ลักษณะสมบัติและการจัดเรียงตัวของยีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์ใน กุ้งกุลาดำ *Penaeus monodon*

นางสาวศิรินิตย์ ธารธาดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

CHARACTERIZATION AND GENE ORGANIZATION OF ANTI-LIPOPOLYSACCHARIDE FACTOR IN THE BLACK TIGER SHRIMP *Penaeus monodon*

Miss Sirinit Tharntada

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Biochemistry

Department of Biochemistry

Faculty of Science

Chulalongkorn University

Academic Year 2007

Thesis Title	CHARACTERIZATION AND GENE ORGANIZATION OF ANTI-LIPOPOLYSACCHARIDE FACTOR IN THE BLACK TIGER SHRIMP *Penaeus monodon*
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Field of Study	Biochemistry
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ศิรินิตย์ ธารธาดา : ลักษณะสมบัติและการจัดเรียงตัวของยีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์ในกุ้งกุลาดำ *Penaeus monodon*. (CHARACTERIZATION AND GENE ORGANIZATION OF ANTI-LIPOPOLYSACCHARIDE FACTOR IN THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษา : ศ.ดร. อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม : รศ.ดร. วิเชียร ริมพณิชยกิจ, PROF. Kenneth Söderhäll, Ph.D., 134 หน้า.

จากการวิเคราะห์ข้อมูลยีนแอนติไลโพพอลิแซกคาไรด์แฟคเตอร์ในฐานข้อมูล *Penaeus monodon* Expressed Sequence Tag database (http://pmonodon.biotec.or.th) โดยทำการเปรียบเทียบลำดับนิวคลีโอไทด์และกรดอะมิโนพบว่า มียีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์ในกุ้งกุลาดำอย่างน้อย 5 ไอโซฟอร์ม (ALF*Pm*1-5) และสามารถแบ่งออกได้เป็น 2 กลุ่ม ได้แก่ กลุ่มเอ ประกอบด้วยไอโซฟอร์ม 1 และ 2 และกลุ่มบี ประกอบด้วย ไอโซฟอร์ม 3 4 และ 5 เมื่อศึกษาการจัดเรียงตัวของยีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์ด้วยวิธี PCR และ genome walking พบว่ายีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์กลุ่มเอ ประกอบด้วย 2 intron และ 3 exon ขณะที่กลุ่มบีประกอบด้วย 3 intron และ 4 exon เมื่อทำการเปรียบเทียบลำดับนิวคลีโอไทด์ของ genomic DNA และ cDNA แสดงให้เห็นว่าไอโซฟอร์มทั้งห้าได้มาจากการเกิด alternative RNA splicing ขึ้นในแต่ละกลุ่ม ส่วนบริเวณปลาย 5′ ของยีนแต่ละกลุ่มนั้นมีความหลากหลายของส่วนที่ทำหน้าที่ควบคุมการแสดงออกของยีนที่สร้างเปปไทด์ด้านจุลชีพ โดยประกอบด้วย activator protein 1 Octamer GATA nuclear factor-kappaB และ GAAA motif จากการ ตรวจสอบการแสดงออกของยีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์แต่ละไอโซฟอร์มเมื่อกุ้งแต่ละตัวติดเชื้อวิบริโอฮาวิอายโดยใช้เทคนิค RT-PCR พบว่ากุ้งแต่ละตัวมีการแสดงออกในเม็ดเลือดของไอโซฟอร์ม 2 และ 3 เป็นหลัก และการแสดงออกของไอโซฟอร์ม 2 และ 3 จะเพิ่มขึ้นเมื่อกุ้งแต่ละตัวติดเชื้อวิบริโอฮาวิอาย ซึ่งแสดงให้เห็นถึงหน้าที่สำคัญของยีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์ในการต่อต้านแบคทีเรีย ดังนั้นเพื่อที่จะศึกษาสมบัติ แอคติวิตี และคุณสมบัติทางชีวภาพของยีนแอนติไลโพพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 2 โปรตีนจึงถูกผลิตในระบบยีสต์ *Pichia pastoris* และผ่านการทำบริสุทธิ์โดยใช้ cation exchange chromatography โปรตีนที่บริสุทธิ์แล้วมีฤทธิ์ยับยั้งการเจริญของแบคทีเรียแกรมลบ *Escherichia coli 363* และแบคทีเรียแกรมบวก *Bacillus megaterium* และนอกจากนี้ยังได้ทำการทดสอบสมบัติของโปรตีนแอนติไลโพพอลิแซ็กคาไรด์แฟคเตอร์ไอโซฟอร์ม 2 และ 3 ในการยับยั้งการติดไวรัสตัวแดงดวงขาวใน hematopoietic stem cell ของ *Pacifastacus leniusculus* โดยใช้เทคนิค RT-PCR ติดตามยีนของโปรตีนที่ผิวไวรัสตัวแดงดวงขาว (VP28) พบว่าที่ความเข้มข้น 20 ไมโครโมลาร์ เฉพาะโปรตีนไอโซฟอร์ม 3 ที่สามารถยับยั้งการ propagation ของไวรัสตัวแดงดวงขาวได้ โดยมีค่าความเข้มข้นของโปรตีนที่สามารถยับยั้งการ propagation ของ WSSV ได้ 50% เมื่อเปรียบเทียบกับสภาวะที่ไม่มีโปรตีนไอโซฟอร์ม 3 (IC_{50}) นั้นน้อยกว่า 2.5 ไมโครโมลาร์

ภาควิชา.....ชีวเคมี..... ลายมือชื่อ นิสิต.....Sirinit Thorntara.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....A. Tassanakajon.....

ปีการศึกษา.....2550..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....K. Rimphanitchayakit.....

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4573839923 : MAJOR BIOCHEMISTRY
KEY WORD: *Penaeus monodon* / ANTIMICROBIAL ACTIVITY / ANTI-LIPOPOLYSACCHARIDE FACTOR / GENE ORGANIZATION / *Pichia pastoris* EXPRESSION SYSTEM

SIRINIT THARNTADA : CHARACTERIZATION AND GENE ORGANIZATION OF ANTI-LIPOPOLYSACCHARIDE FACTOR IN THE BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS COADVISOR : ASSOC. PROF. VICHIEN RIMPANITCHAYAKIT, Ph.D., PROF. KENNETH SÖDERHÄLL, Ph.D. ,134 pp.

Different isoforms of the ALF homologues (ALF*Pm*1 to 5) have been previously identified from *Penaeus monodon* expressed sequence tag (EST) database (http://pmonodon.biotec.or.th). The nucleotide and amino acid sequences of the *P. monodon* ALF homologues were analyzed and categorized into two groups, ALF*Pm*1 and 2 in group A and ALF*Pm*3 to 5 in group B. The genomic sequences of the two ALF gene groups were obtained by using the PCR and genome walking techniques. The ALF group A gene consisted of three exons interrupted by two introns whereas the ALF group B gene contained four exons interrupted by three introns. The alignment of genomic sequences with the ALF cDNA sequences revealed that different transcripts in both groups were generated by alternative RNA splicing of the pre-mRNA transcripts. The 5′ upstream sequences of the two ALF groups contained the putative cis-regulatory elements, including the activator protein 1, the Octamer, the GATA, the nuclear factor-kappaB, and the GAAA motifs, which possibly promoted transcription in response to infection as in other antimicrobial peptide genes. The RT-PCR analysis revealed that although all ALF isoforms were expressed in individual shrimp, the ALF*Pm*2 and 3 were the major or authentic ALFs in the hemocytes. The expression of ALF*Pm*2 and 3 were increased in response to *Vibrio harveyi* infection indicating the important function of the ALFs against bacterial invasion. To further characterize the activity of ALF*Pm*2, the recombinant ALF*Pm*2 protein (rALF*Pm*2) was produced in the *Pichia pastoris* and then purified using cation exchange chromatography. The purified rALF*Pm*2 showed antibacterial activity against *Escherichia coli* 363 and *Bacillus megaterium*. Moreover, 20 μM of rALF*Pm*3 but not rALF*Pm*2 exhibited the antiviral activity against the white spot syndrome virus (WSSV) by inhibiting the propagation of WSSV in hematopoietic stem cells of *Pacifastacus leniusculus*. Analysis of VP28 transcripts by quantitative RT-PCR showed that the concentration of rALF*Pm*3 required for 50% inhibition of viral propagation in vitro (IC_{50}) was less than 2.5 μM.

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Acknowledgements

I would like to express my deepest gratitude to my advisor Professor Dr. Anchalee Tassanakajon, and my co-advisors Associate Professor Dr. Vichien Rimpanitchayakit and Professor Dr. Kenneth Söderhäll for their guidance, supervision, encouragement and supports throughout my study.

My gratitude is also extended to Professor Dr. Aran Incharoensakdi, Associate Professor Dr. Siriporn Sitthipraneed and Assistant Professor Dr. Apinunt Udomkit for serving as thesis committees, for their available comments and also useful suggestions.

Thanks are also expressed to Dr. Irene Söderhäll, Dr. Pikul Jiravanichpaisal and Dr. Kunlaya Somboonwiwat for very useful comments and suggestions. Many thanks to all my friends of the Department of Biochemistry, Chulalongkorn University especially my former colleagues in R728 and of Comparative Physiology, Uppsala University, Uppsala, Sweden for their help in the laboratory and friendships that help me enjoy and happy through out my study.

Special thank to Thai students in Uppsala, Sweden for their help, supporting and encouragement.

Finally, I would like to express my deepest gratitude to my mother, aunts and brother for their love, care, understanding and encouragement extended throughout my study.

I wish to acknowledge to contributions of the Royal Golden Jubilee Ph.D. program, Thailand Research Fund for my fellowship and the National center for Genetic Engineering and biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA).

CONTENTS

Page

Page

Page

Page

List of Tables

List of Figures

Page

Page

Page

List of Abbrevations

ALF	anti-lipopolysaccharide factor
AP-1	activator protein 1
bp	base pair
^{o}C	degree Celcius
Cfu	colony forming units
CPBS	crayfish phosphate buffer
DEPC	diethylpyrocarbonate
dNTP	Deoxyribonucleotide triphosphate
DNA	deoxyribonucleic acid
EF-1 alpha	Elongation factor-1 alpha
EtBr	ethidium bromide
EST	expressed sequence taq
hpi	hour-post injection
hpt	hematopoietic tissue
LPS	lipopolysaccharide
kb	kilobase
kDa	kilodalton
M	molar
MCS	multiple cloning sites
MES	2-(*N*-morpholino)ethanesulfonic acid
MIC	minimum inhibitory concentration
ml	millilitre
$MgCl_2$	magnesium chloride
mg	milligram
mM	millimolar
ng	nanogram
nm	nanometre
NF-kappaB	nuclear factor-kappaB

O.D.	optical density
Oct-1	Octamer-1
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RSL	reactive site loop
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
μg	microgram
μl	microlitre
μM	micromolar
WSSV	white spot syndrome virus
YHV	Yellow-head virus

CHAPTER I

INTRODUCTION

1.1 General introduction

For the trading of aquaculture produces, shrimp and prawn as a group were the second highest reported value worth US$10.6 billion. For single species, the highest reported value was US$5.9 billion for the pacific white shrimp (*Litopenaeus vannamei*), followed by Atlantic salmon (*Salmo salar*), silver crap and black tiger shrimp (*Penaeus monodon*) (Source: FAO Aquaculture Newsletter No. 38, 2007). Thailand is one of the top ten countries in aquaculture production of aquatic animals, and shrimp is one of our highest value productions. Black tiger shrimp, *P. monodon*, and the pacific white shrimp, *L. vannamei,* are the two major shrimp species exported to various countries. Before the year 2000, Thailand was the world's leading shrimp producer of *P. monodon*. In 2002, however, the production, as compared to 2001, decreased about 40 percent to approximately 160,000 tons due to infectious diseases at the beginning of the year, unfavorable weather, high salt concentration in water, slow shrimp growth rates, low yield per area. The worst of it is that the black tiger shrimp production of Thailand fell down from 180,000 tons in 2004 to 19,000 tons in 2005 (Figure 1.1). In 2007, the prediction of black tiger shrimp production will be 12,480 tons while that of white shrimp will be 611,520 tons.

Due to the serious problems of the black tiger shrimp, the species mainly cultured in Thailand had switched to the white shrimp, *L. vannamei*. The exotic species, *L. vannamei*, was imported for aquaculture instead of the native one like *P. monodon* (Figure 1.1). The main reasons behind the importation of *L. vannamei* had been the poor performance, slow growth rate and disease susceptibility of the *P. monodon* shrimp virtually everywhere in Thailand. However, the total Thai shrimp production was not affected as it was compensated by the increased production of the white shrimp (Figure 1.1).

Table 1.1 Thailand's exports of *P. monodon* in various countries

Country	2003		2004		2005		2006	
	Quantity	Value	Quantity	Value	Quantity	Value	Quantity	Value
USA	37,701	12,157	20,593	5,878	12,147	2,992	15,302	3,613
Japan	15,238	5,381	11,672	3,961	6,662	2,261	2,523	802
Canada	5,453	1,767	3,345	1,109	1,966	578	2,019	531
Singapore	2,366	612	1,042	201	379	65	238	38
Korea	5,141	1,345	4,994	1,244	3,704	864	2,769	664
Taiwan	1,886	515	2,825	469	1,469	381	456	145
Australia	2,669	718	1,365	371	296	108	317	107
Hong Kong	516	140	666	217	589	169	618	142
China	361	74	612	136	646	102	582	71
Total	73,334	23,341	49,303	14,307	29,168	7,900	25,420	6,298

Quantity = tons Value = million baht (Source: The Customs Department)

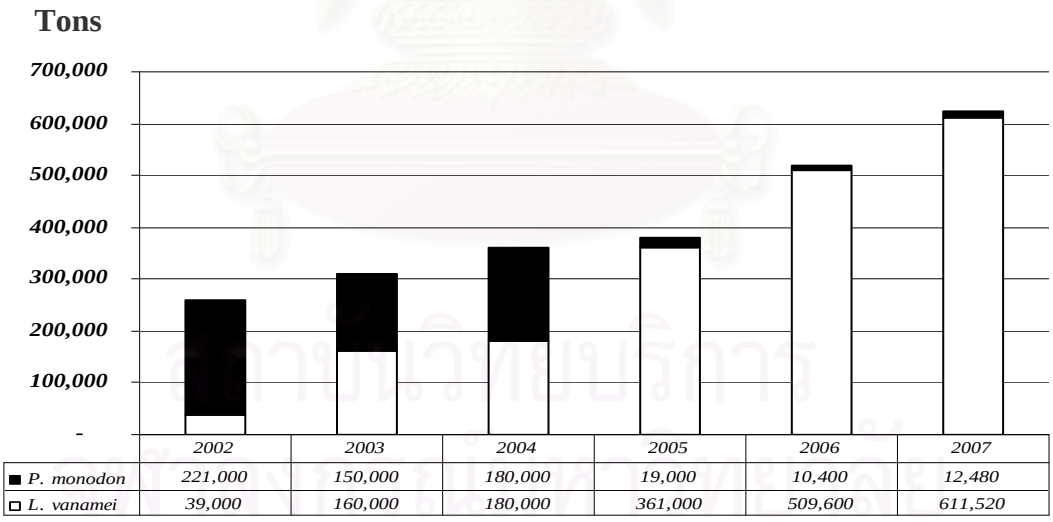

Figure 1.1 Thai shrimp production between 2002 and 2006 and the prediction in 2007 [Source: FAO Fishstat (2006)]

The advantages of *L. vannamei* include its rapid growth rate, tolerance of high stocking density, tolerance of low salinities and temperatures, lower protein requirements (and, therefore, the production costs), certain disease resistance (if specific pathogen resistant stocks are used), and high survival during larval rearing. There are, however, also disadvantages to the importation of *L. vannamei*, including its ability to act as a carrier of various exotic viral pathogens. *L. vannamei* is a known carrier of *Baculovirus penaei* (BP), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Reo-like virus (REO) and Taura Syndrome virus (TSV). These viruses can be transmitted to the native wild penaeid shrimp populations, and thus increases the concern over the spreading of diseases by the release of infected shrimps from culture facilities (Overstreet et al., 1997). Because the first signs of Taura Syndrome Virus (TSV) in Thai *L. vannamei* and *Macrobrachium rosenbergii* has been observed (Briggs et al., 2004), there is also the possibility of viral outbreak on the cultured *L. vanamei* as in Latin America. Although TSV is not reported to have affected indigenous cultured or wild shrimp populations, insufficient time and research have been conducted on this issue and there is a need for caution. TSV is a highly mutable virus, capable of mutating into more virulent strains capable of infecting the other shrimp species. In addition, some other viruses are probably imported along with the *L. vannamei*, for example, a new LOVV-like virus, have been implicated in causing the slow growth problems currently being encountered in the culture of indigenous *P. monodon*.

