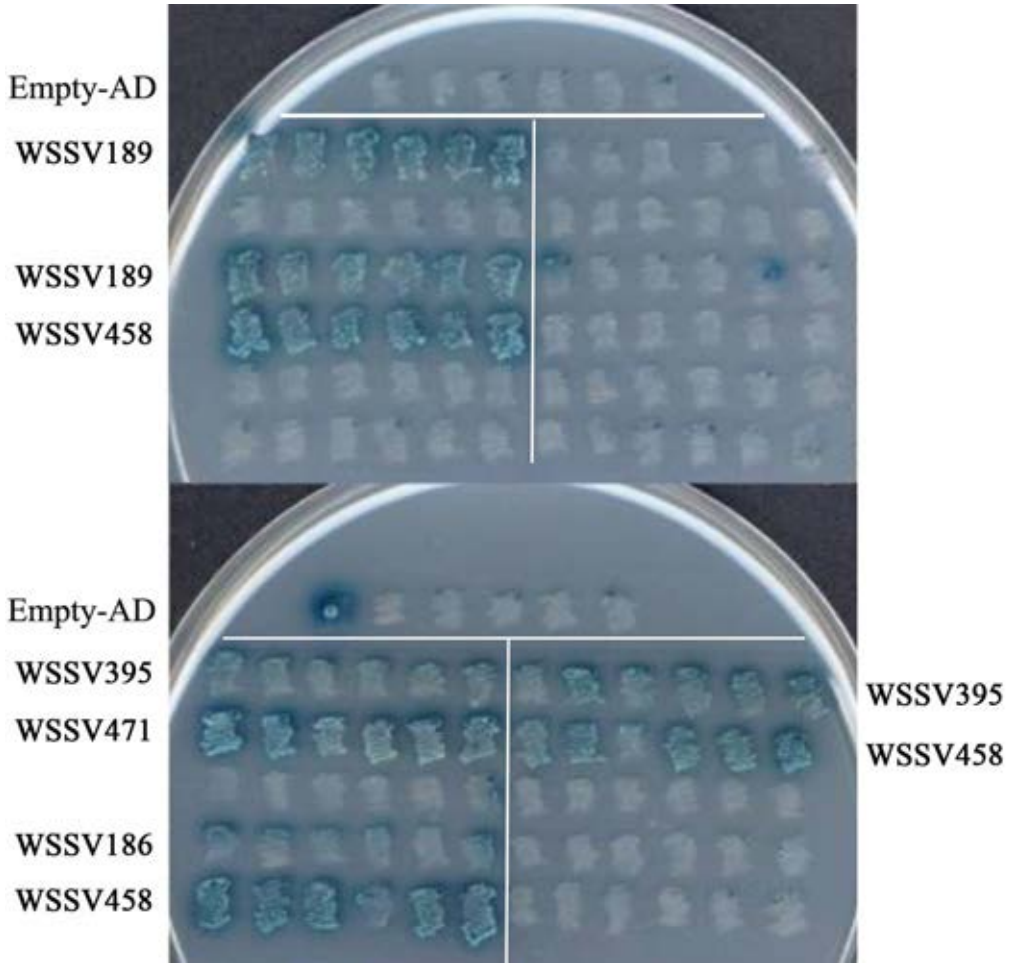


Figure 3.6 Confirmation of the interaction between ALF*Pm*3 and WSSV protein by co-transformation. *S. cerevisiae* AH109 cells were co-transformed with (1) ALF*Pm*3 in the pGBKT7 vector and empty in pGADT7 (Empty-AD); (2) ALF*Pm*3 in the pGBKT7 vector and amino acids residues of WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471 in pGADT7 vector. Transformed cells were selected on SD/-Leu/-Trp/-Ade/-His containing X-alpha-Gal (QDO/X) plates.

WSSV186 (484 amino acid residues)

```
        10         20         30         40         50         60
MSSYLDLLNV AGGGGGGGAN DADNETARRI FQTYGSFYNN NQTVAEEDYK RLVGVTETDD
        70         80         90        100        110        120
LLTPENVVGN LDTGERVTPF LSLDVMLSTC DLKHPSSTDG NVLKNIHFSE SIPANDIISF
       130        140        150        160        170        180
PSSDTEELNK DLLDSVRNQI KFGFDPITET LKNCITTQTL LHSFLKSSLL TLQEKFNEWG
       190        200        210        220        230        240
SIQLEKGGQE MALCASLKIM GQISALIETA KEASMDNKKK NNNACANCRD SKCSASLVTL
       250        260        270        280        290        300
FNKTIDEKYV KQNSSSASAL LANTFTAGAN KPPKEFITKD NAHGNSDTNY TAMSDNLICP
       310        320        330        340        350        360
GKYYSSDITY EVTKQAKERI KNNNKKMRLA TGVEMVMKEL EAENNKEGGR VEVEVEGVEQ
       370        380        390        400        410        420
QQPSTSGEEM QMEIMLPTPP PPDLESLVTE GVDDYPVFSP LPSLLSPMPA SPLPSNGNSA
       430        440        450        460        470        480
LEDGGPFAPS ADIVVDKTSE IMGRTPGSEW VHQRDRNSKM EIRNYGARGS GINTGRYRRN
       490
NTVL
```

WSSV189 (222 amino acid residues)

```
        10         20         30         40         50         60
MEWINQRTSR EDLFNTYTGN AVIRSAAKQA LAIEKHAAER RGEKAWTTSA AAAASSNFNN
        70         80         90        100        110        120
VQQDYTDDDI TQVSIANSVL NNPFLKRYAK LIDNLAISSL PPDIEDDVII HTRDASNSTV
       130        140        150        160        170        180
RVDGANIYFA IIDGDLCVYP KQYISDKVLC GSLNREKALF YNSSKNKWTY GCNLNFDIVD
       190        200        210        220        230
AAIMKHPDYK EETTSTKHIR KILGIGASEK LNITHYLNYF IQ
```

WSSV395 (283 amino acid residues)

```
        10         20         30         40         50         60
MSSNGDEPAV TEAEIASVEA QLGAAHHDNS WITRKSDQLK YRLGAIAYSV AKNASIKYIE
        70         80         90        100        110        120
DQVRQEINSH LTNVMTFEHL YEDAFNPVIC EAIFEKGIPV VMEKVYDVNR RIMEPREDFI
       130        140        150        160        170        180
TEILKEERWR RYIPGFYHTS FSFKYNTIAF TDSSTSFSVP INDKHMLSIT PPGAAQGDLI
       190        200        210        220        230        240
DLSLSFKIDS SAKTLTLEFN RKSTFAGIVN RPKSVVILSN LRNSDSSDNI GDYLKRNDPI
       250        260        270        280        290
YISHDTNGII NPSEDSASLI TIHMPEIENA SDDLYIDFNL FVF
```

WSSV458 (183 amino acid residues)

```
        10         20         30         40         50         60
MFQKWFESFL DSSRPRYLDT TCVCSVYSYF SPCRKHIKFS TSHSHEGIKI HPPSILNHNT
        70         80         90        100        110        120
SSPTSGKMCN HHHKRLYLST DDHTRWYDKN TSCIYLEDIG GVQFMVYEFH LTPKNNQLFS
       130        140        150        160        170        180
FPVHLQIHNR NTEKTSLLVF ENEEDMRVRN IHPKSKILIP VSKDTVLVEN GFRYKVKIVL
       190
SNK
```

WSSV471 (159 amino acid residues)

```
        10         20         30         40         50         60
MEDLKSTIER VYEERVENLE QWTNTVEEEE RTVSAIDSVL EEQKRALDAW EAAIKEREND
        70         80         90        100        110        120
LAVKEGISAL VFNAADAKTR KELINTWIAE RETSEKRRKE ATSTNNQLKN QMSSLVNTTK
       130        140        150        160
TLKEKYNKYY RRSAILNMQY INNKRDYEAS QFWVYTNNA
```

Figure 3.7 **Amino acid sequences of WSSV186 (NP_477653), WSSV189 (NP_477656), WSSV395 (NP_477861), WSSV458 (NP_477921) and WSSV471 (NP_477934) from GenBank database.**

3.2 Expression analysis of the WSSV transcript

To understand the involvement of WSSV proteins in WSSV infected shrimp, we determined the expression of these WSSV genes in shrimp at various time points after WSSV infection by RT-PCR analysis. After WSSV challenge gill was collected for total RNA isolation at 0, 3, 6, 12, 24 and 48 h. The first stranded cDNA was synthesized and the expression of WSSV genes were analyzed by RT-PCR.

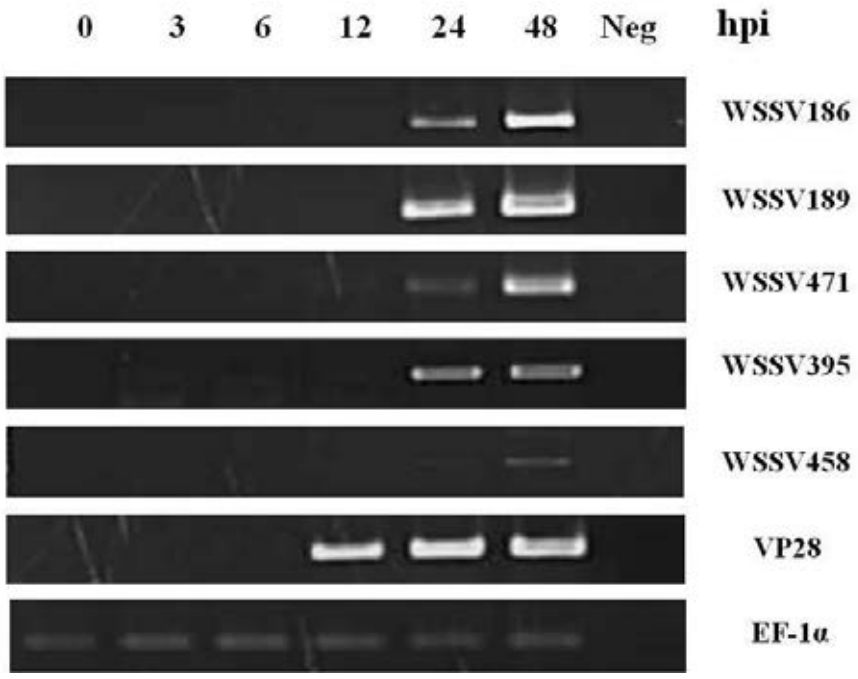


Figure 3.8 Temporal transcription analysis of the WSSV genes from gill of WSSV-infected *P. monodon* by RT-PCR. Total RNA was extracted from gill of 3 individual WSSV-challenged shrimp at 0, 3, 6, 12, 24 and 48 hours post infection (hpi), respectively. cDNA derived from reverse transcription reaction was used as a template for WSSV186, WSSV189, WSSV471, WSSV395 and WSSV458 gene amplification by PCR. The PCR product was analyzed on 1.5% agarose gel electrophoresis with ethidium bromide staining. Lane Neg: negative control for PCR reaction.

From semi-quantitative RT-PCR analysis, the results showed that WSSV186, WSSV189, WSSV471, WSSV395 and WSSV458 genes were detected at 24 hpi (hours post infection). A major structure gene VP28 detected from 12 hpi was used as a positive control and shrimp elongation factor gene as a loading control. This result indicated that five WSSV genes were expressed late stage post-WSSV infection (Fig. 3.8).

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

In order to obtain the rALF*Pm*3 protein to further confirmation of the interaction with WSSV protein by *in vitro* pull-down assay, the rALF*Pm*3 was produced in yeast *Pichia pastoris* system and purified by a cation-exchange column.

The single yeast recombinant clone containing ALF*Pm*3 gene was grown in 3.2 L of BMGY media to increase biomass. After that, cells were collected and transferred to induce rALF*Pm*3 expression in BMMY media. 100% Methanol was added to a final concentration of 0.5% every 24 hours to maintain the induction. During the methanol induction, the rALF*Pm*3 was secreted into the culture medium. The expression culture was collected at day 3 and analyzed by 15% SDS-PAGE (Fig. 3.9). The expected size of rALF*Pm*3 was 11.3 kDa. This result revealed the successful expression of the rALF*Pm*3 protein by the yeast *P. pastoris*. The crude supernatant of rALF*Pm*3 was collected and determined for the antibacterial activity against *Escherichia coli* 363. The results showed that the rALF*Pm*3 had inhibitory activity of the crude supernatant against *E. coli* 363. These results indicated that the functional rALF*Pm*3 was produced.

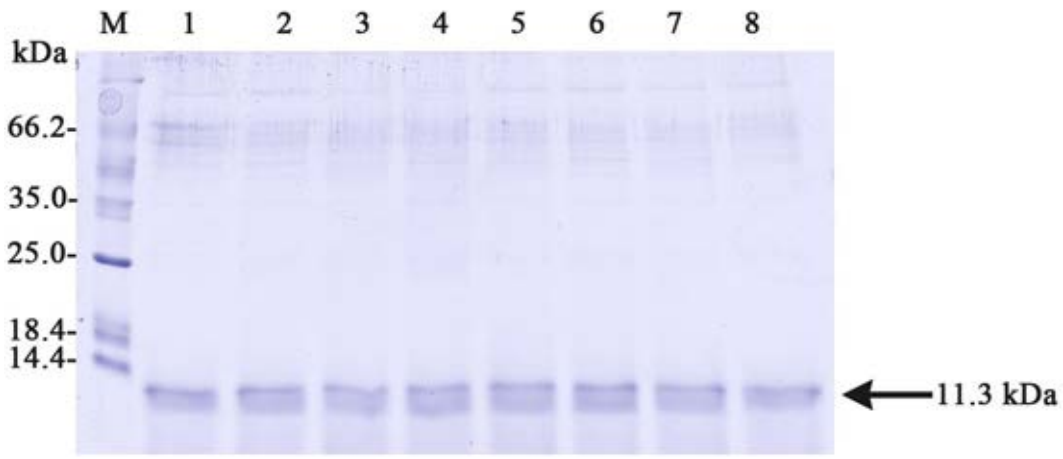


Figure 3.9 The coomassie stained 15% SDS-PAGE gel of the rALF*Pm*3 protein expressed by the yeast *P. pastoris*. Lane M: unstained protein marker (Fermentas); Lanes 1-8: crude supernatant at day 3 after methanol induction.

The crude supernatant was purified by strong cation exchange chromatography (SP Sepharose High Performance) as described in section 2.9.3. The elution fractions containing rALF*Pm*3 were analyzed by 15% SDS-PAGE (Fig. 3.10) and tested for antimicrobial activity against *E. coli* 363. The results showed that the purified rALF*Pm*3 had activity against *E. coli* 363. The purified rALF*Pm*3 fractions with antimicrobial activity was then pooled and dialyzed against deionised water at 4 °C to eliminate salt for overnight. Then, it was concentrated by rotary evaporation and determined for protein concentration by measuring the absorbance at 280 nm. The total purified ALF*Pm*3 protein obtained for this batch was approximately 1.7 mg. The purified protein was then used for the *in vitro* pull-down assay.

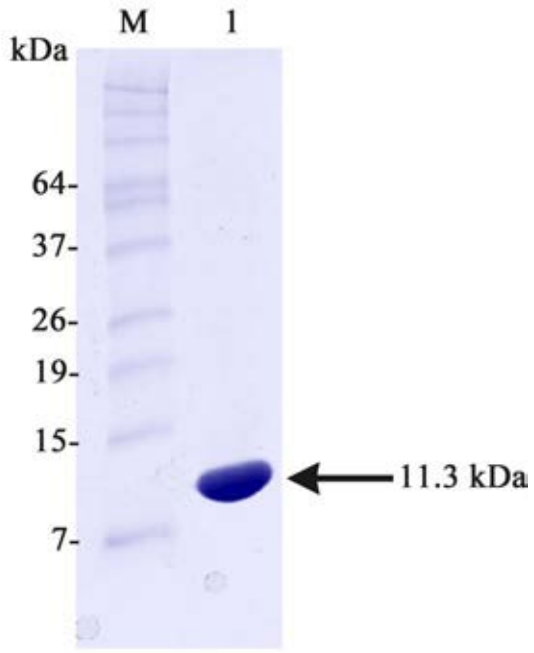


Figure 3.10 The coomassie stained 15% SDS-PAGE analysis of the purified rALF*Pm*3. Lane M: prestained protein marker (BenchMark™ Pre-stained protein ladder, Invitrogen); Lane 1: the purified rALF*Pm*3.

3.4 Expression and purification of the recombinant WSSV189 and WSSV471 proteins

In order to obtain the recombinant WSSV189 (rWSSV189) and WSSV471 (rWSSV471) proteins for *in vitro* pull-down experiments, two recombinants were produced in *E. coli* system and purified by affinity column.

3.4.1 Construction of recombinant plasmids; (pET-19b/WSSV189 and pET-19b/WSSV471)

According to sequence analysis, the WSSV189 and WSSV471 ORF encoded for the proteins containing 222 and 158 amino residues, respectively. Analysis of deduced amino sequence of WSSV189 and WSSV471 using SignalP program revealed that they did not contain signal peptide. Therefore, to produce the rWSSV189 and rWSSV471, the WSSV genes in pGADT7/WSSV189 or pGADT7/WSSV471 were amplified using forward primer and reverse primer containing His_6-tag coding sequence at their 5′-end. The PCR products of expected size; 701 and 512 bp, respectively, obtained were double digested with *Nco*I and *Bam*HI (Fig. 3.11). The purified *Nco*I/*Bam*HI digested fragments were ligated into pET-19b vector cut with the same enzymes. The ligation mixtures were transformed into XL-1blue. The recombinant clones were screened by colony PCR using specific primers. The recombinant plasmids were extracted and verified by restriction enzyme digestion with *Nco*I and *Bam*HI as shown in Fig. 3.12 and subjected to nucleotide sequencing to confirm the result.