Due to the above problems, the native shrimp should be improved to gain resistance to the pathogens and rapid growth rate. Researches in *P. monodon* must be continued in various fields concerning the shrimp immunity, pathology, physiology and genetics, aquaculture, etc.

1.2 Taxonomy of *Penaeus monodon*

Penaeus monodon, the giant tiger shrimp, is a shrimp species that was classified into the largest phylum in the animal kingdom, the Arthropoda. The taxonomic definition of *Penaeus monodon* is as follows (Fast and Lester, 1992):

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Subclass Eumalacostraca
Order Decapoda
Suborder Natantia
Infraorder Penaeidea
Superfamily Penaeoidea
Family Penaeidae Rafinesque, 1985
Genus Penaeus Fabricius, 1798
Subgenus Penaeus
Species monodon

Scientific name: *Penaeus monodon* (Fabricius), 1798

Common name: Jumbo tiger prawn, Giant tiger prawn, Blue tiger prawn, Leader prawn, Panda prawn (Australia), Jar-Pazun (Burma), Bangkear (Cambodia), Ghost prawn (Hong Kong), Jinga (India, Bombay region), Udang windu (Indonesia), Ushi-ebi (Japan), Kamba ndogo (Kenya), Kalri (Pakistan), Sugpo(Philipines), Grass shrimp (Taiwan), Kung kula-dum (Thailand), Timsa (Vietnam).

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

1.3 Morphology

The shrimp body includes three regions: head, thorax, and abdomen (Figure 1.2). Each body region possesses appendages specialized for different functions. The head (five somites) and thorax (eight somites) are fused into a cephalothorax, which is completely covered by the carapace. Many internal organs, such as gills, digestive system, reproductive system and heart located in thorax are protected by carapace, while the muscles concentrate in the abdomen. The pleura of the cephalothorax form the branchiostegite or gill cover. The carapace has characteristic ridges (carinae) and grooves (sulci). The rostrum is always prominent, with a high median blade bearing dorsal teeth and, in some genera, ventral teeth as well. The compound eyes are stalked

and laterally mobile and the somites of the head bear, in order, pairs of antennules, antennae, mandibles, maxillae 1 and maxillae 2 (not visible in Figure1.2). The thorax has three pairs of maxillipeds and five pairs of pereopods (legs), the first three being chelate and used for feeding, and the last two simple (non-chelate) and used for walking. The abdomen consists of six somites, the first five with paired pleopods (walking legs) (Baily-Brook and Moss, 1992; Bell and Lightner, 1988) and the sixth with uropods. The mouth is situated ventrally and the cephalic appendages surrounding it, plus the first and second maxillipeds and sometimes the third as well, may be referred to collectively as the "mouth parts". The anus is on the ventral surface of the telson towards its base (Dall et al., 1990).

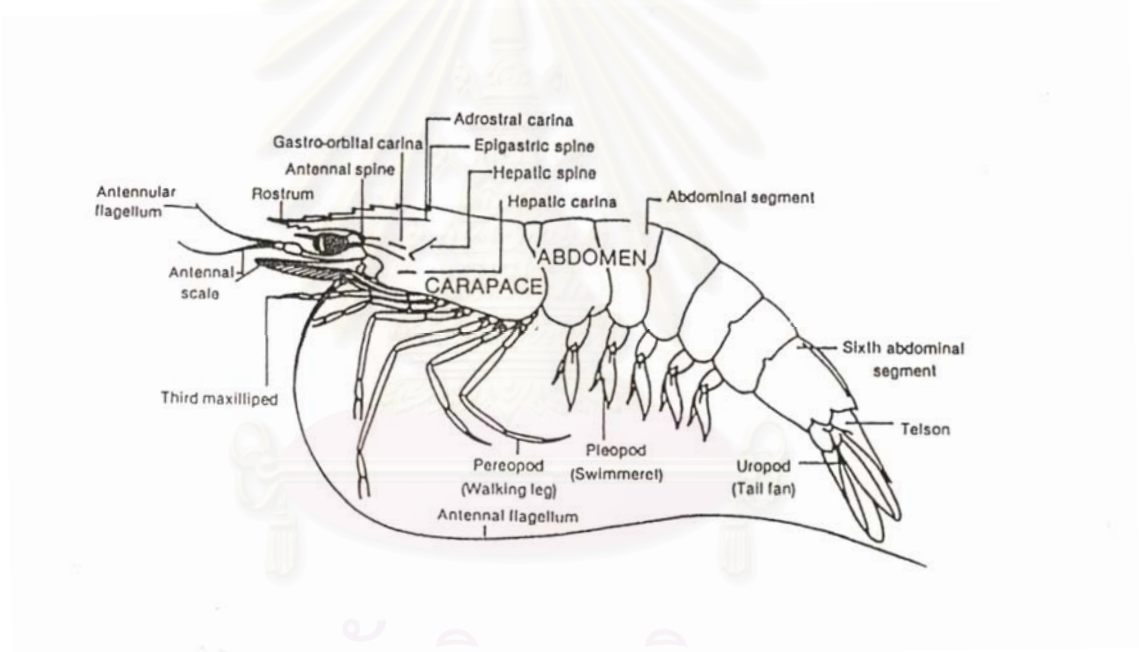

Figure 1.2 Lateral view of the external morphology of *Penaeus monodon* (Anderson, 1993).

The cuticle, which is secreted by an epidermal cell layer, consists of chitin and protein in which calcium carbonate and calcium phosphate have been deposited. The epidermis detaches from the inner cuticle layer and begins to secrete a new cuticle, while the old cuticle is molted. After molting the new cuticle is soft and is stretched to accommodate the increased sized of the shrimp.

The black tiger shrimp has the following characteristic coloration: carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In

shallow brackishwaters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Moton, 1981:cited in (Solis, 1988).

The internal morphology of penaeid shrimp is very well developed (Figure 1.3). Muscular, digestive, circulatory, respiratory, nervous, and reproductive systems are all present. Many sorts of muscles control movements of the body such as walking, crawling, burrowing, swimming, feeding, and breathing. The digestive system is complex, in which parts of the tract are differentiated into a foregut, a midgut, and a hindgut. The circulatory system consists of a heart, dorsally located in the cephalothorax, with branching arteries conducting blood to the various organs. Gills are responsible for respiration process. The nervous system consists of two ventral nerve cords, a dorsal brain, and a pair of ganglia for each somite. Hepatopancreas connects to the gastrointestinal tract via the primary duct. It occupies a large portion of the cephalothorax in penaeid shrimp and functions on absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leave the heart ends in the lymphoid organ where the haemolymph is filtered. This organ consists of two distinct lobes, each located ventro-lateral to the junction of the anterior and posterior stomach chambers. The lymphoid lobes are apposed slightly dorso-anterior to the ventral hepatopancreatic lobe. The haemocytes are produced in haematopoietic tissue. The hematopoietic tissue consists of densely packed lobules located at different parts of the shrimp anterior region. The first one is hematopoietic tissue surrounding the lateral arterial vessel, which joins the anterior recurrent artery at the base of the rostrum. The second one located within the first maxillliped. The third one is the second maxilliped hematopoietic tissue. The last one is epigastric hematopoietic tissue located dorsal to the anterior stomach chamber and just ventral to the dorsal cuticle. Lymphoid organ and haematopoietic tissue are shown in Figure 1.4.

1.4 Distribution and life cycle

The black tiger shrimp is mainly found throughout the Indo-Pacific region. Northern part of Indo-Pacific region ranges from Japan and Taiwan, Eastern region distributes toward Tahiti. For southern and western part, it distributes to Australia and

Africa, respectively. The development of penaeid shrimps includes several distinct stages in various habitats (Figure 1.5). Brackish shore is a preferable area for juveniles. The adults migrate to deeper offshore areas that have higher salinity, where mating and reproduction occur. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). Larvae hatching from the fertilized eggs pass through a series of moults and metamorphic stages before becoming adulting-like (juveniles). After one day, the eggs hatch into the first stage, nauplius. The nauplii feed on their egg-yoke reserves for a few days and develop into the protozoeae. Around 4-5 days, the protozoeae will metamorphose into mysis by feeding on algae. The mysis feed on algae and zooplankton and then develop into early postlarvae (PLs), which the

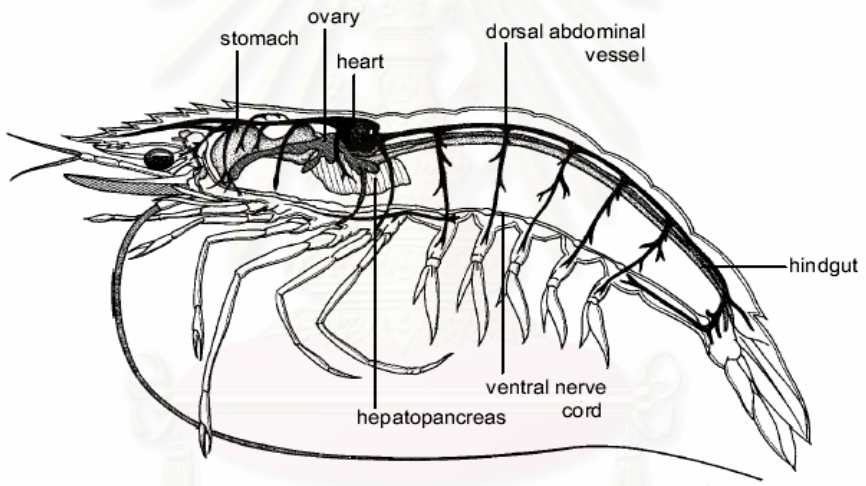

Figure 1.3 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990).

development time is 6-15 days (Solis, 1988). Transition from juvenile to subadult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months. The larvae inhabit plankton-rich surface waters offshore, with a coastal migration as they develop.

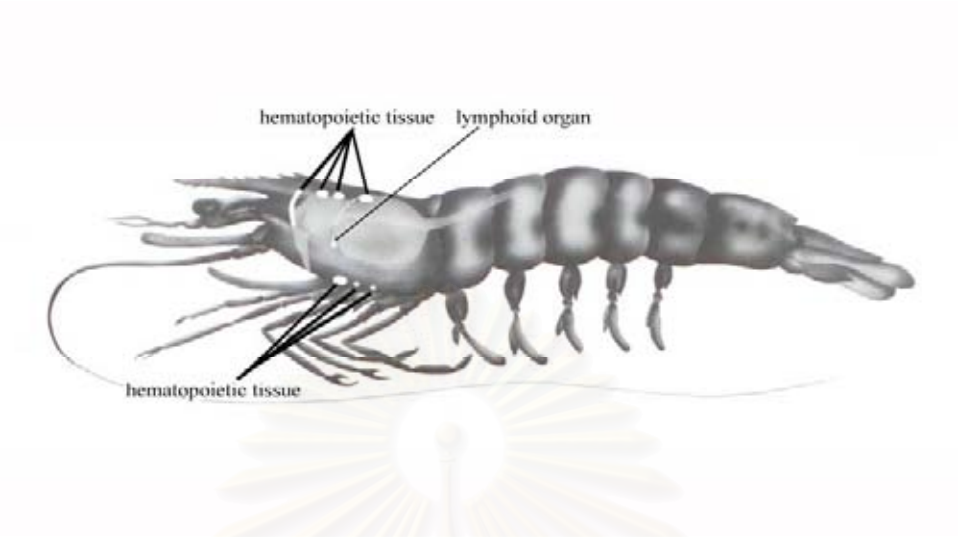

Figure 1.4 Location of hematopoetic tissue and lymphoid organ of penaeid shrimp.

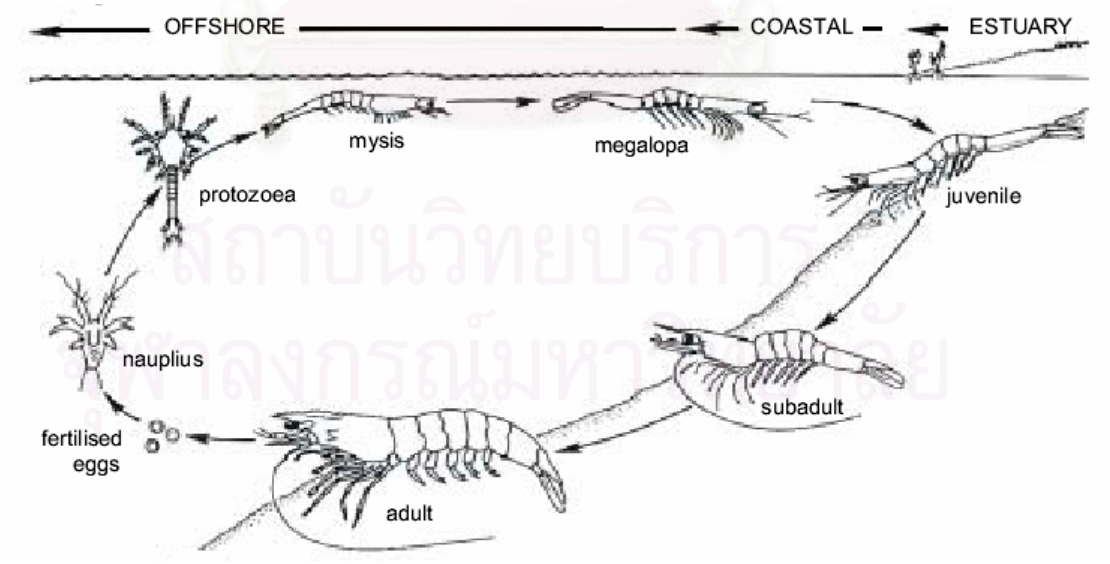

Figure 1.5 The life cycle of *Penaeus monodon* shrimp (Baily-Brook and Moss, 1992).

1.5 Shrimp diseases

Diseases are the biggest obstacle to the future of shrimp farming. Viral and bacterial outbreaks have decimated the industries in many countries. At present, white spot syndrome virus and bacteria in Vibrionacea family are considered significant causative agents of infectious diseases in Thailand. Diseases affect the economic viability and long-term sustainability of the shrimp farming industry. They cause great loss of shrimp production. Therefore, the prevention and control of diseases are a priority for shrimp production. To deal with this problem, besides the development of farm management, shrimp immunity and defense effectors responded to the pathogens should be elucidated.