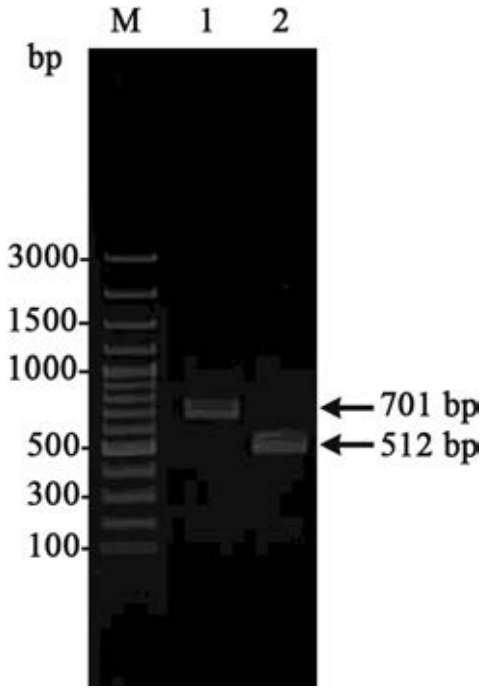


Figure 3.11 **Amplification of the WSSV189 and WSSV471 ORF.** The WSSV189 and WSSV471 ORF were amplified from the corresponding prey plasmid using WSSV189F and WSSV189R, and WSSV471F and WSSV471R primer pairs, respectively. The PCR products were run on 1% agarose gel electrophoresis. Lane M: 100 bp DNA ladder marker; Lane 1: PCR product of the WSSV189 gene; Lane 2: PCR product of the WSSV471 gene. The expected size of WSSV189 (701 bp) and WSSV471 (512 bp) is indicated by arrow.

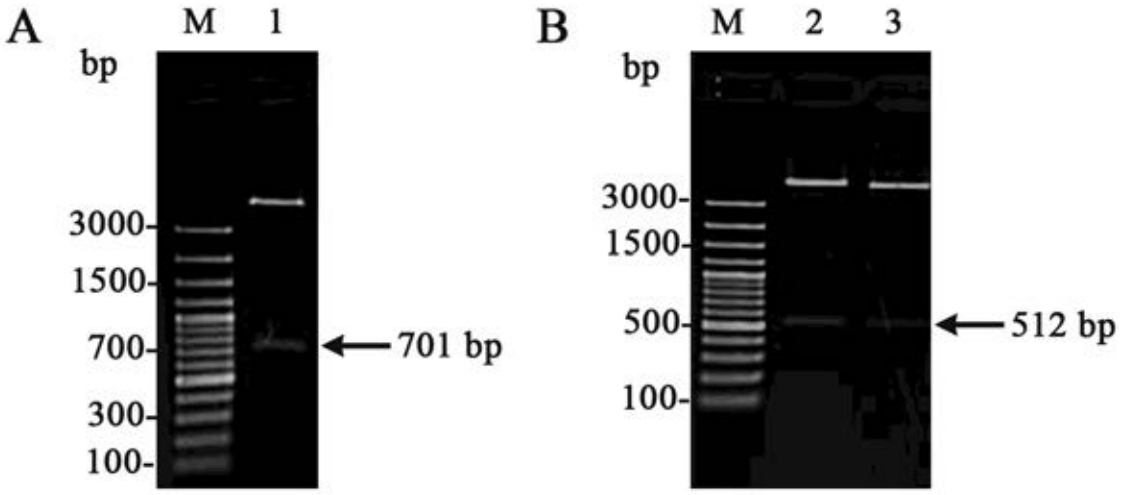


Figure 3.12 Verification of the recombinant plasmid by restriction enzyme digestion. Each recombinant plasmid was digested by *Nco*I/*Bam*HI and analyzed by 1% agarose gel electrophoresis. Lane M: 100 bp DNA ladder marker; the *Nco*I/*Bam*HI digested product of (A) recombinant pET-19b/WSSV189 plasmid and (B) recombinant pET-19b/WSSV471 plasmid. The expected insert products are indicated by the arrow.

3.4.2 Recombinant protein expression of pET-19b/WSSV189 and pET-19b/WSSV471 in *E. coli* system

The pET-19b/WSSV189 and pET-19b/WSSV471 plasmids with correct sequence of inserted gene were transformed into expression hosts, *E. coli* strains BL21(DE3), Rosetta(DE3)pLysS and BL21-CodonPlus(DE3)-RIL, respectively.

Each recombinant clone was grown and the recombinant protein was produced by induction with 1 mM IPTG. The whole cells collected at various times (0-6 h) after IPTG induction were tested for presence of the overproduced recombinant protein (rWSSV189 and rWSSV471) by 15% SDS-PAGE and coomassie staining. The result showed that *E. coli* strain BL21-CodonPlus(DE3)-RIL containing pET-19b/WSSV189 and pET-19b/WSSV471 expression plasmid could overproduce the expected rWSSV189 and rWSSV471 with the molecular weight of about 26 kDa (Fig. 3.13) and 20 kDa (Fig. 3.14), respectively. To determine whether the rWSSV189 and rWSSV471 proteins were expressed as soluble or inclusion bodies forms, an aliquot of whole cells were resuspended in 1×PBS, pH 7.4, repeat frozen-thawed for 3 times and then sonication to break the bacterial cells. Then, the pellet and soluble fractions were analyzed by 15% SDS-PAGE and Western blot using anti-His antibody. The results showed that rWSSV189 and rWSSV471 were expressed in the inclusion bodies form (Fig. 3.15).

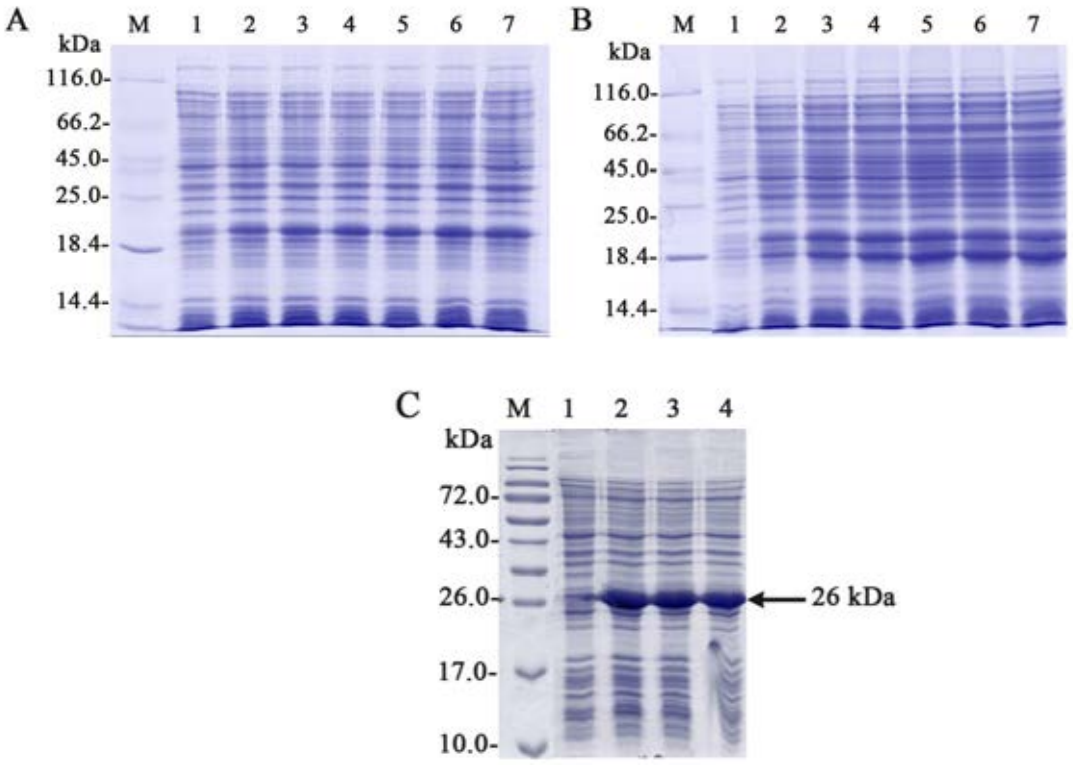


Figure 3.13 **Expression analysis of recombinant WSSV189 protein produced in various host strains.** The transformant in *E. coli* strain (A), BL21(DE3); (B), Rosetta(DE3)pLysS; (C), BL21-CodonPlus(DE3)-RIL were tested for WSSV189 overproduction. The culture of each transformant was induced for the WSSV189 expression by 1 mM IPTG. The cells were collected at 0 to 6 h after induction and analyzed for protein expression by 15% SDS-PAGE. In (A) and (B), Lane M: unstained protein marker (PageRuler^{TM} Unstained protein ladder, Fermentas); Lanes 1-7: whole cell at 0-6 h after 1mM IPTG induction. In (C), Lane M: prestained protein marker (PageRuler^{TM} Prestained protein ladder, Fermentas); Lanes 1-4: whole cell at 0, 2, 4 and 6 h after 1mM IPTG induction, respectively. An arrow indicates the expected 26 kDa recombinant protein.

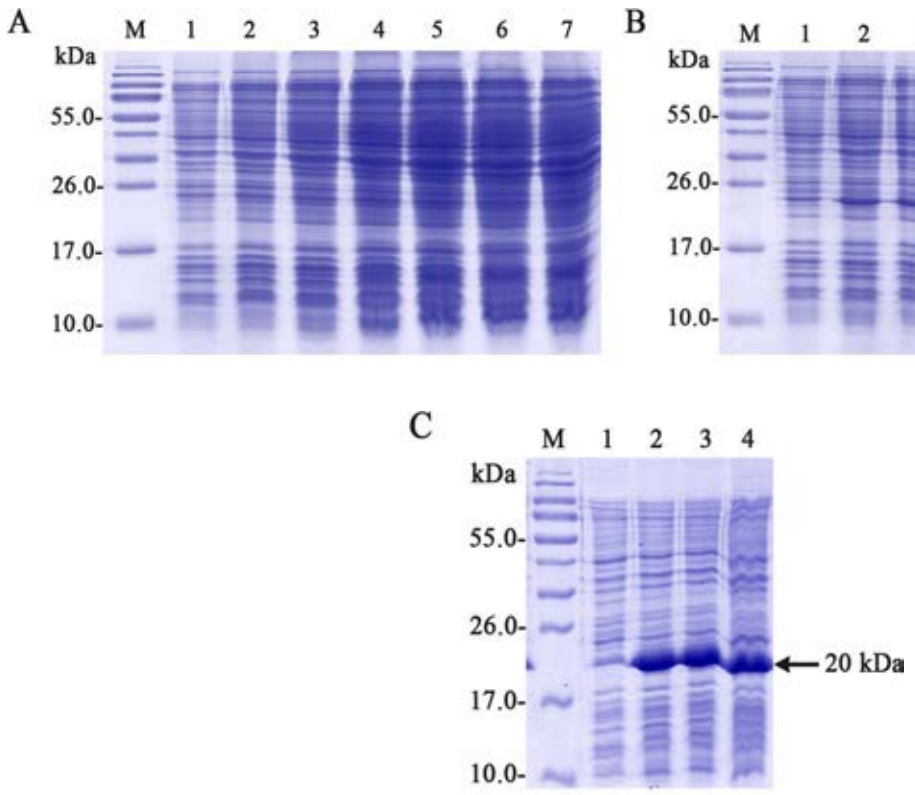


Figure 3.14 Expression analysis of recombinant WSSV471 protein produced in various host strains. The transformant in *E. coli* strain (A), BL21(DE3); (B), Rosetta(DE3)pLysS; (C), BL21-CodonPlus(DE3)-RIL were tested for WSSV471 overproduction. The culture of each transformant was induced for the WSSV471 expression by 1 mM IPTG. The cells were collected at 0 to 6 h after induction and analyzed for protein expression by 15% SDS-PAGE. In (A) and (B), Lane M: unstained protein marker (PageRuler^{TM} Unstained protein ladder, Fermentas); Lanes 1-7: whole cell at 0-6 h after 1mM IPTG induction. In (C), Lane M: prestained protein marker (PageRuler^{TM} Prestained protein ladder, Fermentas); Lanes 1-4: whole cell at 0, 2, 4 and 6 h after 1mM IPTG induction, respectively. An arrow indicates the expected 20 kDa recombinant protein.

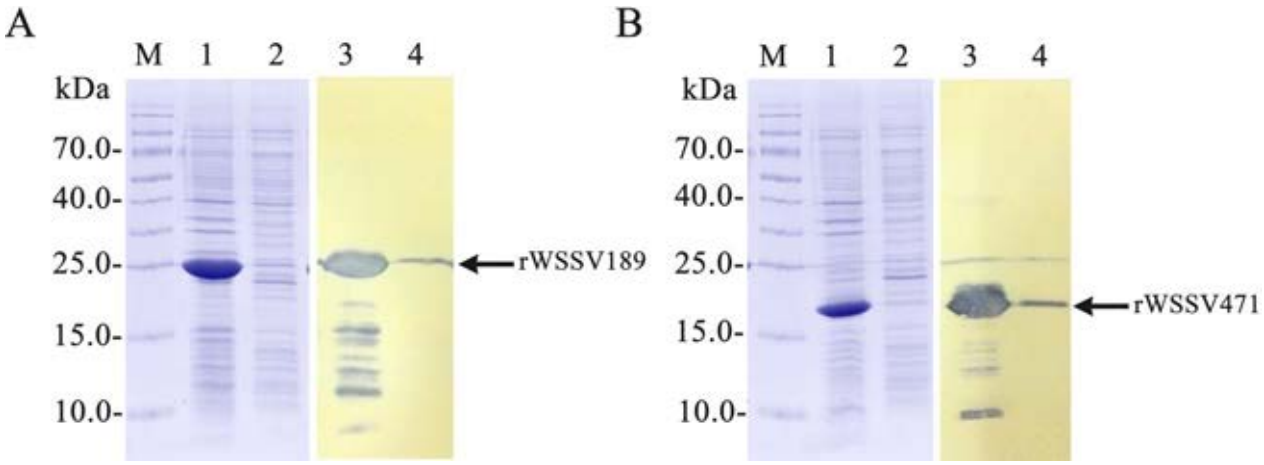


Figure 3.15 **15% SDS-PAGE analysis of recombinant protein expressed in *E. coli* BL21-CodonPlus(DE3)-RIL containing pET-19b/WSSV189 and pET-19b/WSSV471 plasmid at 2 h after IPTG induction.** Transformants for rWSSV189 and rWSSV471 were cultured and the expression of the recombinant proteins, were induced by 1 mM IPTG. After 2 h of induction, cells were collected, resuspended in PBS and then lysed by sonication. The inclusion bodies were subjected to analysis by 15% SDS-PAGE and Western blot analysis. (A), rWSSV189; (B), rWSSV471. Lane M: prestained protein marker; Lanes 1 and 2: coomassie staining of inclusion and soluble fractions, respectively; Lanes 3 and 4: Western blotting of inclusion and soluble fractions using anti-His_6 antibody as a primary antibody. An arrow reveals the expected recombinant protein.

3.4.3 Purification of recombinant protein pET-19b/WSSV189 and pET-19b/WSSV471

From the previous experiment, the rWSSV189 and rWSSV471 proteins containing the His_6-tag were expressed as inclusion bodies. 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. After solubilization, the insoluble and soluble fractions were analyzed by SDS-PAGE (Fig. 3.16). The result indicated that rWSSV189 (Fig. 3.16A) and rWSSV471 (Fig. 3.16B) could be partially solubilized from the inclusion bodies using Tris-HCl buffer, pH 7.4 and 6 M urea.

Both rWSSV189 and rWSSV471 were further purified by Ni Sepharose 6 Fast Flow bead. For the rWSSV189, the solubilized proteins were dialyzed against 20 mM sodium carbonate buffer, pH 10.0 and then purified under non-denaturing condition. The purified rWSSV189 was eluted with 20 mM sodium carbonate, 0.5 M NaCl, pH 10.0 buffer containing 100 mM imidazole. On the other hand, the rWSSV471 was purified under denaturing condition. The purified rWSSV471 was eluted with 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 buffer supplement with 6 M urea and 100 mM imidazole. Then, the purified proteins were analyzed on 15% SDS-PAGE (Fig. 3.17 and 3.18) and dialyzed against 0.1x PBS, pH 7.4. The purified proteins were concentrated by evaporation under vacuum and used as antigen for mouse immunization in order to produce the specific anti-WSSV189 and anti-WSSV471 polyclonal antibody.

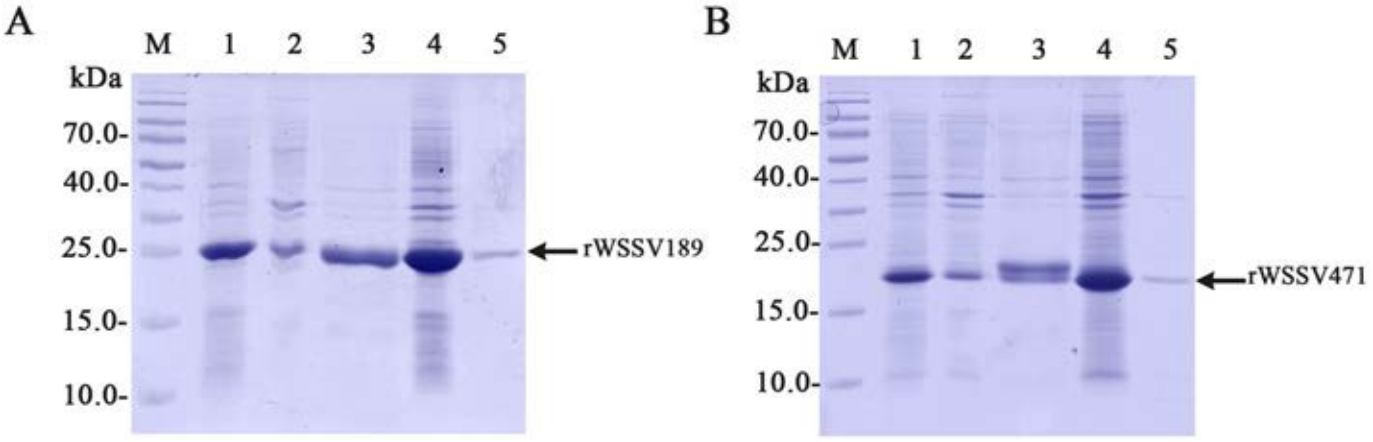


Figure 3.16 Solubilization test for rWSSV189 and rWSSV471 from inclusion bodies. The inclusion bodies of (A), rWSSV189 and (B), rWSSV471 were mixed with Tris-HCl buffer, pH 7.4 and 6M urea for overnight. Then, the soluble and insoluble fractions were separated by centrifugation and analyzed by 15% SDS-PAGE.