1.5.1 Viral disease

The shrimp farming industry in Thailand has encountered a severe problem from viral infectious disease for over a decade. There are 7 families of viral pathogens including Parvoviridae, Baculoviridae, Iridoviridae, Picornaviridae, Rhabdoviridae, and Togaviridae identified in penaeid shrimp (Jittivadhna, 2000). Four major viruses, in order with the greatest to the least economic impact, are white spot syndrome virus (WSSV), yellow head virus (YHV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvo-like virus (HPV) and monodon baculovirus (MBV). HPV, IHHNV and MBV infections are related to the impeding of shrimp growth (Chayaburakul et al., 2004; Sukhumsirichart et al., 2002). The virus species that cause high mortality of *P. monodon* are WSSV and YHV, causative agents of white spot syndrome (WSS) and yellow-head (YH) diseases, respectively (Kiatpathomchai et al., 2004; Mohan et al., 1998; Sithigorngul et al., 2000; Soowannayan et al., 2003; Sukhumsirichart et al., 2002; Wongteerasupaya et al., 2003). The outbreak of these viruses results in a great deal of loss in the shrimp industry in several countries including Thailand.

1.5.2 White spot syndrome (WSS)

White spot syndrome (WSS) is one of the most important viral diseases, which affects most of the commercially cultivated marine shrimp species, not just in Asia but globally (Chou et al., 1995; Flegel, 1997; Lightner, 1996; Lotz,

1997; Span, 1997). Lightner (1996) called this virus white spot syndrome baculovirus (WSSV). This virus belongs to a new virus family, the Nimaviridae, and the genus *Whispovirus* (Mayo, 2002; van Hulten et al., 2001b). WSSV has a large circular double-stranded DNA genome of 292,967 bp (van Hulten et al., 2001a); however, different genome sizes have been reported from diverse virus isolates (Chen et al., 2002). The virion contains one nucleocapsid with a typical striated appearance and five major and at least other 39 structural proteins (Huang et al., 2002; Sanchez-Martinez et al., 2007; Tsai et al., 2006; van Hulten et al., 2001a; van Hulten et al., 2001b). It is believed that the envelop proteins play important roles in virus infection. Therefore, many researches have now focused on the analyses of envelop proteins (Wu et al., 2005; Zhang et al., 2004).

The affected shrimp exhibit white patches or spots on the inside surface of the carapace and the shell accompanied by reddish to pinkish red discoloration of the body. The disease affects the on-growing juvenile shrimp of all ages and sizes. Histological changes observed from the affected shrimp show wide spread cellular degeneration and severe nuclear hypertrophy in most tissue derived from ectodermal and mesodermal origin, especially from subcuticular shell epithilum, gill epitelium, sublcuticular stomach epithelium, lymphoid organ, connective tissue, hematopoietic tissue and nervous tissues (The ASEAN Fisheries Sub-Working group, 1998). The other clinical sign of this disease is a rapid reduction in food consumption of shrimp. Mortality was low during the initial two to three days. Mass mortality occurs within seven to ten days of the first clinical signs. Very few of the WSSV-infected shrimp survived the disease. Shrimp, which survive, are suspected to be life-long carriers of WSSV. Nevertheless, mechanism of WSSV infection is still unknown. Recently, WSSV envelope protein VP187 was found to interact with the shrimp intergrin. It is believed that β-integrin may function as cellular receptor for WSSV infection (Li et al., 2007).

To minimize damage from WSSV infection, detection of virus at an early stage is necessary. Several diagnostic methods have been described such as PCR (Tapay et al., 1999), in situ hybridization (Wang et al., 1998), miniarray (Quere et al., 2002), observation of tissues subjected to fixation or negative staining (Inouye et al., 1994), immunological methods using monoclonal and polyclonal antibodies to WSSV

or their component proteins and recently developed method (Poulos et al., 2001), a reverse passive latex agglutination (RPLA) method (Okumura et al., 2005).

Recently, the WSSV subunit vaccine, the WSSV envelop proteins VP19 and VP28, had been tested. The VP19 and VP28 fused to maltose binding protein (MBP) gave significantly better protection against infection (Witteveldt et al., 2005). The other researches to control of WSSV infection is the use of antiviral proteins. A 170-amino acid peptide with a C-type lectin-like domain from a *PmAV* cDNA of WSSV-resistant *P. monodon* was shown to possess a strong antiviral activity in virus infected fish cell line (Luo et al., 2003). In 2004, Zhang and co-workers showed that hemocyanin from the *P. monodon* had non-specific antiviral properties. In addition, interferon-like protein from hemocytes of the virus-resistant *M. japonicus* was found to have also the non-specific antiviral ability by inhibiting the SGIV (grouper iridovirus) in GP cells (grouper embryo cells) (He et al., 2005).

1.5.3 Bacterial disease

Species of *Vibrio* are commonly found in marine environments as the bacterial flora and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). They exist in high number in both the water and sediment of shrimp ponds, especially in an intensive culture system (Direkbusarakom et al., 1998). The virulence of this species has been recognized in a small but expanding list of cultured marine animal particularly penaeids in Asia and Australia (Vandenberghe et al., 1998). Disease outbreaks attributed to *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus* and *V. penaeicida* have been observed in nursery or grow-out ponds of *P. monodon*, *L. vannamei*, *P. japonicus* and *P. stylirostris* (Saulnier et al., 2000). Disease signs range from localized cuticular lesions, oral and enteric infections to septicaemia (Lightner, 1996). The gross signs of Vibriosis are light or dark brown focal lesion and necrosis of appendage tips. Melanin produced by the host hemocytes involved in the inflammatory process causes the change of color. Affected shrimp exhibit decreased appetite. Some also have a darker, larger of shrunken hepatopancreas.

The bioluminescent marine bacterium, *V. harveyi*, is considered as the most devastating pathogen that causes extreme loss of cultured *P. monodon* in

hatcheries and shrimp farms. This bacterial outbreak causes mortality of the affected shrimps up to 100%, whether they are larvae, post-larvae, juveniles, sub-adults or adults (Lightner, 1983). *V. harveyi* is a rod shape, Gram-negative bacterium with 0.5-0.8 μm width and 1.4-2.6 μm in length. Presumptive diagnosis is made on the basis of clinical sign and culture of the suspension of hepatopancreas or blood on tryptic plate supplemented with 2% (w/v) NaCl. After incubation at 30 °C overnight, colonies of *V. harveyi* grow and show strong luminescence in dim light.

For sometimes, the antibiotic was used to control the bacterial pathogen and had caused problem of antibiotic resistance. The use of probiotics, a marine bacterial strain *Pseudomonas* I-2, which can produce a compound with inhibitory property against *Vibrio* spp. is an interesting strategy for controlling the shrimp pathogenic *Vibrio* spp. in cultured system (Chythanya et al., 2002). Another potential probiotic bacterium is *Bacillus subtilis* BT23 whose cell free extract has inhibitory effect on the growth of *V. harveyi* (Vaseeharan and Ramasamy, 2003).

1.6 The invertebrate immune system

The invertebrates accounted for at least 95% of animals live in different environments. For defending themselves against microbial attack or other foreign substances, they have adopted their own immune system. The immune system can be divided into two types, namely adaptive or acquired and innate or natural immunity. Vertebrates possess both adaptive and innate immune system, whereas the invertebrates have only innate immunity. The adaptive immune system produces receptors which recognize specific antigens, namely antibodies. The system can memorize foreign molecules following the first time of exposure. The innate immune system involves a large number of generalized effectors molecules.

The innate immunity is essential for the first line of defense and interprets the biological context of antigens and instructs the adaptive immune system to make the appropriate antibody or T-cell response (Medzhitov and Janeway, 1999). Phylogenetic analysis of the protein families functioning in the invertebrate and vertebrate immunities suggests an evolutionary discontinuity between vertebrate and invertebrate innate immunity. The question is how invertebrates survive in a

pathogen-laden environment without an adaptive immune system. The answer is that the innate immune system is adequate. It includes phagocytosis, nodulation and encapsulation, synthesis of antimicrobial pepteides (AMPs) and activation of proteolytic cascades that lead to melanization, blood coagulation, release of stress-reponsive proteins and molecules believed to function in opsonization and iron sequestration (Jiravanichpaisal et al., 2006). The innate immunity can distinguish between self and non-self. They recognize non-self through a series of pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). PRRs recognize conserved pathogen-associated molecular patterns (PAMPs), which are essential and unique components of virtually all microorganisms, but absent in higher organisms.

The powerful model for studying on invertebrate innate immunity is the dipteran insect *Drosophila melanogaster*. The efficacy of *Drosophila* host defense results from the recognition of various classes of infectious microorganisms and selective activation of intracellular signaling cascade. The signaling pathways, the Toll pathway and the immuno-deficiency (IMD) pathway control the AMP gene expression upon microbial infection (Hetru et al., 2003). The current complete genomic data of *D. melanogaster* combining with new technologies on biochemistry, genetics, and molecular biology will facilitate study on innate immunity.

1.7 The crustacean immune system

In crustacean, the innate immune system is based on cellular and humoral components of the circulatory system. The hard cuticle which covers all external surfaces of the crustaceans is the first line of defense between them and the environment. However, the innate immunity responses rapidly if microorganisms can invade the animals. The major defense system of crustacean is carried in the hemolymph, which contains cells called hemocytes. Hemocytes and plasma proteins recognize a large number of pathogens by means of common molecular patterns of particular microbes. The molecules being recognized including the lipopolysaccharides (LPS) of Gram-negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram-positive bacteria, the mannans of yeast, the β–1,3-glucan of fungi and double-stranded RNA of viruses (Hoffmann et al., 1999), may interact with and activate the hemocytes. Several pattern recognition proteins (PRPs)

have been isolated and characterized in several invertebrates (Lee et al., 2004; Lee and Soderhall, 2001). Some PRPs are lectins which can promote the agglutination. Therefore, they are likely to have a potential role in invertebrate non-self-recognition reactions. In crustaceans, examples of PRPs are lectins (Kopacek et al., 1993; Vargas-Albores et al., 1996) and β–1,3-glucan binding protein (BGBP) in many crustaceans. The crustacean BGBPs were also found in freshwater crayfish, *Pacifastacus leniusculus* (Duvic and Soderhall, 1992) and three marine shrimp species, *P. californiensis* (Vargas-Albores et al., 1996), *L. vannamei* (Jimenez-Vega et al., 2002; Vargas-Albores et al., 1997) and *P. monodon* (Sritunyalucksana et al., 2002). Hemocyte activation results in the release of various types of the immune effectors, such as antimicrobial peptides, coagulation factors, proteinases and proteinase inhibitors, which function to elimination the invading pathogens. The current knowledge of crustacean immunity was summarized in Figure 1.6.

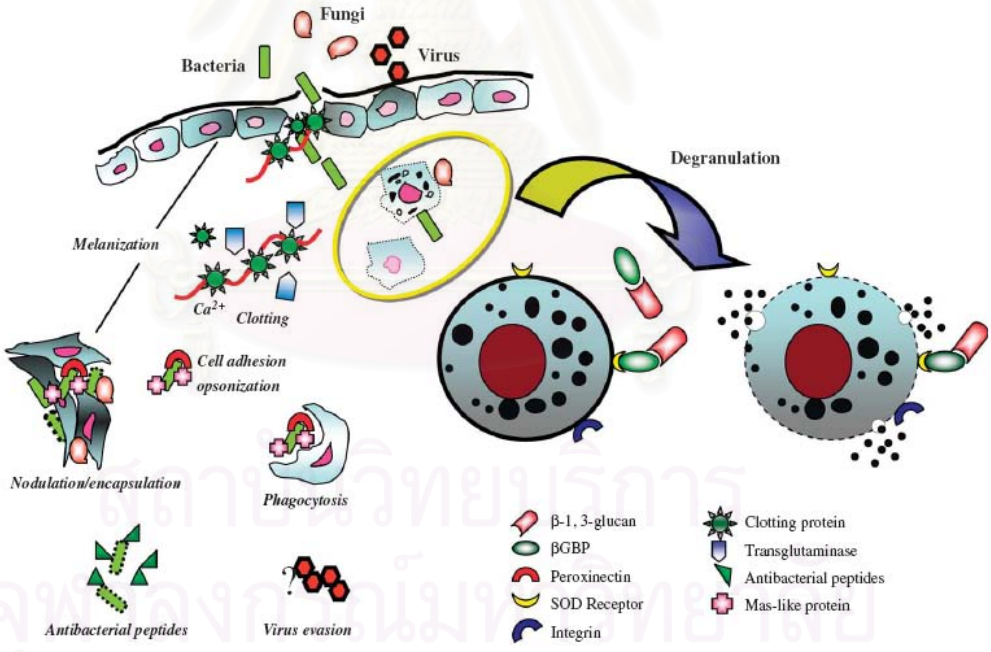

Figure 1.6 An illustration of the innate immune reactions (Jiravanichpaisal et al., 2006)

1.8 The prophenoloxidase activating system (proPO system)

The proPO system is an important part of the innate immunity, and contains several proteins that are involved in melanization, cytotoxic reactions, cell adhesion encapsulation and phagocytosis. This system is triggered by the presence of minute amounts of microbial components such as LPS and peptidoglycans (PGN) from bacteria and β-1,3 glucans from fungi. The microbial components are recognized by the pathogen-associated molecular patterns (PAMPs) including LPS and β-1,3 glucan binding protein (LGBP), β-1,3 glucan binding protein (βGBP) and peptidoglycan binding protein (PGBP). Moreover, this system is composed of several serine proteases and their zymogens and proPO as well as proteinase inhibitors which are important regulatory factors to avoid activation of system where it is not appropriate (Cerenius and Soderhall, 2004). According to the proPO system in crayfish, when βGBP binds to β-1,3 glucan, it becomes activated and binds specifically to a cell-surface associated protein, a superoxide dismutase (SOD) (Johansson, 1999) or binds to a β-integrin on the hemocyte surface through its RGD motif (Arg–Gly–Asp) The recognition of non-self triggers degranulation of the semigranular cells (SGCs) and the granular cells (GCs). Among the released proteins are components of the proPO activating system, for example the proprophenoloxidase activating enzyme (proppA), which is further activated to ppA by the presence of PAMPs. Active ppA converts the proPO to PO (monophenol dihydroxyphenylalanine: oxidoreductase; EC1.14.18.1). PO is a bifunctional copper containing enzyme, which is known as a tyrosinase and catalyzes the early steps in the pathway to melanin formation. The active enzyme catalyzes the oxygenation of monophenols to *o*-diphenols and further the oxidation of *o*-diphenols to *o*-quinones and eventually the synthesis of melanin. In addition, PO is also important for pigmentation, sclerotisation in many tissues, wound healing, and encapsulation of foreign materials (Jiravanichpaisal et al., 2006).

Another function of ppA is found in crayfish whose ppA contains one clip domain. Its clip domain contains homologous amino acid sequences to the horseshoe crab-derived big defensin and mammalian β-defensin. The recombinant clip-domain of ppA has antibacterial activity in vitro against Gram-positive bacteria suggesting a dual function of the crayfish ppA which may be true also for other ppAs (Wang et al., 2001). Since this part of the ppA is cleaved during the proteolytic processing of pro-

ppA into ppA, it is possible that the antimicrobial activity is produced concomitantly with proPO activation (Jiravanichpaisal et al., 2006).

1.9 Antimicrobial peptides or proteins

Antimicrobial peptides (AMPs) are effector molecules that play an important role in innate immune system. In all kingdoms, from bacteria to human, a wide variety of AMPs have been identified and characterized (Bulet et al., 1999; Hancock and Diamond, 2000). AMPs share common biochemical features such as small size, generally less than 150-200 amino acid residues, amphipathic structure and cationic property. However, the anionic peptides also exist. AMPs have wide variety and diversity in amino acid sequences, structure, and range of activity. AMPs are active against a wide spectrum of microorganisms such as bacteria and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Hancock and Diamond, 2000; Murakami et al., 1991; Pan et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991). In arthropods, a broad activity of AMPs against bacteria and filamentous fungi, have been reported in insects, horseshoe crab, and shrimp (Bachere et al., 2004; Bulet et al., 1999; Vizioli and Salzet, 2002). Moreover, depending on their distribution, antimicrobial peptide expression appears to be regulated by different tissue-specific pathways and these effectors may consequently participate in either a local or a systemic reaction. Some AMPs are constitutively expressed within secretory cells, others are induced upon microbial stimulation (Hancock and Diamond, 2000). In 2004, Bulet et al. evaluated that over 1000 AMPs had been isolated and characterized from multicellular organisms at the level of their primary structure and most of them had been identified in insects.