Lane M : prestained protein marker (PageRuler^{TM} Prestained protein ladder, Fermentas);

Lane 1 : inclusion bodies

Lane 2 : insoluble fraction of inclusion bodies in 20 mM Tris-HCl buffer, pH 7.4 and 6 M urea

Lane 3 : soluble fraction of inclusion bodies in 20 mM Tris-HCl buffer, pH 7.4 and 6 M urea

Lane 4 : insoluble fraction of inclusion bodies in 20 mM Tris-HCl buffer, pH 7.4

Lane 5 : soluble fraction of inclusion bodies in 20 mM Tris-HCl buffer, pH 7.4

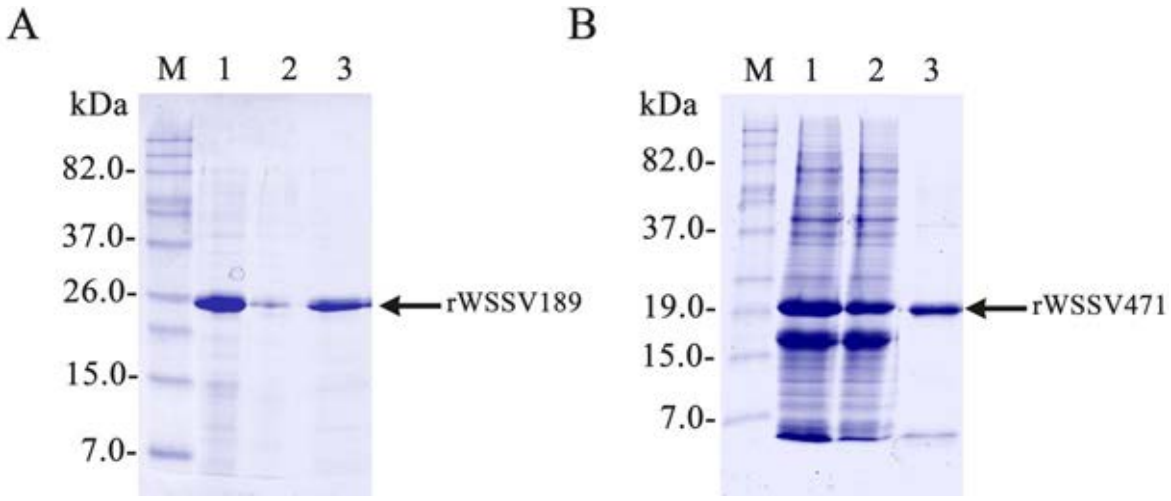


Figure 3.17 Purification of rWSSV189 and rWSSV471 by Ni affinity chromatography. The crude soluble fractions of (A), rWSSV189 and (B), rWSSV471 were purified through Ni Sepharose 6 Fast Flow column under non-denaturing and denaturing condition, respectively. For rWSSV189 the purified protein was eluted with 20 mM sodium carbonate, 0.5 M NaCl, pH 10.0 buffer containing 100 mM imidazole. Whereas, the purified rWSSV471 was eluted with 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 buffer supplement with 6 M urea and 100 mM imidazole. The purified proteins were then run on 15% SDS-PAGE. Lane M: prestained protein marker (BenchMark™ Pre-stained protein ladder, Invitrogen); Lane 1: crude rWSSV in carbonate buffer, pH 10; Lane 2: flowthrough fraction; Lane 3: purified rWSSV fraction. The arrows indicate the expected band of purified proteins.

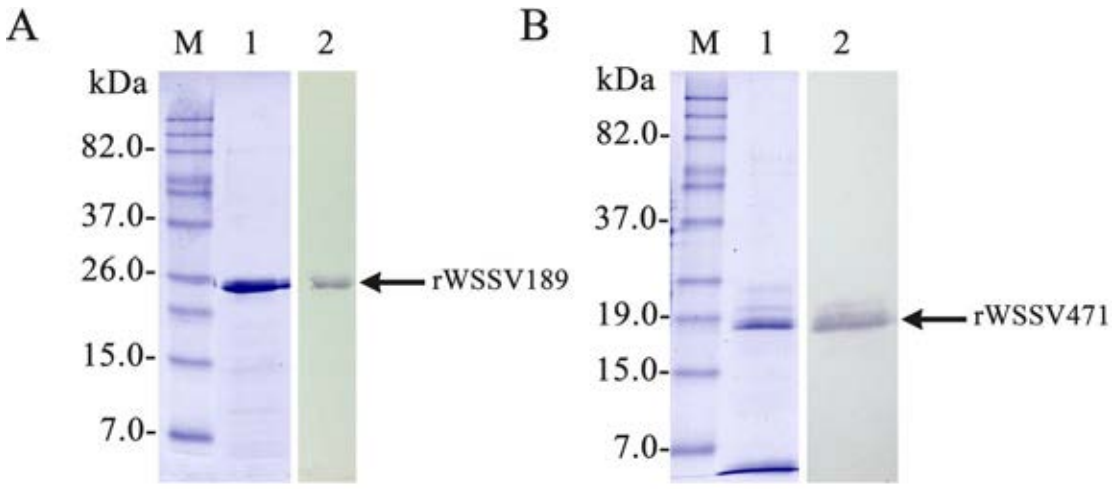


Figure 3.18 Western blotting of the purified recombinant proteins using anti-His_6 antibody. The purified protein of (A), rWSSV189 and (B), rWSSV471 were analyzed by 15% SDS-PAGE and Western blot analysis using anti-His as a primary antibody and anti-mouse as a secondary antibody. Lane M: prestained protein marker (BenchMark™ Pre-stained protein ladder, Invitrogen); Lane 1: coomassie stained 15% SDS-PAGE gel; Lane 2: Western blot analysis.

3.5 Confirmation of the ALF*Pm*3 and WSSV protein interactions by *in vitro* pull-down assay

The crude proteins of rWSSV189 and rWSSV471 as well as the purified rALF*Pm*3 were used to confirm the interactions between rWSSV and rALF*Pm*3 proteins by *in vitro* pull-down assay. In this experiment, rWSSV189 and rWSSV471 protein containing His_6-tag were used as bait. They were incubated with a cobalt chelate resin. Wash fraction was collected and checked on SDS-PAGE to ensure that there is no excess rWSSV protein left in the column before adding the prey protein (rALF*Pm*3) (Fig. 3.19-3.20, lane 3). Then rALF*Pm*3 protein was added to the column and allow it bind to WSSV. Proteins wash fraction was collected and checked on SDS-PAGE to ensure that there is no excess rWSSV and rALF*Pm*3 protein left in the column (Fig. 3.19-3.20, lane 6). After incubation, the protein complexes were eluted. They were resolved by SDS-PAGE followed by the silver staining. As shown in Fig. 3.19 and 3.20, cobalt chelate beads effectively pulled down protein giving band for both WSSV189 containing His_6-tag (~26 kDa) and ALF*Pm*3 (~11 kDa) and WSSV471 containing His_6-tag (~20 kDa) and ALF*Pm*3 (~11 kDa), respectively, but not in the prey control (ALF*Pm*3 only; lane 8) because ALF*Pm*3 could not bind with cobalt chelate beads. The results indicate that the WSSV189 and WSSV471 proteins could specifically bind to rALF*Pm*3 *in vitro*.

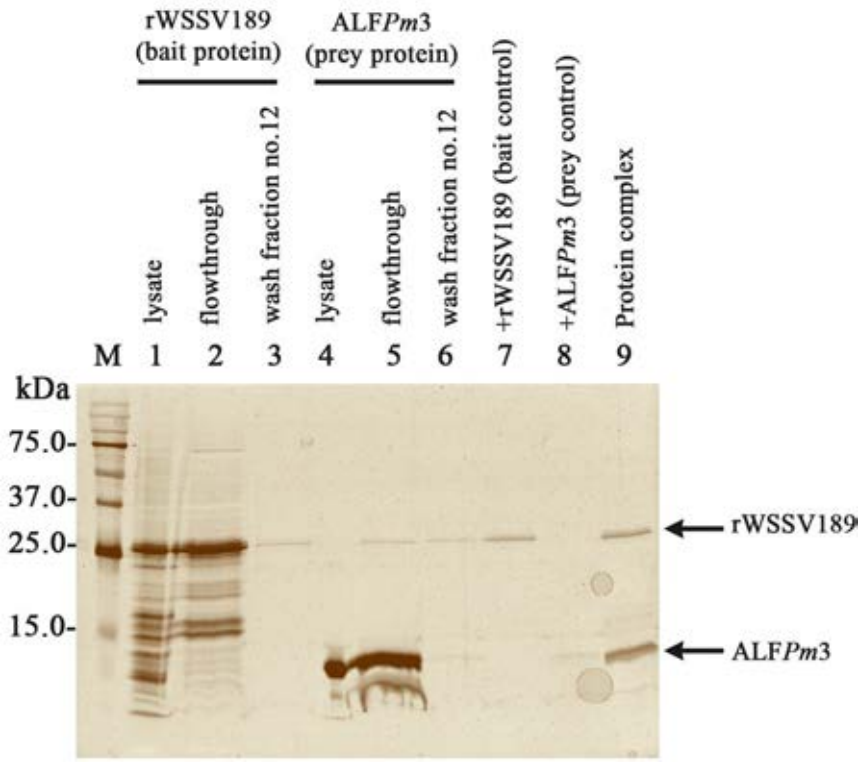


Figure 3.19 ***In vitro*** **pull-down assay between rWSSV189 and rALF*Pm*3 proteins.** rWSSV189 was immobilized on the cobalt chelate resin. After extensive washing, the rALF*Pm*3 was added and incubated for 3.5 h. The excess protein was extensively washed and the bound proteins were eluted. The elution fraction was then analyzed for the presence of the protein complex by 15% silver-stained SDS-PAGE. Lane M: prestained protein marker (Bio-RAD); Lane 1: crude rWSSV189 protein in 20 mM sodium carbonate buffer, pH 10 (bait protein); Lane 2: flowthrough fraction of rWSSV189 after binding to cobalt agarose for 1.50 h at 4 °C; Lane 3: the wash fraction of rWSSV189; Lane 4: crude rALF*Pm*3 protein (prey protein); Lane5: flowthrough fraction of rALF*Pm*3 after incubation with immobilized bait for 3.5 h at 4 °C; Lane 6: the wash fraction of rALF*Pm*3; Lane 7: bait control (rWSSV189 only). Bait treated as described in lanes 1-6 and subsequently eluted. No prey added-just binding buffer; Lane 8: prey control (ALF*Pm*3 only). Prey treated as described in lanes 1-6 and subsequently eluted. No bait added-just binding buffer and Lane 9: elution of bait:prey complex (prepared in lanes 1-6) from the agarose beads with 290 mM imidazole.