AMPs are diverse group of molecules, which can be divided into sub-groups on the basis of their amino acid compositions and structures: (i) anionic peptides including maximin H5 from amphibians, small anionic peptides rich in glutamic and aspartic acids from sheep, cattle and human; (ii) linear cationic α–helical peptides including cecropin, magainin, pleurocidin, CAP18 and LL37; (iii) cationic peptides enriched for specific amino acids including bactenecins, hymenoptaecin, coleoptercin and indolicidin; (iv) anionic and cationic peptides that contain cysteine and form disulfide bonds including defensin, protegrin and brevinin; and (v) anionic and

cationic peptide fragments of larger proteins including lactoferricin and casocidinI (Brogden, 2005).

Two mechanisms of antimicrobial activity of AMPs have been proposed: transmembrane pore-forming and intracellular killing (Brogden, 2005). There are three proposed models of transmembrane pore-forming mechanisms that explain peptide insertion and membrane permeability (Figure 1.7): (i) the barrel-stave model - the attached peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions align with the lipid core regions and the hydrophilic peptide regions form the interior region of the pore; (ii) the carpet model - the peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive layer or carpet; (iii) the toroidal model - the attached peptides aggregate and induce the lipid monolayers to bend continuously through the pore so that the water core is in line by both the inserted peptides and the lipid head groups.

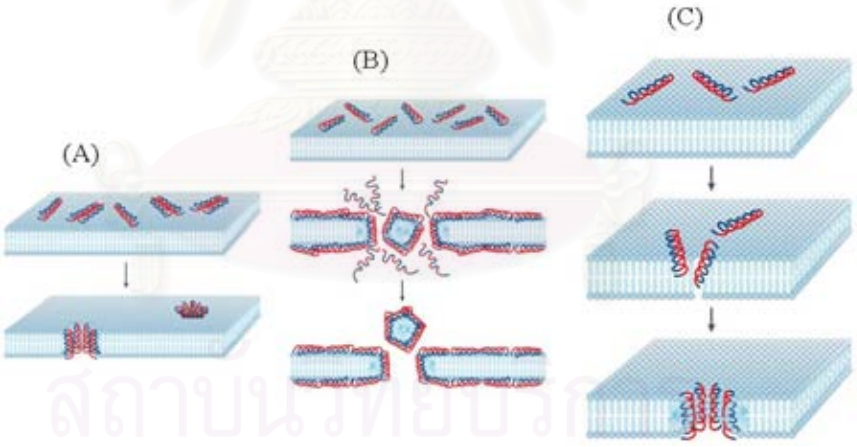

Figure 1.7 Transmembrane pore-forming mechanisms of antimicrobial-peptide-induced killing. Hydrophilic regions of the peptide are shown colored red, hydrophobic regions of the peptides are shown colored blue (Brogden, 2005).

(A) The barrel-stave model,

(B) The carpet model, and

(C) The toroidal model.

Although the formation of ion channels, transmembrane pores and extensive membrane rupture eventually leads to lysis of the microbial cells, there is increasing speculation that these effects are not the only mechanisms of microbial killing. There is increasing evidence indicating that antimicrobial peptides have other intracellular targets (Figure 1.8), for example, inhibition of cell wall synthesis, alteration of cytoplasmic membrane (inhibition of septum formation), activation of autolysin, binding to DNA, inhibition of DNA, RNA, protein syntheses and inhibition of enzymatic activity.

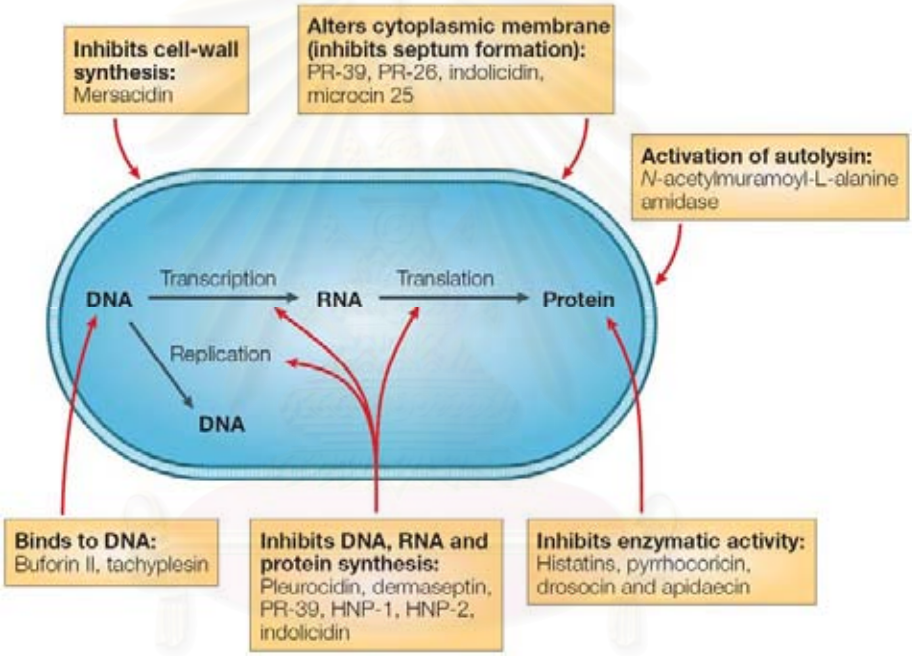

Figure 1.8 Mode of action for intracellular antimicrobial peptide activity. In the figure, *Escherichia coli* is shown as the target microorganism (Brogden, 2005).

In arthropods, several antimicrobial peptides were isolated and characterized, mainly in insects especially *Drosophila* and chelicerates (horseshoe crabs) (Iwanaga et al., 1998). In horseshoe crabs, these proteins are mainly synthesized in hemocyte and are stored within the cytoplasmic granules. The cells are highly sensitive to LPS, a major outer membrane component of Gram-negative bacteria, and respond by degranulating these granules after stimulation by the LPS. This system differs from that described in insects, where the fat body is the main site for the antimicrobial peptide synthesis (Engström, 1999; Hoffmann and Reichart, 1997), and upon injury

antimicrobial peptide gene transcription is induced, resulting in their immediate synthesis and subsequent secretion into the blood circulation.

There are few reports on antimicrobial peptides in crustaceans. Tachyplesin family and anti-LPS factors which act against Gram-negative bacteria are observed in horseshoe crab (Aketagawa et al., 1986; Muta et al., 1990; Nakamura et al., 1988; Ohashi et al., 1984). In 1997, a small peptide named callinectin was reported to be responsible for the majority of antimicrobial activity observed in the hemolymph of blue crab, *Callinectes sapidus* (Khoo et al., 1999) and penaeidins, a family of antimicrobial peptide which acted against Gram-positive bacteria and fungi were reported in many penaeid shrimp including *L. vannamei* (Destoumieux et al., 1997), *P. japonicus* (Rojtinnakorn et al., 2002), *L. setiferus* (Cuthbertson et al., 2002), *L. stylirostris* (Munoz et al., 2004), *P. monodon* (Supungul et al., 2004). These peptides contain both a proline rich domain at the N-terminal and a C-terminal domain containing the six cysteines which form 3 disulfide linkages. The cDNA clones of penaeidin isoforms were also isolated from the hemocytes of *L. vannamei* and *L. setiferus* (Gross et al., 2001) and *P. monodon* (Supungul et al., 2004). A cysteine-rich 11.5 kDa antibacterial protein was purified and characterized from hemolymph of shore crab, *Carcinus maenas* (Relf et al., 1999). Crustins, antimicrobial peptide homologues of an 11.5 kDa antibacterial peptide were identified from two species of *Penaeid* shrimp, *L. vannamei* and *L. setiferus* (Bartlett et al., 2002; Vargas-Albores et al., 2004). Astadidin1 produced by a proteolytic cleavage of hemocyanin inhibits growth of both Gram-positive and Gram-negative bacteria (Lee et al., 2003). Peptides derived from the hemocyanin of *L. vannamei and P. stylirostris* exhibited antifungal activity (Destoumieux-Garzon et al., 2001). Moreover, the hemocyanin of *P. monodon* with antiviral activity had been also identified (Zhang et al., 2004). Recently, the histones and histone derived peptides of *L. vannamei* had been reported as an innate immune effectors because they inhibited the growth of Gram-positive bacteria (Patat et al., 2004).

Animal and plant organisms possess an innate immune system for immediate protection from invading foreign microorganisms. Besides many other peptides and proteins in the system, antimicrobial peptides (AMPs) are one of the key elements (Bulet et al., 1999). The AMPs have broad spectra of antimicrobial activity, an ability

to kill or neutralize Gram-negative and Gram-positive bacteria, fungi, parasites and viruses (Hancock and Scott, 2000). Lacking the adaptive immunity, crustaceans as well as the shrimp rely on only the innate immunity. Therefore, the AMPs are crucial for them to fight the pathogenic invasion. The AMPs reported in shrimp include anti-lipopolysaccharide factor (ALF), crustin, lyzozyme and penaeidin. 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).

1.10 Anti-lipopolysaccharide factor

The anti-lipopolysaccharide factor (ALF) was originally isolated from the hemocytes of horseshoe crabs, *Tachypleus tridentatus* (TALF) and *Limulus polyphemus* (LALF) (Tanaka et al., 1982). Thereafter, the ALF was identified and characterized in many shrimp species including *Penaeus monodon* (Supungul et al., 2002), *Litopenaeus setiferus* (Gross et al., 2001), *Fenneropenaeus chinensis* (Liu et al., 2005), *Marsupenaeus japonicus* (Nagoshi et al., 2006), *Pacifastacus leniusculus* (Liu et al., 2006) and *Litopenaeus vannamei* (de la Vega et al., 2008). Recently, an ALF from *Scylla paramanosian* was characterized and shown to be phylogenetically related to the ALFs from the horseshoe crabs, shrimps and crabs (Imjongjirak et al., 2007).

The function of the ALF lies on the positively charged cluster within a disulfide loop in its primary structure as suggested by Aketagawa et al. (1986). From the three-dimensional structure of LALF, the amphipathic disulfide loop binds the lipid A and neutralizes the biological effects of LPS (Hoess et al., 1993; Pristovsek et al., 2005). The ALFs from horseshoe crabs, shrimps and crabs also contain the two highly conserved-cysteine residues and the highly conserved cluster of positively charged residues within the disulfide loop. The synthetic disulfide loops from ALF*Pm*3 and ALF*Sp* has been shown unequivocally to be antimicrobial active, though the actual mechanism is not known (Imjongjirak et al., 2007; Somboonwiwat et al., 2005). Recently, the structure of ALF*Pm*3 was published in Protein Data Bank as shown in Figure 1.9.

The ALF is a small basic protein that has the ability to inhibit the endotoxin or lipopolysaccharide (LPS) mediated coagulation system and, thus, exhibits strong anti-bacterial activity against the Gram-negative bacteria in particular (Morita et al., 1985). Other ALFs from shrimp (ALF*Pm*3, *Lv*ALF) and mud crab (ALF*Sp*) have much broader anti-microbial activity against bacterial and fungal (de la Vega et al., 2008; Imjongjirak et al., 2007; Somboonwiwat et al., 2005). Another possibly role of the shrimp ALF as an antiviral molecule has been shown in *P. leniusculus* as it interferes with the replication of white spot syndrome virus (Liu et al., 2006). ALF RNA interferences (RNAi) experimert in crayfish showed that ALF can prevent crayfish from WSSV infection because ALF knockdown significantly resulted in higher rate of viral propagation.

Figure 1.9 Structure of an anti-lipopolysaccharide factor from shrimp and its possible lipid A binding site (PDB: 2JOB).

In *P. monodon*, ALF was identified from *Penaeus monodon* expressed sequence tag (EST) database (http://pmonodon.biotec.or.th) (Tassanakajon et al.,

2006). 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% to 65% amino acid homology with those of the horseshoe crab *Tachypleus tridentatus* and *Limulus polyphemus*. The nucleotide and amino acid sequences alignment revealed the existence of at least 5 different isoforms (ALF*Pm*1-5) (Supungul et al., 2004). They contain open reading frames of 252, 360, 369 396 and 465 bp coding for proteins of 84, 120, 123 132 and 155 amino acids, respectively. Furthermore, the alignment shows two groups of LPS binding sites. The first group consists of ALF*Pm*1 and 2. The others are ALF*Pm*3 and 4. The differences between LPS binding site of both groups probably correlate with their antimicrobial activity. It was suggested that ALF*Pm* does not arise from a single copy nuclear gene but may be encoded by 2 different loci (Supungul et al., 2004). ALF*Pm*3, the predominant isoform, has been expressed in the yeast *Pichia pastoris* expression system (Somboonwiwat et al., 2005) and the recombinant ALF*Pm*3 protein exhibits antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria and fungi. Immunohistochemistry revealed that the ALF*Pm*3 protein is primarily localized in shrimp hemocytes and the production of protein increases rapidly in the *V. harveyi* challenged shrimp suggesting a likely important function of the protein in defense against invading pathogens. ALF*Pm*3 was shown to be able to bind to Gram-negative and Gram-positive bacterial cells and their major cell wall components, lipopolysaccharide (LPS) and lipoteichoic acid (LTA), respectively (Somboonwiwat et al.).

1.11 Objectives of the dissertation

In this dissertation, we further characterized the *P.monodon* ALFs. The gene organization of different ALF isoforms was determined. The transcriptional levels of the major ALF isoforms were examined in the non-challenged and *V. harveyi*-challenged shrimp. To investigate the antimicrobial activity of ALF*Pm*2, the recombinant protein was over-produced in the yeast *Pichia pastoris* expression system. The recombinant protein was purified and further characterized its antimicrobial activity. The anti-WSSV activity of ALF*Pm*2 and 3 were also examined in the cell culture.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

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

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

Balance PB303-S (Mettler Teledo)

Biological safty Cabinets (Nuaire)

Biophotometer (Eppendrof)

Centrifuge 5804R (Eppendorf)

Centrifuge Avanti™ J-30I (Beckman Coulter)

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Thermo Electron Corporation)

Dry bath incubator (Major Science)

Gel document (Syngene)

GelMate2000 (Toyobo)

Gene pulser (Bio-RAD)

Hitrap SP HP (Amersham Biosciences)

Hoefer™ miniVE (Amersham Biosciences)

PD-10 Desalting column (GE Healthcare)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Microplate reader: FLUOstar OPTIMA (BMG Labtech)

Microscope eclipse TS100 (Nikon)

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

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

Real-time rotary analyzer: Rotor-Gene™ 3000 (Corbett Life Science)

Refrigerated incubator shaker (New Brunswick Scientific)

Thermal Cycler Matercycler Gradient (Eppendrof)

Touch mixer Model # 232 (Fisher Scientific)

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

2.1.2 Chemicals and reagents

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Amplicilin (Biobasic)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

Biotin (Sigma)

Boric acid, BH_3O_3 (Merck)

5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, X-gal (Fermentus)

Bromophenol blue (BDH)

Calcium chloride, $CaCl_2$ (Merck)

Chloroform, $CHCl_3$ (Merck)

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

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

Ethidium bromide (Sigma)

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

Formaldehyde, CH_2O (BDH)

G418-sulfate (USB)

GeneRuler™ 100bp DNA Ladder

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine, NH_2CH_2COOH (Scharlau)

Hydrochloric acid, HCl (Merck)

Isoamylalcohol, $C_5H_{12}O$ (Merck)

Isopropanol, C_3H_7OH (Merck)

Isopropyl-beta-D-thiogalactopyranoside, IPTG (Fermentus)

Maganese (II) chroride, $MnCl_2$ (Merck)

Magnesium chloride, $MgCl_2$ (Ajax)

Magnesium sulfate, $MgSO_4$ (Carlo Erba)

Methanol, CH_3OH (Merck)

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

2-mercaptoethanol, C_2H_6OS (Fluka)

0.22 μm millipore membrane filter (Millipore)

2-(*N*-morpholino)ethanesulfonic (Sigma)

Phenol:chloroform:isoamyl alcohol (Sigma)

Prestained protein molecular weight marker (Fermentus)

Potassium chloride, KCl (Ajax)

Potassium dihydrogen phosphate, KH_2PO_4 (Ajax)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

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

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

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

Sodium hydroxide, NaOH (Eka Nobel)

Ticine, $C_6H_{13}NO_5$ (National diagnostics)

TRIreagent (Molecular biology)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH2OH)_3$ (USB)

Tryptic soy broth (Difco)

Whatman 3 MM™ filter paper (Whatman)

Yeast nitrogen base without amino acid (Difco)

2.1.3 Enzymes

Advantage 2 Polymerase Mix (Clontech)

DNaseI (Promega)

*Dra*I (Biolabs, UK)

DyNazyme II DNA™ polymerase (Finnzymes, Finland)

*Eco*RI (Biolabs, UK)

Lyticase (Sigma)

*Not*I (Biolabs, UK)

Proteinase K (Sigma)

RNase A (Sigma)

T4 DNA ligase (Promega)

*Xho*I (Biolabs, UK)

2.1.4 Microorganisms

Escherichia coli strain JM 109

E. coli strain XL-I blue

E. coli strain 363

Salmonella thyphimurium

Klebsiella pneumoniae

Enterobacter cloacae

Erwinia carotovora

Micrococcus luteus

Bacillus megaterium

Pichia pastoris KM71

Staphylococcus aureus

Staphylococcus haemolyticus

Vibrio anguillarum

Vibrio harveyi 1526

2.1.5 Kits

GenomeWalker™ Universal Kit (Clontech)

GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich)

HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Genomics)

ImProm-II™ Reverse Transcription System (Promega)
QIAprep® Spin Miniprep Kit (QIAGEN)
QuantiTect SYBR green PCR Kit (QIAGEN)

2.1.6 Vectors

pGEM®-T Easy vector (Promega)
pPIC9K (Invitrogen)

2.2 Samples

Crayfish *Pacifastacus leniusculus* were purchased from Berga Kräftodling and were maintained in tanks with aerated running water at 10 ºC. Only intermolt healthy crayfish were used for the experiment. Freshwater crayfish were used for the preparation of cell culture. The hematopoietic tissue was isolated from the dorsal part of cardiac stomach of crayfish.

Black tiger shrimp *Penaeus monodon* juveniles (16 to 20 g body weight) were purchased from local market and separated into 3 groups. The first group was the unchallenged or normal shrimp. The second group was *P. monodon* experimentally injected with saline solution [0.85% (w/v) NaCl] as a control. The last group was *P. monodon* injected with live 10^3 CFU of *V. haveyi* 1526. All groups were acclimatized in aquaria at the ambient temperature (28 ± 4 °C) and the salinity of 15 ppt for at least 1 day before used in the experiments.

2.3 Genomic organization of ALF*Pm*2 and 3 genes

2.3.1 Preparation of *P. monodon* gemonic DNA

Genomic DNA was prepared from the pleopods of *P. monodon* using phenol-chloroform extraction. The pleopods were homogenized in 700 µl of extraction buffer (100 mM Tris buffer pH 8.0, 100 mM EDTA pH 8.0, 250 mM NaCl, 1% (w/v) SDS, and 100 µg/ml Proteinase K) and incubated overnight at 65 ºC. Then, 5 µg of RNase A were added into the lysate and incubated for 30 min at 37 ºC, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) by inverting and mixing for 20 min and centrifuged for 10 min at 5,000 rpm. The upper phase was transferred to a new tube, extracted with chloroform:isoamyl alcohol (24:1) by inverting and

mixing for 20 min and centrifuged for 10 min at 5,000 rpm. The upper phase was transferred to a new tube. The genomic DNA was precipitated with two volumes of cold absolute ethanol. The mixture was centrifuged at 5,000 rpm for 1 min. The genomic DNA was washed with 70% ethanol, air-dried, and then dissolved in 50 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0).

2.3.2 Quality of genomic DNA

The quality of genomic DNA was checked by 0.6% agarose gel electrophoresis. One µl of experimental genomic DNA (0.1 µg/µl) and 1 µl of control genomic DNA (0.1 µg/µl) were loaded and run on 0.6% agarose gel in 1× TBE buffer. The gel was stained with ethidium bromide and visualized under the UV light to estimate the size of product. A good genomic DNA preparation should contain DNA larger than 50 kb with minimum smearing.

2.3.3 Amplification of genomic ALF*Pm* genes

The primers, ALF*Pm*2F, ALF*Pm*2R, ALF*Pm*3F and ALF*Pm*3R (Table 2.1), designed from the cDNA sequences were used for the amplification of the corresponding genomic DNA sequences. The genomic DNA (50 ng) was used for the PCR amplification in 50 µl reaction containing 1 unit Advantage 2 Polymerase Mix (Clonetech), 1× Advantage 2 PCR buffer, 200 µM of dNTP, 0.2 µM each of the appropriate forward and reverse primers. The initial denaturation was at 94 ºC for 3 min, followed by 5 cycles of denaturation at 94 ºC for 45 sec, annealing at 65 ºC for 45 sec, elongation at 72 ºC for 2 min; 25 cycles of denaturation at 94 ºC for 45 sec, annealing at 55 ºC for 45 sec, elongation at 72 ºC for 2 min; and the final extension at 72 ºC for 10 min. The 5 µl of PCR products were analyzed on 1.5% agarose gel in 1× TBE buffer. The gel was stained with ethidium bromide and visualized under the UV light to determine the size of PCR product. The expected PCR product was purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Genomics). The expected DNA band was excised and removed extra agaorse to minimize the gel slice. The gel slice was added with 500 µl of DF buffer and mixed by vortexing. The

mixture was incubated at 55 ºC for 10-15 min with occasional inverting the mixture until the gel slice was completely dissolved. The sample mixture was loaded into the DF column and centrifuged at 13,000 rpm for 30 sec. The flow-through was discarded. The 500 µl of wash buffer was added to the DF column and centrifuged 13,000 rpm for 30 sec. After the flow-through was discarded, the wash buffer was added and repeated the previous step. Then, the column matrix was dried by centrifuge for 2 min. The dried DF column was transferred into a new microcentrifuge tube and added 30 µl of the elution buffer to the center of the column matrix. The column was allowed to stand for 2 min until the elution buffer was absorbed by matrix. The DF column was finally centrifuged at 13,000 rpm for 2 min. The eluted DNA was kept at -20 °C until used.

2.3.4 Preparation of competent cells

To prepare the starter inoculums, a single colony of either *E. coli* XL-1 blue or JM109 was cultured in 10 ml of LB broth [1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl] and incubated at 37 °C with shaking at 250 rpm overnight. One percent of the microbial starter was inoculated into 1,000 ml of LB broth and the culture was incubated at 37 °C with vigorous shaking for 3-5 h until the optical density at 600 nm (OD_{600}) of the culture reached 0.5-0.8. Cells were then chilled on ice for 15-30 min and harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. The supernatant was removed as much as possible. The cell pellet was washed by resuspending in a total volume of 1000 ml of cold sterile water, gently mixing and centrifugation. The pellet was washed further with different solutions, first with 500 ml of cold sterile water, followed by 20 ml of ice cold sterile 10% (v/v) glycerol, and resuspended to a final volume of 2-3 ml ice cold sterile 10% (v/v) glycerol. This cell suspension was divided into 40 µl aliquots and stored at -80 °C until used. The cells are good for at least 6 months under these conditions.

Table 2.1 Nucleotide sequences of primers for gene organization

Primer	Sequence (5'-3')
Primers for genomic amplification	
ALF*Pm*2F	CTCAGCCTGATTGCACTTATGCCACGGT
ALF*Pm*2R	CTGGCGTCTTCCTCCGTGATGAGATTAC
ALF*Pm*2AF	TGGTATAACATATATTTCGTATATCACG
ALF*Pm*2AR	ATTTATGATTTACAAATATCACTCTTGC
ALF*Pm*3F	CAAGGGTGGGAGGCTGTGG
ALF*Pm*3R	TGAGCTGAGCCACTGGTTGG
ALF*Pm*3AF	AAAAGGGCTCGCGAGGCCATTCGGGTC
ALF*Pm*3AR	CAAAAGAGCCTAAGGATCCTTCCAGGAC
Primers for genomic walking	
ALF*Pm*2GSP1R	ACCGTAAGATTAATGGGCAGTGGTGAC
ALF*Pm*2GSP2R	TGGCACCGTGGCATAAGTGCAATCAG
ALF*Pm*3GSP1R	TAGTACACCTGGAATCTCTTCAAATAAGGC
ALF*Pm*3GSP2R	TAAGTGCCACACCCTACGATCTTGCTG
ALF*Pm*3GSP1R2	ACTGAGTTGCCAGGAGCCTATTTAACG
ALF*Pm*3GSP2R2	GACGTGGAAACTATAGTAACAGCAACG
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT

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2.3.5 Cloning of the amplified genomic DNA fragments

The purified amplified genomic DNA fragments were cloned into the pGEM®-T Easy vector (Promega). The reaction component contained 5 µl of 2× rapid ligation buffer, 1 µl of pGEM®-T Easy Vector (50 ng), proper amount of PCR product to achieve 1:3 insert:vector molar ratio, 1 µl of T4 DNA ligase (3 units/µl), and deionized water to a final volume of 10 µl. The reactions were mixed by pipetting and incubated overnight at 4 °C.

To calculate the appropriate amount of insert for the ligation reaction, the following equation was used:

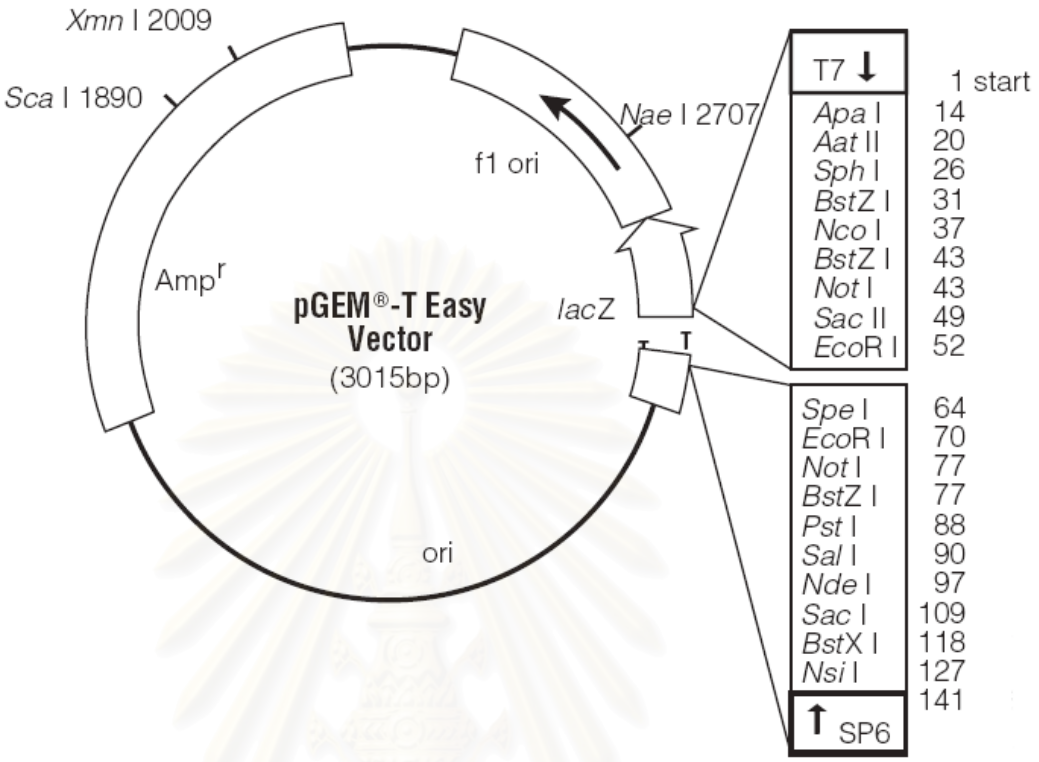
$$\frac{\text{ng of vector} \times \text{kb size of insert} \times \text{insert:vector molar ratio}}{\text{kb size of vector}} = \text{ng of insert}$$

Two microliters of each ligation reaction were added to 40 µl of *E. coli* strain XL-I blue competent cells. The reaction was mixed, placed on ice for 1 min and transferred to a cold elctroporation cuvette. The apparatus was set at 25 µF of the Gene pulser, 200 Ω of the Pulse controller unit, and 2.50 kV of the Gene pulser apparatus (Bio-RAD). After one pulse was applied at the above setting, one milliliter of SOC medium [2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose] was added to the cuvette and quickly resuspended the cells with a pasture pipette. The cell suspension was transferred to a microcentrifuge tube and incubated at 37 °C with shaking at 250 rpm for 1 h. One hundred microliters of each transformation was then plated onto a LB/ampicillin/IPTG/X-Gal plate.

2.3.6 Determination of insert size by colony PCR

After cloning of the PCR fragments, 4-10 recombinant clones were randomly selected for each DNA fragment and screened by colony PCR. The pGEM®-T Easy vector is 3,015 bp in length (Figure 2.1). This plasmid has unique restriction sites in the multiple cloning region flanked by T7 and SP6 RNA promoters, therefore T7 and SP6 primers can be used to amplify and analyze the size of the DNA insert cloned into the multiple cloning region. Colony PCR was performed in a 25 µl reaction volume containing 2.5 µl of 2.5 mM of dNTP mix, 0.5 µl of each 50 µM T7 and SP6 primers, 2 units of DyNAzyme™ II DNA polymerase. For the DNA template, a single colony was picked using a sterile toothpick and resuspended in the reaction mixture. The cycling parameters were one cycle at 95 °C for 2 min, 30 cycles at 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.2% agarose gel electrophoresis. The clone containing an expected size of insert was selected and the recombinant plasmid was isolated.