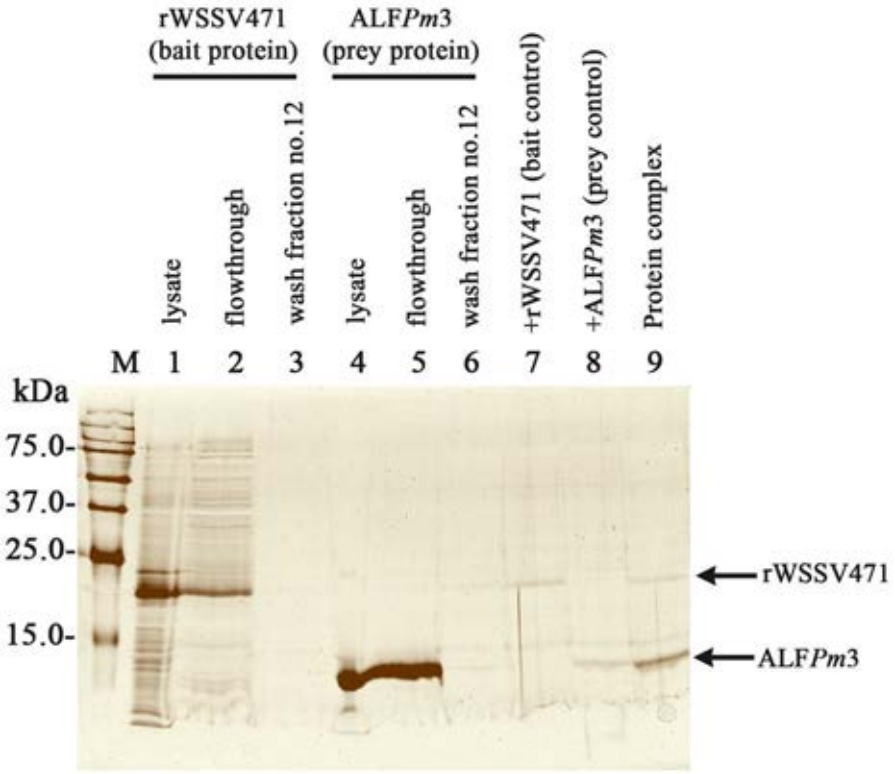


Figure 3.20 ***In vitro*** **pull-down assay between rWSSV471 and rALF*Pm*3 proteins.** rWSSV471 was immobilized on the cobalt chelate resin. After extensive washing, the rALF*Pm*3 was added and incubated for 3.5 h. The excess protein was extensively washed and the bound proteins were eluted. The elution fraction was then analyzed for the presence of the protein complex by 15% silver-stained SDS-PAGE. Lane M: prestained protein marker (Bio-RAD); Lane 1: crude rWSSV471 protein in 20 mM sodium carbonate buffer, pH 10 (bait protein); Lane 2: flowthrough fraction of rWSSV471 after binding to cobalt agarose for 1.50 h at 4 °C; Lane 3: the wash fraction of rWSSV471; Lane 4: crude rALF*Pm*3 protein (prey protein); Lane5: flowthrough fraction of rALF*Pm*3 after incubation with immobilized bait for 3.5 h at 4 °C; Lane 6: the wash fraction of rALF*Pm*3; Lane 7: bait control (rWSSV471 only). Bait treated as described in lanes 1-6 and subsequently eluted. No prey added-just binding buffer; Lane 8: prey control (ALF*Pm*3 only). Prey treated as described in lanes 1-6 and subsequently eluted. No bait added-just binding buffer and Lane 9: elution of bait:prey complex (prepared in lanes 1-6) from the agarose beads with 290 mM imidazole.

CHAPTER IV

DISCUSSION

Antimicrobial peptides (AMPs) are effector molecules that play important role in innate immune system and function as a first line of defense against invading microorganisms (Hancock et al., 2006). AMPs are typically small size, generally less than 150-200 amino acid residues, and have amphipathic structure and cationic property. AMPs are active against a large spectrum of microorganisms; bacteria, virus, yeast, parasite, fungi and even tumor cells (Hancock and Diamond et al., 2000). The antimicrobial agents found in the innate immune system of shrimp are potentially useful for aquaculture. To date, the protein-protein interaction techniques have become a widely-use routine method for the identification of proteins that interact with the known protein and are an essential for protein functional characterization. These include; yeast two-hybrid (Y2H) system, co-immunoprecipitation, *in vitro* pull-down assay, surface plasmon resonance etc (Phizicky and Fields, 1995).

ALF*Pm*3 was previously identified as an important antimicrobial peptide in *P. monodon* (Somboonwiwat et al., 2005; Ponprateep et al., 2009; Ponprateep et al., 2012). The purified rALF*Pm*3 protein exhibited a broad spectrum antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, fungi, and WSSV (Somboonwiwat et al., 2005; Tharntada et al., 2009). To better understand the role of ALF*Pm*3 in shrimp immune response to *V. harveyi* and WSSV infection, the hemocyte proteins of infected shrimp and pathogen proteins that could interact with ALF*Pm*3 were screened by Y2H technique. A cDNA fragment corresponding to ALF*Pm*3 mature protein was used as a bait to screen the libraries from hemocyte of *V. harveyi*- and WSSV-infected shrimp and cDNA libraries from *V. harveyi* and WSSV. From our results, the Y2H screening showed that ALF*Pm*3 could not interact with other shrimp proteins from the hemocytes of *V. harveyi*-infected and WSSV-infected shrimp. In

mammalian models, the antimicrobial peptides play a modulating role and directly kill pathogens (for review, see Hancock and Sahl, 2006; Risso, 2000). Defensins, the cationic amphipathic peptides are subdivided into three types of α, β, and θ defensins based on the pattern of disulfide bonding. Defensins could enhance the bacterial clearance and control inflammation and sepsis. They assembled neutrophils ceasing granule synthesis. Then, they released into the blood and enter tissues after exposure to pathogens (Ganz, 2003). For human cathelicidin LL-37 attracts neutrophils, monocytes, T cells, and mast cells, using formyl peptide receptor-like 1 (FPRL-1) after inhibit the pathogens (Zanetti, 2004). The penaeidin, an AMP from *P. monodon* was reported to play role as a modulator of the immune system. It exhibits cytokine activity in response to inflammation (Li et al., 2010; Li and Song, 2010). Nevertheless, there is no report on ALF immunodulation function in shrimp. So far, the results suggested that ALF*Pm*3 might not be the modulator of shrimp immunity but it directly killed the pathogens.

Screening of ALF*Pm*3-interacting protein on *V. harveyi* Y2H library showed that ALF*Pm*3 had likewise no interaction with *V. harveyi* proteins. The result implied that the mechanism of ALF*Pm*3 against *V. harveyi* might not interact with the bacterial protein. In the previous study, the rALF*Pm*3 was found to bind to LPS (lipopolysaccharide), which is the cell wall component of Gram-negative bacteria (Somboonwiwat et al., 2008). In 2009, Yang et al. demonstrated that the ALF*Pm*3 binds to LPS, lipid A and to OM®-174, a soluble analogue of lipid A using Surface Plasmon Resonance. Moreover, they demonstrated the possible lipid A-binding site by NMR on the basis of striking structural similarities to the FhuA/LPS complex. Two kinds of antimicrobial mechanisms of AMPs: transmembrane pore forming mechanism and intracellular killing have been reported (Brogden, 2005). Currently, it has been shown that the rALF*Pm*3 can permeabilize the outer and inner membrane of *V. harveyi* cell. The effect of rALF*Pm*3 on bacterial cell morphology was clearly shown. Treating bacterial cells with the rALF*Pm*3 caused the membrane disruption and damaged as well as the leakage of cytoplasmic content. Therefore, the rALF*Pm*3 acts on bacterial membrane causing membrane disruption and cell lysis (Jaree et al., submitted). According to our study, it was suggested

that rALF*Pm*3 causes membrane permeabilization, induce physical change on the cell surface but it cannot bind to the intracellular protein (Brogden, 2005).