(A)



(B)

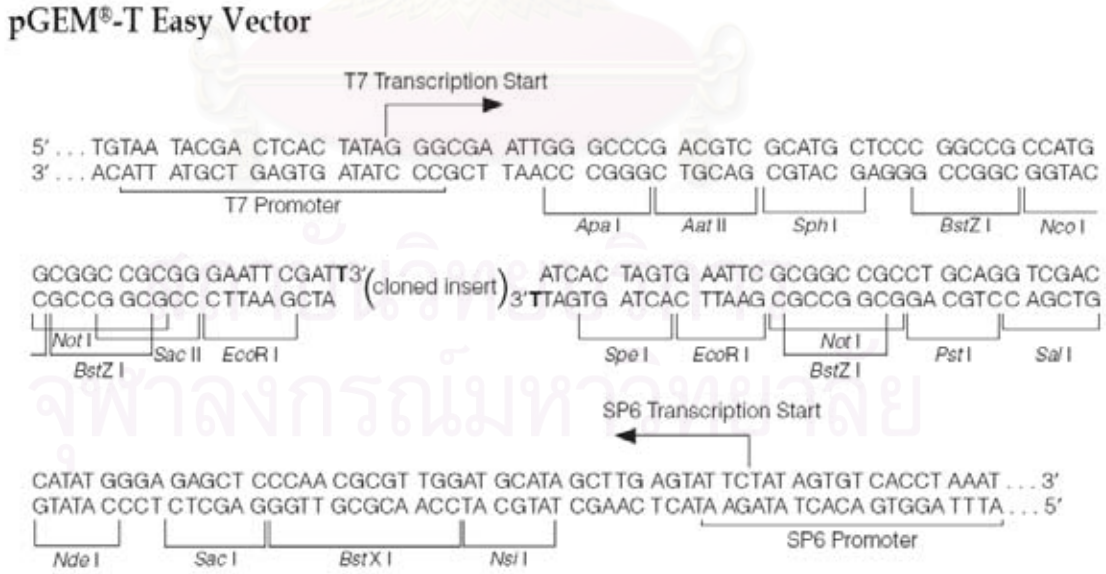


Figure 2.1 The circular map of the pGEM®-T Easy vector showing the sequence reference points (A) and the linear map showing the promoter and multiple cloning sites (B). (Source: www.promega.com)

2.3.7 Preparation of the recombinant plasmid

A colony was inoculated into 1.5 ml of LB broth and incubated at 37 °C with shaking overnight. The culture was transferred into a 1.5-ml microcentrifuge tube and spun at 8,000 rpm for 1 min. The plasmid was purified using QIAprep® Spin Miniprep Kit. The bacterial cell pellet was resuspended in 250 μl of Buffer P1, added 250 μl of Buffer P2 and mixed thoroughly by inverting the tube 4-6 times (the solution turned blue). Then, 350 μl Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4-6 times (the solution turned colorless). The mixture was centrifuged at 13,000 rpm for 10 min. The supernatant was applied to the QIAprep spin column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded. The column was washed by adding 0.5 ml Buffer PB and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded. The column was washed by adding 0.75 ml of Buffer PE and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep spin column was placed in a new 1.5-ml microcentrifuge tube, 50 μl Buffer EB was added to the center of QIAprep spin column, the column was let stand for 1 min and centrifuge at 13,000 rpm for 1 min to collect the DNA eluate. The DNA insert was sequenced by the service of Macrogen.

2.3.8 Genomic DNA library construction

Four genomic DNA libraries were constructed by digesting the genomic DNA separately with four blunt-end restriction enzymes. In each reaction, 2.5 μg of genomic DNA was digested in 100-μl reaction with 80 units of restriction enzyme (*Dra*I, *Eco*RV, *Pvu*II or *Stu*I) and 1× restriction enzyme buffer. The digestion mixtures were incubated for 2 h at 37 ºC. The reactions were then vortexed at slow speed for 5-10 min and incubated further at 37 ºC overnight. Each digestion reaction was analyzed by running 5 μl on 0.6% agarose gel electrophoresis to determine whether the digestion was completed.

Then, each reaction tube was added an equal volume (95 μl) of phenol and vortexed at slow speed for 5-10 sec. The mixture was briefly spun at room temperature to separate the aqueous and organic phases. The upper aqueous phase

was transferred into a new tube. The upper phase was then added an equal volume (95 µl) of chloroform and vortexed at slow speed for 5-10 sec. The mixture was briefly spun at room temperature to separate the aqueous and organic phases. The upper phase was transferred into a new tube. The upper phase was added two volumes (190 µl) of ice cold 95% ethanol, 1/10 volume (9.5 µl) of 3 M NaOAc (pH 4.5) and 20 µg of glycogen. The mixture was vortex at slow speed for 5-10 sec and centrifuged at 14,000 rpm for 15 min at 4 ºC. The supernatant was decanted and the pellet was washed in 100 µl of ice cold 80% ethanol. The supernatant was decanted and air-dried the pellet. The pellet was dissolved in 20 µl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5) and vortexed at slow speed for 5-10 sec. To determine the approximate quantity of DNA after purification, 1 µl of each reaction was run on 0.6% agarose gel electrophoresis.

The genomic DNA fragments from the four digestion reactions were ligated with GenomieWalker™ adaptors. For each ligation reaction, the digest was added to 1.9 µl of 25 µM GenomeWalker adaptor, 1.6 µl of 10× ligation buffer and 0.5 µl of T4 DNA ligase (6 units/µl). The reaction mixture was incubated overnight at 16 ºC. The reactions were stopped by incubating at 70 ºC for 5 min. Each reaction was added 72 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and vortexed at slow speed for 10-15 sec. The four libraries were named *Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries.

2.3.9 Determination of the 5' upstream sequences of ALF genes

To “walk” along the genomic DNA, two PCR amplifications were done for each library. The first or primary PCR uses the outer adaptor primer (AP1) provided in the kit and an outer, gene-specific primer (GSP1) designed from the known sequences. The primary PCR products were, then, used as template for the secondary or nested PCR using the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2). The secondary PCR product begins from the known sequence at the 5' end of GSP2 and extends into the unknown adjacent genomic DNA (Figure 2.2).

The two gene-specific primers (ALF*Pm*GSP1 and 2) were designed from the sequences close to the 5' end of the known genomic sequence. For primary

PCR amplification, 0.4 µl of each library was used in a 20 µl reaction volume containing 1 unit DyNAzyme II DNA polymerase (Finnzyms), 1× buffer, 0.2 mM of dNTP, 0.2 µM of AP1 and ALF*Pm*GSP1. The initial denaturation was at 94 ºC for 2 min, followed by 5 cycles at 94 ºC for 30 sec, and 65 ºC for 3 min; 25 cycles of denaturation at 94 ºC for 30 sec, annealing at 60 ºC for 30 sec, elongation at 72 ºC for 2 min; and the final extension at 72 ºC for 7 min. The primary PCR product was diluted 50 folds (1 µl into 49 µl) with water. The secondary PCR amplification used 0.4 µl of the diluted primary PCR product in a 20 µl reaction volume containing 1 unit DyNAzyme II DNA polymerase (Finnzyms), 1× buffer, 0.2 mM of dNTP, 0.2 µM of AP2 and ALF*Pm*GSP2. The initial denaturation was at 94 ºC for 2 min, followed by 35 cycles at 94 ºC for 30 sec, 68 ºC for 3 min, and 72 ºC for 7 min. The secondary PCR products were cloned into the pGEM®-T easy vector (Promega) and subsequently sequenced.

The primers ALF*Pm*2AF, ALF*Pm*2AR, ALF*Pm*3AF and ALF*Pm*3AR were designed to amplify the whole ALF genomic DNA segments containing both the 5' regulatory sequences and the genes. The PCR products were cloned, sequenced and analyzed in order to confirm the contiguous nature of the DNA fragments obtained from the genome walking technique.

The putative promoters, 5' *cis*-regulatory elements in the 5' upstream sequences and the putative start sites of the ALF genes were predicted by using the MATCH™/TRANFAC program (http://www.gene-regulation.com) (Biobase GmbH) and the Neural Network Promoter Prediction (http://www.fruitfly.org /seq_tools/promoter.html) (Reese, 2001), respectively.

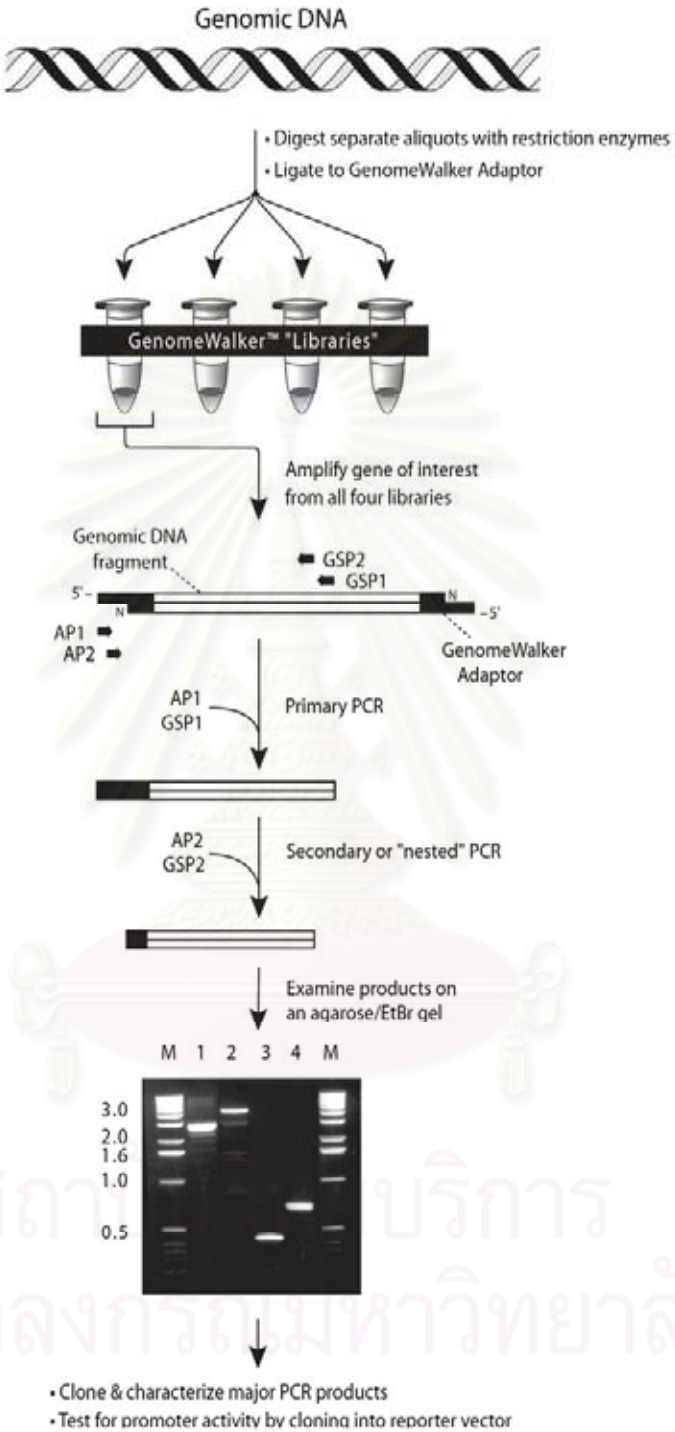


Figure 2.2 Flow chart of the BD GenomeWalker™ protocol. The genomic libraries were constructed for use as templates for nested PCR.
(Source: www.bdbiosciences.com)

2.4 Differential expression of ALF isoforms

2.4.1 Preparation of *V. harveyi* infected shrimp (Supungul, 2002)

A single colony of *V. harveyi* 1526 (kindly provided by Charoenpokaphand Group of Companies) was inoculated in the tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl and cultured at 30 °C for 12-16 h. The overnight culture was diluted (1:100) with the same medium and grown at 30 °C for 2 h. The culture was then diluted 1:1000 with a sterile 0.85% (w/v) NaCl solution. The titer of this dilution was monitored by plate count method using tryptic soy agar (TSA) supplemented with 2% (w/v) NaCl (modified from Austin, 1988). The 10^3 CFU diluted culture was intramuscularly injected into the forth abdominal segment, whereas the control group was injected with 100 μl of 0.85% (w/v) NaCl solution.

At 0, 6, 24, 48 and 72 h post-injection, hemolymph was collected and shrimp were tested whether the infection was successful by culturing the suspensions of hepatopancreas on TSA plates supplemented with 2% (w/v) NaCl at 30 °C overnight. Colonies of *V. harveyi* 1526 from infected shrimps should show strong luminescence in the dark.

2.4.2 Hemocyte collection and total RNA preparation

Hemolymph was collected from the ventral sinus of each shrimp using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 μl of anti-coagulant (10% sodium citrate, w/v). Hemolymph was immediately centrifuged at 800*g* for 10 min at 4 °C to separate hemocytes from the plasma. The hemocyte pellet was resuspended in 1 ml of Trizol reagent (Gibco BRL) and briefly homogenized. The homogenate was stored at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes. Then, 200 μl of chloroform was added and vigorously shaken for 15 sec. The resulting mixture was stored at room temperature for 2-5 min and centrifuge 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. RNA was precipitated by the addition of 500 μl of isopropanol. The mixture was left at room temperature for 5–10 min and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was removed. The RNA pellet was washed with 500 ml of 75% ethanol. The RNA pellet

was kept in 75% ethanol until used. When required, the samples were 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.

The concentration of total RNA was determined by measuring the OD_{260} and estimated in μg/ml using the following equation:

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

One OD_{260} unit corresponds to approximately 40 μg/ml of RNA (Sambrook et al., 1989).

2.4.3 First strand cDNA synthesis

The synthesis of first strand cDNA was performed using the ImProm-II™ Reverse Transcription System (Promega). The 2 μg of total RNA was mixed with the oligo$(dT)_{15}$, incubated at 70 ºC for 5 min and chilled on ice. Then, the reverse transcription reaction mix (4 μl of 5× reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of 10 mM each of the dNTP, 1 μl of ImProm-II reverse transcriptase and 20 units of RNasin) was added and gently mixed. The reaction was incubated at 25 °C for 5 min and at 42 °C for 60 min. Then, the reaction was incubated at 70 °C for 15 min to terminate reverse transcriptase activity.

2.4.4 RT-PCR

For PCR amplification, 0.5 μl of each cDNA preparation was used in a 15 μl reaction volume containing 0.3 unit DyNAzyme II DNA polymerase (Finnzymes), 1× buffer, 200 μM of dNTP, and 0.2 μM each of the appropriate forward and reverse primers (Table 2.2). The reactions were carried out using the following conditions: an initial denaturation step of 94 ºC for 2 min; followed by 21 cycles for EF-1 alpha, 27 cycles for ALF*Pm*2, 19 cycles for ALF*Pm*3, 24 cycles for ALF*Pm*4 and 36 cycles for the detection of two ALF isoforms simultaneously (ALF*Pm*1-2 and ALF*Pm*3-4) of 94 ºC for 30 sec, 50 ºC for 1 min and 72 ºC for 1 min; and the final extension phase of 72 ºC for 10 min. The PCR products of ALF genes were analyzed by electrophoresis using the 1.2% agarose gels. The band intensity of the PCR products were recorded and analyzed using the Genetools analysis software.

The ratios between the ALF*Pm* genes and the internal control (Elongation factor-1 alpha) were determined. Statistical analysis of the RT-PCR results was performed using the Independent Samples *t*-test as indicated in the results. Data differences were considered significant at $P<0.05$.

Table 2.2 Nucleotide sequences of primers for differential expression

Primer	Sequence (5'-3')
Primers for RT-PCR	
ALF*Pm*1RTF	CTCACGGAATTTCTCAGAAGTGCCAG
ALF*Pm*1RTR	TCACTCTTGCCTTAACCGTCTCAAC
ALF*Pm*2RTF	CAAGCGGTGCAGGACCTCC
ALF*Pm*2RTR	TTAGTGCTCAAGCCAAATCCTGG
ALF*Pm*3RTF	CAAGGGTGGGAGGCTGTGG
ALF*Pm*3RTR	TGAGCTGAGCCACTGGTTGG
ALF*Pm*4RTF	AGGGTGTGGCACTTACTCCATTCC
ALF*Pm*4RTR	GGAGGAGAACGGACGAACAAATTAG
EF-1alphaF	GGTGCTGGACAAGCTGAAGGC
EF-1alphaR	CGTTCCGGTGATCATGTTCTTGATG

2.5 Expression and characterization of anti-lipopolysaccharide factor

2.5.1 Recombinant expression of anti-lipopolysaccharide factor isoform 2 in *Pichia pastoris* expression system

The full-length cDNA clone of anti-lipopolysaccharide factor isoform 2 (ALF*Pm*2) was identified in *V. harveyi*-challenged *P. monodon* hemocyte cDNA library (Supungul et al., 2004). To characterize the ALF*Pm*2, the gene was subjected to express by *Pichia pastoris* methylotrophic yeast system (Invitrogen).

2.5.2 Construction of expression plasmid pALF*Pm*2

The *P. pastoris* multi-copy expression vector, pPIC9K (Figure 2.3), was chosen for the ALF*Pm*2 expression. It carries kanamycin resistance gene which

confers resistance to G418 in *P. pastoris*. Spontaneous generation of multiple insertion events, which occur in *P. pastoris* at a frequency of 1-10%, can be identified by the level of resistance to G418. *P. pastoris* transformants are selected on histidine deficient medium and screened for their level of resistance to G418. The increased level of resistance to G418 indicates multiple copies of the kanamycin resistance gene as well as the cloned gene. Increasing the copy number of the cloned gene in *P. pastoris* transformants increases the level of protein expression. The pPIC9K vector also allows the secretion of the expressed gene.

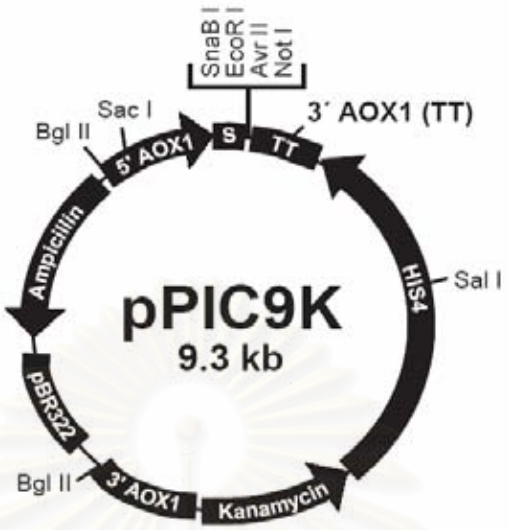
2.5.2.1 Primer design

To subclone the the ALF*Pm*2 gene into the pPIC9K, the forward and reverse primers were designed from the cDNA sequence encoded mature peptide of ALF*Pm*2 for the construction of an expression cassette. The *Xho*I and *Not*I sites were included at the 5' and 3' ends of forward and reverse primers, respectively, for the cloning into the *Xho*I/*Not*I sites of pPIC9K vector. The primer sequences were:

ALF*Pm*2YF: 5'-TCTCGAGAAAAGACAAGGCGTGCAGGACCTC-3'
ALF*Pm*2YR: 5'-AATTATTGCGGCCGCTTAGTGCTCAAGCCAAATC-3'

The primers were used to amplify the mature ALF*Pm*2 gene, cloned in-frame into the pPIC9K downstream of the sequence for the α-mating factor signal peptide from *Saccharomyces cerevisiae* and then transformed the *E. coli* JM109.

(A)



(B)

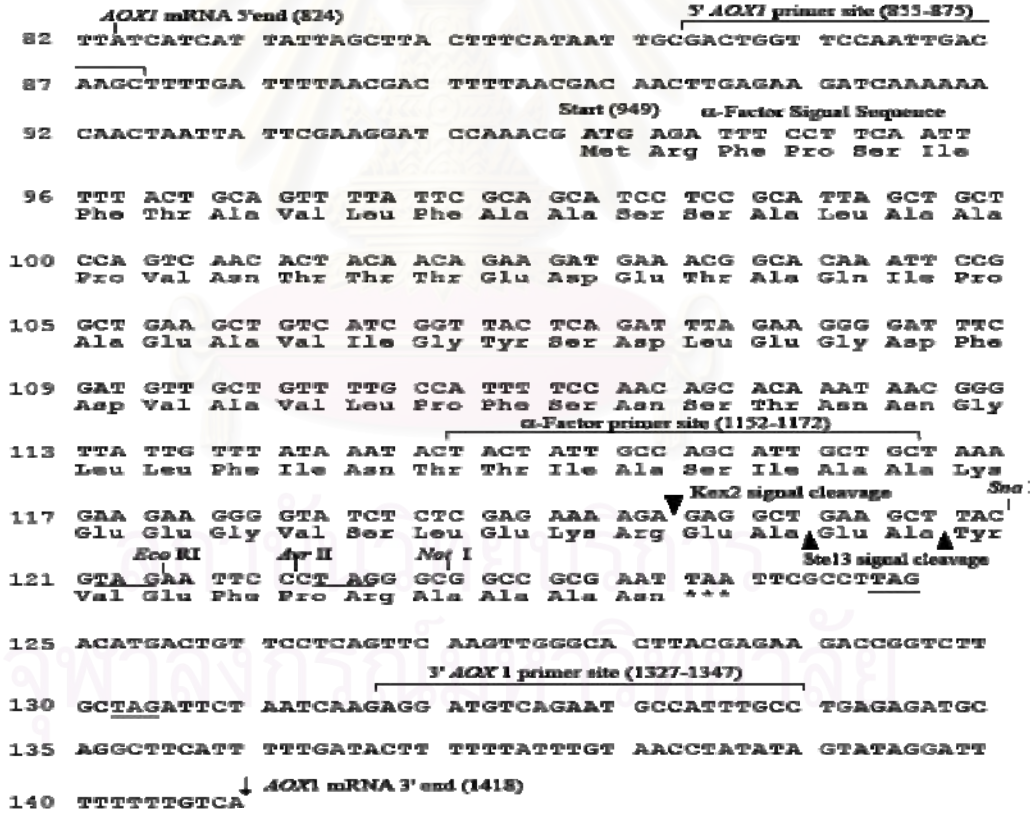


Figure 2.3 The circular map (A) and the P_{AOX1} and multiple cloning sites (B) in pPIC9K vector. (Source: www.invitrogen.com)

2.5.2.2 Amplification of ALF*Pm*2 gene by PCR

ALF*Pm*2 cDNA was amplified using *Pfu* DNA polymerase (Fermentas) and PCR profile as follows: heat denature at 94 °C for 1 min, 30 cycles of amplification; denature at 94 °C for 1 min, annealing at 55 °C for 30 sec and extension at 72 °C for 45 sec, and the last additional extension at 72 °C for 5 min. The PCR reaction was performed in the total 50 µl reaction consisting of 0.4 µg of plasmid containing ALF*Pm*2 gene, 1× reaction buffer, 2 µM of each forward and reverse primers, 0.2 mM dNTPs and 1 unit of *Pfu* DNA polymerase (Fermentas). After PCR amplification, the expected 325 bp PCR product was purified by HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Genomics).

2.5.2.3 Restriction enzyme digestion of the purified PCR product and expression vector

The pPIC9K vector was digested with *Xho*I and *Not*I. In the first step, 3 µg of vector was cut at 37 °C for an overnight with *Not*I in the reaction of 1× reaction buffer, 1× BSA, and 15 unit of *Not*I (New England Biolab). The reaction was stopped by incubating the reaction at 65 °C for 20 min. An equal volume of sterile water was then added to the reaction. In the second step, the reaction was partially digested for 1 min with *Xho*I at 37 °C in the reaction of 1× reaction buffer and 15 units of *Xho*I (New England Biolab). The reaction was stopped by incubating at 65 °C for 20 min. The correctly digested pPIC9K was separated by agarose gel electrophoresis and purified by HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Genomics). The purified PCR product of ALF*Pm*2 gene was double digested with *Xho*I and *Not*I, and the reaction was stopped at 65 °C for 20 min. The digested PCR product was separated by agarose gel electrophoresis and purified by HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Genomics).

2.5.2.4 Ligation

The *Xho*I/*Not*I-digested PCR fragment and pPIC9K were ligated prior to transformation into *P. pastoris*. The ligation reaction of 20 µl contained 2 µl of 10× ligation buffer, 2 µl of digested PCR product, 8 µl of *Xho*I/*Not*I

digested pPIC9K vector, and 2 μl of T4 ligase (New England Biolab). The molar ratio of insert:vector was 7:1. The ligation reaction was then incubated at 16 °C overnight.

2.5.2.5 Transformation into *E. coli* and plasmid preparation

The ligation reaction was transformed into *E. coli* JM 109 cells by electroporation as described in 2.3.5. One hundred microliters of each transformation was then plated onto a LB/ampicillin plate. The transformants were screened by colony PCR as described in 2.3.6. A few transformants were selected for sequencing in order to verify the sequence and orientation of ALF*Pm*2 gene in the expression plasmid. The α-signal peptide and the 3'-AOX primers were used in sequencing reactions.

2.5.3 *P. pastoris* transformation

The *P. pastoris* electrocompetent cells were prepared for transformation. A single colony of *P. pastoris* KM71 was cultured and used as starter in 5 ml of YPD [2% (w/v) peptone, 1% (w/v) bacto yeast extract and 2% glucose] at 30 °C overnight. One hundred μl of the overnight culture was inoculated in 500 ml of fresh medium in a 2-liter flask and grown overnight again to an OD_{600} of 1.3-1.5. The cells were collected by centrifugation at 3,500 rpm for 5 min at 4 °C. The cell pellet was washed by resuspending in 500 ml of cold sterile water, gently mixing and centrifugation. The pellet was washed further with 250 ml of cold sterile water followed by 20 ml of ice-cold sterile 1 M sorbitol and resuspended in 1 ml of ice-cold sterile 1 M sorbitol. The final volume including the cell pellet was approximately 1.5 ml. This cell suspension was divided into 80 μl aliquots and stored at -80 °C until used.

Before transformation, the ALF*Pm*2 expression clone was linearized with *Sac*I in AOX1 sequence. When *P. pastoris* KM71 was transformed with the *Sac*I-linearized plasmid, Mut^S transformants would be generated. Both the pALF*Pm*2 construct and the parent vector were digested by mixing 10 μg of plasmid, 10 units of *Sac*I, 1× reaction buffer, and incubating at 37 °C for 12-16 h. A small portion of the digest was analyzed by 1.0% agarose gel electrophoresis to confirm the complete

digestion of plasmids. The digested DNA was purified using HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Genomics) and stored at -20 °C until used.

An aliquot of *P. pastoris* KM71 competent cells was gently thawed on ice, mixed with 5 μg of *Sac*I-linearized plasmid and placed on ice for approximately 5 min. The mixture of cell and DNA was electroporated in a cold 0.2 cm cuvette with the apparatus setting as follows; 25 μF of the Gene pulser, 200 Ω of the Pulse controller unit and 1.50 kV of the Gene pulser apparatus (Bio-RAD). After one pulse was applied at the above setting, the cells were immediately resuspended with 1 ml of ice cold sterile 1 M sorbitol and transferred to test tube. The cell suspension of 200-600 μl was spread on the MD agar plates and incubated at 30 °C until the colonies appeared.

2.5.4 Screening for yeast high expression transformants

The yeast transformants were screened for G418 resistance. The transformants on MD plates were pooled by adding sterile water over each plate and running sterile spreader across the top of the agar to resuspend them. The transformants from all plates were pooled. The cell density was determined using a spectrophotometer assuming that 1 OD_{600} was equal to 5×10^7 cells/ml. Spread 10^5 cells on the YPD plates containing G418 at final concentrations of 0, 0.25, 0.75, 1, 1.5, and 2 mg/ml. The plates were incubated at 30°C. The G418 resistant colonies appear after 2 - 5 days. The resistant transformants on each YPD-G418 plate were randomly selected. They were streaked on the YPD plates to isolate the single colonies and to confirm the level of G418 resistance.

2.5.5 Determination of the integrated ALF*Pm*2 gene in *P. pastoris* genome by PCR

To check the transformants for the presence of the integrated ALF*Pm*2 gene, yeast genomic DNA was prepared by picking a single colony and resuspending in 10 μl of water. The cells were lysed by adding 5 μl of a 5 U/μl solution of lyticase (Sigma), incubating at 30 °C for 10 min, and freezing at -80 °C for 10 min. The cell lysate of 5 μl were mixed with the hot start PCR reaction mixture containing 1 μl each of the 10 μM α-factor and 3'-AOX primers, 5 μl of 25 mM $MgCl_2$, and 1 μl of 25 mM

dNTP mixture. Sterile distilled water was added to make the final volume to 49.5 µl. After incubating the mixture at 95 °C for 5 min, about 0.1 unit of DyNazyme II DNA™ polymerase was added. The PCR are performed for 30 cycles as follows: denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min. The resulting PCR reaction was analyzed by electrophoresis on a 1.2% agarose gel to determine whether the DNA fragment was successfully amplified. The size of the DNA product was 464 bp which was the α-factor signal sequence, the ALF*Pm*2 gene and the 3'sequence of alcohol oxidase (AOX) gene.

2.5.6 Expression of recombinant clones

A single colony of each clone was grown in YPD broth at 30 ºC overnight. The starter was inoculated into 100 ml of BMGY and grown at 30 ºC with 300 rpm shaking until the OD_{600} reached 4-6. The cells were harvested by centrifuging at 3,500 rpm for 5 min at room temperature. The cell pellet was resuspended in 20 ml of BMMY medium. To induce the expression, 100% methanol was added to the final volume concentration of 0.5% every 24 h to maintain the induction. The expression culture was collected 1 ml at each time points (0, 1, 2, 3, 4, 5 and 6 days) and centrifuged at 9,000 rpm for 2 min at room temperature. The supernatant and cell pellet were kept at -80 ºC until ready to use. The ALF expression was analyzed by 15% Coomassie-stained and silver stained SDS-PAGE. In addition, the ALF expression was then analyzed by MALDI-TOF mass spectrometer to determine the molecular mass.

2.5.7 Antimicrobial activity assay

The antibacterial activity was assayed against Gram-positive bacteria: *Bacillus megaterium, Micrococcus luteus, Staphylococcus aureus* and *Staphylococcus hamolyticus* and Gram-negative bacteria: *Enterobacter clocae*, *Erwinia carotovora*, *Escherichia coli 363, Klebsiella pneumoniae*, *Salmonella thyphimurium*, *Vibrio anguillarum and Vibrio Harveyi* 1526.

The expressed protein in crude preparation and during the different purification steps was monitored for its antibacterial activity by the liquid growth inhibition assay (Shafer, 1997). A colony of bacteria was cultured overnight in LB

medium (1% tryptone, 0.5% yeast extract and 1% NaCl) at appropriate temperature. The starter was inoculated in LB medium to obtain a fresh exponential phase culture. Poor broth medium (1% bactotryptone, 0.5% NaCl, pH 7.5) was used for bacterial dilution to a starting OD_{600} of 0.001. In each well of a 96-well microtiter plate, the protein preparation was incubated with 100 µl of exponential phase bacterial suspension. The culture was grown overnight (12-16 h) under gentle shaking at 30 and 37 ºC depending on the microorganism. Growth of bacteria was evaluated by measuring the absorbance at 600 nm using a microplate reader.

2.5.8 SDS-PAGE

The SDS-PAGE system was performed according to the method of Bollag et al. (1996). The slab gel (10 × 10 × 0.75 cm) system consisted of 15% (w/v) separating gel and 5% (w/v) stacking gel, containing 0.1% (w/v) SDS. Tris-glycine buffer, pH 8.3 containing 0.1% (w/v) SDS was used as electrode buffer. The gel preparation was described in Appendix A. After induction, the cell pellets were suspended with sample buffer and boiled for 10 min before loading into the gel. The electrophoresis was carried out at constant current of 20 mA per slab at room temperature from cathode towards anode. The gel was stained with Coomassie blue gel staining solution (0.1% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol, and 10% (v/v) glacial acetic acid) at room temperature for at least 30 min with gently shaking. After staining, the gel was destained by soaking in destaining solution (10% (v/v) methanol and 10% (v/v) glacial acetic acid) with gently shaking and changed the destaining solution three or four times in 24 h.

The silver staining gel was also used to detect the protein bands (Bollag et al., 1996). The advantage of this method was the sensitivity of as little as 2 ng of protein in a single band. After electrophoresis, the gel was soaked in 50% methanol and 10% acetic acid for at least 1 h with 2-3 changes of methanol/acetic acid solution. The gel was rinsed with water for 30 min with at least 3 changes. The solution for sliver staining was described in Appendix A. The gel was stained in Solution C for 15 min with gentle and constant agitation. The water was used for rinsing 2 times and then soaking for 2 min. The gel was developed using Solution D. After the protein bands were appeared in less than 10 min, the gel was rinsed with 1%

acetic acid to stop the development. The stopping solution was decanted and the gel was washed with ultra pure water for three times.