The antiviral properties of several peptides have been reported in various crustaceans including tachyplesin (Murakami et al., 1991; Yasin et al., 2000), polyphemusin (Morimoto et al., 1991; Masuda et al., 1992; Nakashima et al., 1992; Tamamura et al., 1993), an antiviral gene from shrimp *P. monodon* (PmAV) (Luo et al., 2003), a novel C-type lectin (LvCTL1) (Zhao et al., 2009), hemocyanin (Zhang et al., 2004; Lie et al., 2008), mytilin (Dupuy et al., 2004; Roch et al., 2008), PmRab7 (Sritunyalucksana et al., 2006) and ALF (Liu et al., 2006; Tharntada et al., 2009). In *P. monodon,* the ALF*Pm*3 is the only shrimp AMP which has been reported to exhibit an anti-WSSV activity. The rALF*Pm*3 can efficiently protect *P. monodon* from WSSV infections (Tharntada et al., 2009; Ponprateep et al., 2012). Recently, RNAi knockdown of ALF*Pm*3 gene caused an increase in mortality of WSSV-infected shrimp (Ponprateep et al., 2012). To better understand ALF*Pm*3 function in shrimp viral responses, we screened the WSSV library for ALF*Pm*3-interacting protein using Y2H technique. Five WSSV proteins including WSSV186, WSSV189, WSSV395, WSSV458 and WSSV471 were found to interact with ALF*Pm*3. Although we identified the interaction between ALF*Pm*3 and WSSV protein, the biological functions of candidate ALF*Pm*3 binding proteins have not been reported. Only structural localization has been reported for some WSSV proteins. The shotgun proteomic study revealed that WSSV186 and WSSV189 were structural proteins (Li et al., 2007b). WSSV395 or VP39 was determined by western blot and immunoelectron microscopy (IEM) as an envelope protein (Tsai et al., 2004).

In previous study, the transcriptional profiling of the WSSV genes in WSSV-infected crayfish by DNA microarray, revealed that WSSV189 and WSSV471 might be the early expressed genes (Lan et al., 2006). In 2006, Zhu et al. showed that the WSSV395 gene expression in hepatopancreas of WSSV-infected crayfish increased at the late stage post-WSSV infection. In contrast, microarray analysis of the WSSV genes expressed in the gill of WSSV-infected *P. monodon*, showed that WSSV189 was an

intermediate-early (IE) gene which were expressed at 2 hpi. Instead, WSSV186, WSSV395, WSSV458 and WSSV471 were late gene expressing at 24 hpi (Wang et al., 2004). In this study, we analyzed the mRNA transcript expression of these five WSSV genes by semi-quantitative RT-PCR in the WSSV-infected *P. monodon* gill. The result indicated that all five WSSV genes were expressed at late stage post-WSSV infection. The disparity of WSSV189 gene result might cause by different dose of WSSV infection in shrimp. Transcription analysis using RT-PCR revealed that WSSV genes such as VP24, VP95, WSSV010, VP26, VP15, VP124, VP466, VP31, VP39, VP19, and VP28 were expressed at late stage. It is noteworthy that several of those have been identified to encode the structural proteins (Huang et al., 2002; Zhang et al., 2002; Zhu et al., 2005; Li et al., 2005; Zhu et al., 2006; Marks et al., 2006; Chen et al., 2007). It is known that the structural proteins play very important roles in virus infection and morphogenesis (For review see; Sánchez-Paz, 2010). Therefore, we hypothesized that five ALF*Pm*3-interacting WSSV protein might be proteins necessary for virion assembly.

Among five ALF*Pm*3-binding proteins, we expressed the recombinant proteins for WSSV189 and WSSV471 and used for confirmation of their interaction with ALF*Pm*3 by *in vitro* pull-down assay. The recombinant WSSV proteins were expressed in the *E. coli* strain BL21-CodonPlus(DE3)-RIL. Both rWSSV189 and rWSSV471 with molecular mass of about 26 kDa and 20 kDa, respectively, were obtained. *In* vitro pull-down assays showed that the WSSV189 and WSSV471 proteins could specifically bind to rALF*Pm*3 *in vitro*. The true interaction between WSSV189 and WSSV471 with rALF*Pm*3 provided clues for further study on anti-WSSV mechanism of rALF*Pm*3.

In mammalian, it has been reported that lactoferrin, an iron binding glycoprotein exhibiting also the antimicrobial activity, is involved in antiviral mechanism. Lactoferrin prevents viral infection of the host cell either by direct binding to virus particles or binding to host cell molecules that the virus uses as a receptor or co-receptor (For review see; van der Strate et al., 2001). The AMP, Indolicidin, inactivates HIV-1 by damaging the virion membrane (Robinson et al., 1998). The α-defensin peptides are able to inhibit

assembly of polyoma virus particles (Dugan et al., 2008). Many reports have studied proteins involved in antiviral response in shrimp. Integrins are transmembrane proteins of *M. japonicas* that recognize a large variety of extracellular and cell surface proteins emerging as receptors or co-receptors for a large number of viruses (Li et al., 2007a). Several studies have reported that RGD (Arg-Gly-Asp) motifs containing viral proteins serve as binding ligands. These viral proteins bind to the integrins on the host cell surface and then gain entry into the cells. Envelope proteins of WSSV such as VP281, VP31, VP36A, VP110, VP136A, VP664 and VP187 contained the RGD motif (Huang et al., 2002; Li et al., 2006a, 2006b, 2006c; Tsai et al., 2004). 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). Besides, PmRab7 could bind to VP28, an envelope protein of WSSV, and might be a receptor for VP28 in *P. monodon* shrimp suggesting that PmRab7 is a common cellular factor required for WSSV replication in shrimp (Sritunyalucksana et al., 2006). Until now, it was known how shrimp ALF*Pm*3 interact with WSSV protein. According to the ALF*Pm*3 structure, the LPS binding site is the cluster of positively charge amino acid residues. Whereas the primary structure of WSSV189 and WSSV471 protein contains a series of aspartate and glutamate negative charge residues (Asp^{103}-Ile^{104}-Glu^{105}-Asp^{106}-Asp^{107}) and (Glu^{27}-Glu^{28}-Glu^{29}-Glu^{30}), respectively. Hence, the rALF*Pm*3 probably bound directly to the WSSV by charge interaction. Further experiments should be done to characterize the anti-WSSV mechanism of ALF*Pm*3. From our study, the antibody specific to rWSSV189 and rWSSV471 will be raised and used for immunogold labeling on viral particle.

CHAPTER V

CONCLUSIONS

ALF*Pm*3 is an antimicrobial peptide playing an important role in *P. monodon* immunity against pathogenic infection. The antimicrobial activity and the possible antiviral activity of ALF*Pm*3 have been reported. To better understand the ALF*Pm*3 response upon bacterial and viral infection, we used Y2H technique to screen for the ALF*Pm*3 partner protein in the *V. harveyi*- and WSSV-infected shrimp hemocyte libraries. The results showed that the ALF*Pm*3 did not interact with other shrimp protein during pathogenic infection suggesting that it might not play roles as an immune modulator but solely as an antimicrobial peptide.

No *V. harveyi* protein was found to interact with ALF*Pm*3 suggesting that the antibacterial mechanism of ALF*Pm*3 against *V. harveyi* might not involve in binding to bacterial proteins.

For WSSV library, five putative ALF*Pm*3 binding proteins including WSSV186, WSSV189, WSSV395, WSSV458, WSSV471, were identified as ALF*Pm*3-interacting proteins by Y2H assay. Their transcripts were expressed at the late stage post-WSSV infection in shrimp gill. The recombinant WSSV189 and WSSV471 proteins were successfully produced in *E. coli* strain BL21-CodonPlus(DE3)-RIL system. The purified recombinant WSSV189 and WSSV471 proteins had estimated molecular mass of 26 kDa and 20 kDa, respectively. *In vitro* pull-down assay confirmed that rWSSV189 and rWSSV471 could bind to ALF*Pm*3. We can conclude that these interactions might involve in anti-WSSV activity of ALF*Pm*3.

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Appendices

Appendix A

1. Preparation for SDS-PAGE electrophoresis

Stock reagents

30% Acrylamide, 0.8% bis-acrylamide, 100 ml

Acrylamide	29.2	g
N, N´-methylene-bis-acrylamide	0.8	g

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8, 100 ml

Tris (hydroxymethyl)-aminomethane	18.17	g

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

1.0 M Tris-HCl pH 6.8, 100 ml

Tris (hydroxymethyl)-aminomethane	18.17	g

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

10% SDS, 10 ml

Sodium dodecyl sulfate	0.1	g

Adjust volume to 10 ml with distilled water.

10% ammonium persulfate (APS), 5 ml

Ammonium persulfate	0.5	g

Adjust volume to 5 ml with distilled water and store for month in the refrigerator.

SDS-PAGE

15% Seperating gel, 5 ml

30% Acrylamide mix	2.50	ml
1.5 M Tris-HCl pH 8.8	1.27	ml
10% SDS	50	µl
10% APS	50	µl
TEMED	2	µl
Distilled water	1.13	ml

5.0% Stacking gel, 3 ml

30% Acrylamide mix	500	µl

1.0 M Tris-HCl pH 6.8	380	µl
10% SDS	30	µl
10% APS	30	µl
TEMED	3	µl
Distilled water	2.06	ml

5× Sample buffer

1.0 M Tris-HCl pH 6.8	0.6	ml
50% Glycerol	5.0	ml
10% SDS	2.0	ml
2-Mercaptoethanol	0.5	ml
1% Bromophenol blue	1.0	ml
Distilled water	0.9	ml

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

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

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

2. Preparation for silver staining solution

Solution A

Silver nitrate	0.8	g
Distilled water	4.0	ml

Solution B

0.36% NaOH	21.0	ml
14.8 M Ammonium hyroxide	1.4	ml

Solution C

Add solution A to solution B dropwise with constant vigorous stirring, allowing brown precipitate to clear.

Solution D

Mix 0.5 ml of 1% citric acid with 50 µl of 3.8% formaldehyde, add water to 100 ml. Solution must be fresh.

Appendix B

Table B1 Homology proteins hit to wssv186 from the BlastX software.

Sequence homology (Accession no.)	Organism	% identity to WSSV186	Expect values	Score (bits)
WSV131 (NP_477653)	Shrimp white spot syndrome virus	460/460 (100%)	0.0	794
Unknown (ABQ12771)	Shrimp white spot syndrome virus	399/399 (100%)	0.0	673
WSV132 (NP_477654)	Shrimp white spot syndrome virus	79/79 (100%)	6e-37	137
hypothetical protein Sfri_1957 (YP_750641)	Shewanella frigidimarina NCIMB 400	21/62 (34%)	5.3	38.1
ABC transporter, ATP-binding/permease protein (ZP_05329475)	Clostridium difficile QCD-63q42	21/66 (31%)	5.9	38.1
metal dependent phosphohydrolase (ZP_05393297)	Clostridium carboxidivorans P7	39/164 (24%)	6.8	37.0
ABC transporter, ATP-binding protein (EHJ37330)	Clostridium difficile 70-100-2010	20/66 (30%)	7.8	37.7
ABC transporter, ATP-binding/permease protein (ZP_05350610)	Clostridium difficile ATCC 43255	20/66 (30%)	7.8	37.7
ABC transporter, ATP-binding/permease protein (ZP_05271498)	Clostridium difficile QCD-66c26	20/66 (30%)	7.8	37.7

Table B2 Homology proteins hit to wssv189 from the BlastX software.

Sequence homology (Accession no.)	Organism	% identity to WSSV189	Expect values	Score (bits)
WSV134 (NP_477656)	Shrimp white spot syndrome virus	222/222 (100%)	5e-154	432
CMGC family protein kinase (XP_001325215)	Trichomonas vaginalis G3	32/107 (30%)	1.0	38.5
unnamed protein product (YP_004259192)	Bacteroides salanitronis DSM 18170	28/81 (35%)	1.3	38.5
extracellular protease (ZP_05367759)	Rothia mucilaginosa ATCC 25296	32/127 (25%)	2.0	37.7
ApaG (ZP_02162797)	Kordia algicida OT-1	19/62 (31%)	8.0	35.8

Table B3 Homology proteins hit to wssv395 from the BlastX software.

Sequence homology (Accession no.)	Organism	% identity to WSSV395	Expect values	Score (bits)
WSV339 (NP_477861)	Shrimp white spot syndrome virus	283/283 (100%)	0.0	537
VP 39 (ABQ44210)	Shrimp white spot syndrome virus	283/283 (99%)	0.0	536
PREDICTED: KIF1-binding protein homolog (XP_001380645)	Monodelphis domestica	30/140 (21%)	2.6	38.1
PREDICTED: major histocompatibility complex class I-related gene protein-like (XP_003459376)	Oreochromis niloticus	39/177 (22%)	3.3	37.7
Tripartite motif-containing protein 15 (EGW13273)	Cricetulus griseus	35/122 (29%)	5.8	36.2
PREDICTED: tripartite motif-containing protein 15-like (XP_003507480)	Cricetulus griseus	35/122 (29%)	8.6	36.2
hypothetical protein HMPREF1019_00975 (ZP_09350292)	Campylobacter sp. 10_1_50	15/35 (43%)	8.7	35.4
periplasmic nitrate reductase, diheme cytochrome C subunit (YP_001466509)	Campylobacter concisus 13826	15/35 (43%)	8.7	35.4
PREDICTED: major histocompatibility complex class I-related gene protein-like (XP_003460221)	Oreochromis niloticus	17/41 (41%)	9.2	36.2

Table B4 Homology proteins hit to wssv458 from the BlastX software.

Sequence homology (Accession no.)	Organism	% identity to WSSV458	Expect values	Score (bits)
wsv399 (NP_477921)	Shrimp white spot syndrome virus	183/183 (100%)	4e-138	389
wsv401 (NP_477923)	Shrimp white spot syndrome virus	77/77 (100%)	6e-55	175
wsv400 (NP_477922)	Shrimp white spot syndrome virus	67/67 (100%)	9e-48	156
wsv476 (NP_477998)	Shrimp white spot syndrome virus	15/23 (65%)	0.058	38.9
ribosomal protein S3 (ACN23341)	Halimeda gracilis	20/64 (31%)	0.072	40.0
bgaD gene product (YP_004735699)	Zobellia galactanivorans	18/48 (38%)	0.99	38.1
conserved hypothetical protein (XP_002792956)	Paracoccidioides brasiliensis Pb01	29/92 (32%)	1.4	37.7
ribosomal protein S3 (ACN23345)	Halimeda lacrimosa	20/65 (31%)	1.7	36.2
ribosomal protein S3 (AAV83652)	Halimeda gracilis	20/65 (31%)	1.9	36.2
ribosomal protein S3 (ACN23343)	Halimeda gracilis	20/65 (31%)	2.0	36.2
SJCHGC04165 protein (AAX30499)	Schistosoma japonicum	26/80 (33%)	3.4	34.7
ribosomal protein S3 (ACN23466)	Halimeda cuneata	19/66 (29%)	3.9	35.4

Table B4 (Cont.) Homology proteins hit to wssv458 from the BlastX software.

Sequence homology (Accession no.)	Organism	% identity to WSSV458	Expect values	Score (bits)
ribosomal protein S3 (AAV83544)	Halimeda cuneata	19/66 (29%)	3.9	35.4
ribosomal protein S3 (ACN23613)	Halimeda discoidea	18/66 (27%)	4.0	35.0
hypothetical protein HMPREF9022_03671 (ZP_08858014)	Erysipelotrichaceae bacterium 2_2_44A	26/87 (30%)	5.5	35.8
hypothetical protein HOLDEFILI_01368 (ZP_03634087)	Holdemania filiformis DSM 12042	19/65 (29%)	5.7	35.4
hypothetical protein AOL_s00043g647 (EGX51913)	Arthrobotrys oligospora ATCC 24927	21/59 (36%)	6.1	35.4
conserved Plasmodium protein, unknown function (XP_001352135)	Plasmodium falciparum 3D7	29/126 (23%)	6.2	35.8
ribosomal protein S3 (ACN23631)	Halimeda discoidea	18/66 (27%)	6.8	34.7
ribosomal protein S3 (AAV83612)	Halimeda discoidea	18/66 (27%)	7.2	34.7
ribosomal protein S3 (AAV83608)	Halimeda discoidea	18/66 (27%)	7.2	34.7
ribosomal protein S3 (AAV83606)	Halimeda discoidea	18/66 (27%)	7.2	34.7
SJCHGC04165 protein (AAX30499)	Schistosoma japonicum	26/80 (33%)	3.4	34.7

Table B4 (Cont.) Homology proteins hit to wssv458 from the BlastX software.

Sequence homology (Accession no.)	Organism	% identity to WSSV458	Expect values	Score (bits)
hypothetical protein KAFR_0H00150 (CCF59424)	Kazachstania africana CBS 2517	21/82 (26%)	7.5	35.4
beta-galactosidase (ZP_09496870)	Flavobacteriaceae bacterium S85	17/48 (35%)	7.8	35.4
Protein of unknown function DUF788,domain-containing protein (CAX69495)	Schistosoma japonicum	26/80 (33%)	8.4	34.7
ribosomal protein S3 (ACN23575)	Halimeda discoidea	18/66 (27%)	9.2	34.3
ribosomal protein S3 (ACN23321)	Halimeda micronesica	24/78 (31%)	9.5	34.3
ribosomal protein S3 (AAV83610)	Halimeda discoidea	18/66 (27%)	9.7	34.3
ribosomal protein S3 (AAV83592)	Halimeda discoidea	18/66 (27%)	9.8	34.3

Table B5 Homology proteins hit to wssv471 from the BlastX software.

Sequence homology (Accession no.)	Organism	% identity to WSSV471	Expect values	Score (bits)
wsv412 (NP_477934)	Shrimp white spot syndrome virus	159/159 (100%)	4e-109	314
ORF181 (AAK77850)	Shrimp white spot syndrome virus	65/67 (97%)	1e-42	142
predicted protein (XP_001629293)	Nematostella vectensis	35/132 (27%)	3e-06	53.1
viral A-type inclusion protein (XP_001579764)	Trichomonas vaginalis G3	32/172 (19%)	7e-04	47.0
hypothetical protein HMPREF0987_01374 (ZP_08335071)	Lachnospiraceae bacterium 9_1_43BFAA	28/112 (25%)	9e-04	46.6

BIOGRAPHY

Miss Sivalee Suraprasit was born in November 20, 1986 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2008. She has studied for the degree of Master of Science, Program in Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2009. In her third year of research work, she presented a poster entitle "Identification of ALF*Pm*3-interacting proteins from hemocytes of *Vibrio harveyi*- and WSSV-infected of *Penaeus monodon*" at the 1st ASEAN Plus Three Graduate Research Congress (AGRC 2012) and published in e-proceedings of AGRC 2012, Organized by Consortium of the Graduate Studies Administrators of Public and Autonomous Universities (CGAU) Thailand at the Empress hotel, Chiang Mai in March 1-2, 2012.