2.5.9 Tricine SDS-PAGE

The Tricine-SDS-PAGE gel electrophoresis was used to achieve the resolution of proteins in the range of 5 to 20 kDa (Schagger and von Jagow, 1987). The slab gel (10 × 10 × 0.75 cm) system consisted of 16.5% (w/v) separating gel and 4% (w/v) stacking gel, containing 0.1% (w/v) SDS. Two kinds of running buffers were used: the anode (0.2 M Tris buffer pH 8.9) and cathode (0.1 M Tris buffer pH 8.25, 0.1 M Tricine, and 0.1% SDS) buffers. The gel preparation was described in Appendix A. The protein samples were mixed with sample buffer and boiled for 10 min before loading the gel. The electrophoresis was started at 30 V. After about 1 h, when the sample had completely entered the stacking gel, the running condition was set at 100 V.

2.5.10 Purification of anti-lipopolysaccharide factor

The recombinant ALF*Pm*2 was purified by ion exchange chromatography. This technique is based on the ionic absorption of charged protein to the oppositely charged groups attached on an insoluble matrix. The cation exchanger was chosen for the purification. The running buffer was 50 mM MES, pH 5.6. At this pH, ALF*Pm*2 with a calculated pI of 6.8 carried a positive net charge and could be purified by cation exchanger.

The PD-10 Desalting column (GE Healthcare) contained Sephadex™ G-25 medium for group separation of high (MW>5000) from low molecular weight substances (MW<1000) was used to exchange the buffer system to 50 mM MES, 200 mM NaCl, pH 5.6 (start buffer). The column was equilibrated with 25 ml of start buffer. The crude protein was adjusted the volume to 2.5 ml with start buffer, loaded into the column and eluted with 3.5 ml of start buffer. The flow-through was collected for cation exchange chromatography.

The Hitrap SP HP (cation exchange) column (GE Healthcare) was washed with 5 column volumes of 50 mM MES buffer, 200 mM NaCl, pH 5.6 (start buffer) with the flow rate of 1 ml/min to remove the preservatives. Then, the column

was washed with 50 mM MES buffer pH 5.6 containing 1 M NaCl, (elution buffer) and equilibrated with 10 column volumes of start buffer. The crude recombinant ALF*Pm*2 in start buffer was applied, and the column was washed with 10 column volumes of start buffer or until the A_{280} read 0. The ALF*Pm*2 were eluted with 10 column volumes of elution buffer. All fractions collected from washing and elution steps were measured the A_{280} to approximate the amount of protein. The fractions were also analyzed by 15% SDS-PAGE and tested for antibacterial activity.

2.5.11 Antiviral activity assay

The antiviral activity assay was performed using cell culture of hematopoietic tissue of *Pacifastacus leniusculus*. The antiviral activity assay was to investigate the inhibition of white spot syndrome virus (WSSV) propagation (Jiravanichpaisal et al., 2006). The detectable WSSV infection was demonstrated by the RT-PCR of a viral protein VP28 expression in the host cells. The presence of the VP28 message indicated viral propagation.

2.5.11.1 Preparation of primary crayfish cell culture

The hematopoietic tissues (hpt) were obtained from *P. leniusculus* for culturing the cells (Soderhall et al., 2005). Briefly, the hematopoietic tissue (hpt) was dissected from the dorsal side of the cardiac stomach (Chaga et al., 1995). The tissue was washed in crayfish phosphate buffer saline (CPBS; 10 mM Na_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 10 μM $CaCl_2$, 10 μM $MnCl_2$, pH 6.8) and then gently shaken in 600 μl of 0.1% collagenase (type I and IV) in CPBS at room temperature to separate the hematopoietic cells. After 40 min, the hpt cells were separated by gently pipetting up and down for 10 times. The mixture was centrifuged at 4,000 rpm for 5 min at room temperature. The hpt cells were washed for 2 times with the CPBS buffer and centrifuged at 4,000 rpm for 5 min at room temperature. The supernatant and tissues were removed from the hpt cells. The cell pellet was resuspended in a modified L15 medium (Sigma-Aldrich) supplemented with 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin (Sigma-Aldrich), 2 mM L-glutamine, 5 μM mercaptoethanol and 1 μM phenylthiourea and then subsequently seeded at 10^5 cells per 150 μl in a 96-well plate. The hpt cells were supplemented with

a crude astakine fraction (Soderhall et al., 2005) after about 30 min of attachment at room temperature. The hpt cells were grown at 16 ºC and the culture medium was changed every 2 days.

2.5.11.2 White spot syndrome virus preparation

WSSV stock solution in plasma or in TN buffer (20 mM Tris–HCl, 400 mM NaCl, pH 7.4) was diluted to 200-µl suspension with a viral titer low enough to ensure that the crayfish stayed alive for 8-10 days after injection. The diluted WSSV was injected intramuscularly into a healthy crayfish in the lateral area of the fourth abdominal segment. About 5–6 days later, hemolymph from the moribund crayfish was collected and centrifuged at 4,000 rpm to remove the hemocytes. The supernatant was filtered through a 0.22 µm filter and kept at -80 ºC for further use.

The intact WSSV viral particles were purified from the hemolymph for use in the experiment according to (Xie et al., 2005). The hemolymph from WSSV-infected crayfish was mixed with TNE buffer (50 mM Tris-HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5) containing a combination of protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine and 1 mM $Na_2S_2O_5$) and then centrifuged at 3,500*g* for 5 min at 4 ºC. The supernatant was centrifuged at 30,000*g* for 30 min at 4 ºC. The pellet was rinsed with TM buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) and centrifuged at 3,500*g* for 5 min. The pellet was suspended in TM buffer and the supernatant was centrifuged at 30,000*g* for 30 min at 4 ºC. The pellet was suspended in TM buffer was divided into aliquots and stored at -80 °C until used.

2.5.11.3 Antiviral activity test

The anti-lipopolysaccharide factor isoforms 2 and 3 exhibited antibacterial activity, especially the broad antimicrobial activity of ALF*Pm*3. With this antimicrobial activity, it was interesting to test the antiviral activity of the ALF*Pm*2 and 3. The antiviral activity of the proteins was investigated by determining the extent of viral propagation in cell culture. The viral coat protein VP28 of WSSV was used as marker for the WSSV propagation test. The 40S ribosomal RNA of

crayfish housekeeping gene was used as internal control (Jiravanichpaisal et al., 2006).

The 20, 10, 5 and 2.5 μM of purified ALF*Pm*3 or 20 μM of purified ALF*Pm*2 were mixed with WSSV and immediately added to the cell culture. After incubated at 20 ºC for 2 h, the medium was replaced with new culture medium. The incubation was continued for 39 h. The total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Briefly, the cell cultures were lysed and homogenized in lysis solution containing 2-mercaptoethanol to release the RNA and inactivate RNases. Lysates were spun through a filtration column to remove cellular debris and shear the DNA. The filtrate was then applied to a high capacity silica column to bind total RNA, followed by washing and elution. The genomic DNA was removed from the total RNA by digesting with DNaseI. The cDNA was synthesized from the mRNA as described above. The VP28 primers were used to detect the WSSV propagation, and the 40S ribosomal RNA primers were used to amplify the housekeeping gene (Table 2.3). Each cDNA preparation of 1 μl was used for PCR amplification in a 50 μl reaction volume containing 0.25 units Paq5000™ DNA Polymerase (Stratagene), 1× reaction buffer, 200 μM of dNTP, 0.6 μM each of the appropriate forward and reverse primers. The initial denaturation was at 94 ºC for 3 min, followed by 28 cycles of denaturation at 94 ºC for 30 sec, annealing at 60 ºC for 30 sec, elongation at 72 ºC for 40 sec, and the final extension at 72 ºC for 10 min. The PCR products of ALF genes were analyzed by electrophoresis using the 1.5% agarose gels. The expected size of VP28 and 40S ribosomal RNA fragments were 506 and 359 bp, respectively.

2.5.11.4 Comparative quantitative RT-PCR

The detection and comparative quantification of WSSV propagation in crayfish hpt cell cultures were performed by quantitative RT-PCR using the QuantiTect SYBR green PCR Kit (QIAGEN) (Liu et al., 2006). The expression of WSSV VP28 gene was normalized against the expression of 40S ribosomal gene for each sample. The primers used for qRT-PCR were shown in Table 2.3. The SYBR green quantitative RT-PCR amplification was performed by a Rotor-Gene 3000 (Corbett Robotics). The RNA extraction and cDNA synthesis were

described above. The cDNA samples were diluted 1:10 with RNase-free sterilized water. The amplification was done in a 25 µl reaction volume containing 12.5 µl of 2× QuantiTect SYBR green PCR master mix, 0.4 µM each forward and reverse primers, and 5 µl of diluted cDNA template. The RNase-free distilled water was added to adjust the total volume to 25 µl per reaction. All runs employed a negative control without target DNA. The PCR profile was as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 sec, 60 °C for 20 sec and 72 °C for 20 sec. Each sample was done in triplicates.

Table 2.3 Nucleotide sequences of primers for assay antiviral activity

Primer	**Sequence (5'-3')**
Primers for reverse transcription (RT)-PCR	
VP28RTF	TCACTCTTTCGGTCGTGTCG
VP28RTR	CCACACACAAAGGTGCCAAC
40SRTF	CCAGGACCCCCAAACTTCTTAG
40SRTR	GAAAACTGCCACAGCCGTTG
Primers for quantitative RT-PCR	
VP28QRTF	GGGAACATTCAAGGTGTGGA
VP28QRTR	GGTGAAGGAGGAGGTGTTGG
40SQRTF	GACGAATGGCATACACCTGAGAGG
40SQRTR	CAGGACTCTGCAGTTCAAGCTGATG

2.5.11.5 Trypan blue exclusion test of cell viability

The dye exclusion test was used to determine the number of viable cells in hpt cultures that were incubated with ALF*Pm*3 or ALF*Pm*3 and WSSV. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. The hpt cultures were incubated with 20, 10 and 5 µM of purified ALF*Pm*3 or 20, 10 and 5 µM of purified ALF*Pm*3 mixed with WSSV. After incubating at 20 ºC for 30 min, the 100 µl of medium was removed and 8 µl of trypan blue solution (0.4% of trypan

blue in 0.81% of sodium chloride and 0.06% of potassium phosphate, dibasic) was added. The viable cells had clear cytoplasm whereas nonviable cells had blue cytoplasm.

2.5.11.6 Antiviral mechanism of ALF*Pm*3

To investigate the antiviral mechanism of ALF*Pm*3, the effect of ALF*Pm*3 on WSSV attachment to hpt cells was studied. The mixture of WSSV and 5 μM of ALF*Pm*3 was incubated with hematopoietic cell cultures at 11 ºC and 20 ºC. For a control, only WSSV was incubated with hpt cell culture. After 2 h of incubation, the medium was removed completely and washed 2 times with CPBS. The CPBS buffer was replaced completely with fresh medium. The incubation was continued at 20 ºC for 36 hours. The infection of WSSV was then determined by RT-PCR as described above.

Additional experiment was performed for observe antiviral mechanism. The ALF*Pm*3 was incubated with hpt cell cultures at 20 ºC for 30 min. The cultures were either washed or not washed with CPBS buffer. The WSSV in fresh medium was added after the old medium or the CPBS buffer was removed. After incubated at 20 ºC for 2 h, the medium was removed and replaced by fresh medium. The cultures were incubated further at 20 ºC for 36 h. The infection of WSSV was then determined by RT-PCR as described above.

CHAPTER III

RESULTS

3.1 Genomic organization of *Penaeus monodon* ALF genes

3.1.1 Analysis of *Penaeus monodon* ALF cDNAs

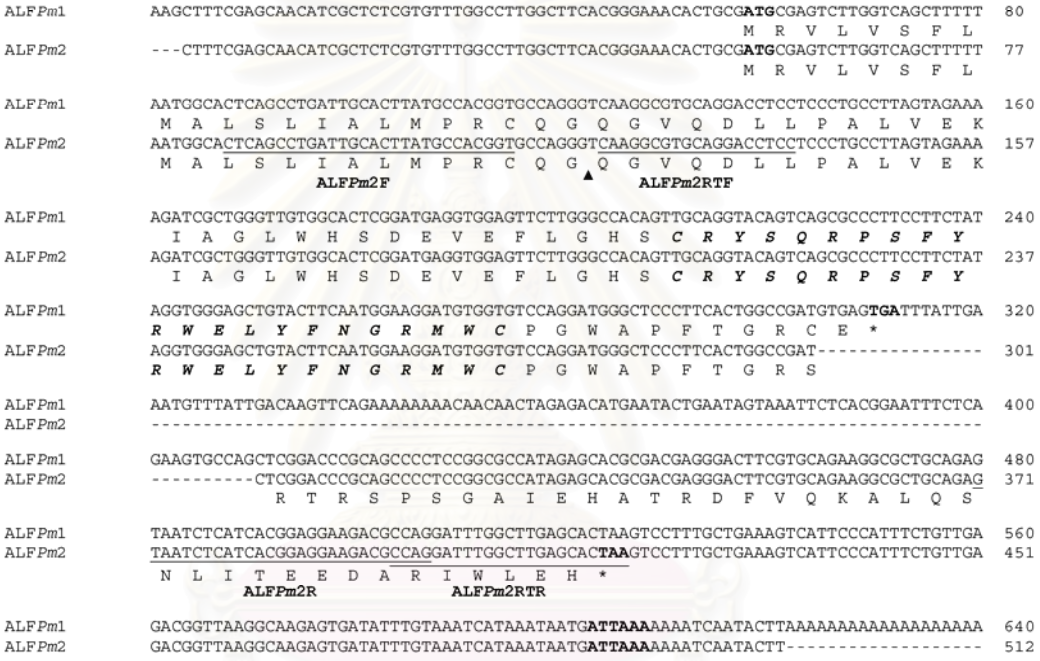
Previously, the deduced amino acid sequences of *Penaeus monodon* ALF homologues identified from the EST libraries were compared, and the analysis suggested that there were at least five isoforms (Supungul et al., 2004). In this study, the same sequences were re-evaluated. The nucleotide sequence comparison undoubtedly revealed two different groups of ALF homologues, designated group A consisting of ALF*Pm*1 and 2 (GenBank accession nos. **BI784448,** and **BI784449**), and group B consisting of ALF*Pm*3, 4 and 5 (GenBank accession nos. **BI018071, BI784451,** and **CF415871**).

In group A, the nucleotide sequence of ALF*Pm*2 totally matched that of ALF*Pm*1. The ALF*Pm*1, however, contained an additional sequence of 104 bp at positions 535–638 (Figure 3.1A). Due to the stop codon (TGA) at the beginning of the extra sequence, the reading frame of ALF*Pm*1 contained only 84 amino acid residues, compared to the 120 amino acid reading frame of ALF*Pm*2. Nevertheless, both isoforms contained a sequence of the disulfide loop CRYSQRPSFYRWELYFNGRMWC that was proposed to be the LPS-binding sequence (Supungul et al., 2004).

Similar to the nucleotide sequence of ALF*Pm*1, the ALF*Pm*4 cDNA contained an additional sequence of 342 bp at positions 426–767 (Figure 3.1B). The reading frame of ALF*Pm*4 encountered a stop codon (TGA) when it read not very far into the extra sequence. The encoding protein was only 52 amino acid residues in length. This additional sequence was missing in ALF*Pm*3 and 5; therefore, their reading frames could read through into the putative LPS binding sequence, CKFTVKPYLKRFQVYYKGRMWC, and coded for a protein of 123 amino acid residues. However, comparing the nucleotide sequences of ALF*Pm*3 and 5, it was

quite certain that they were from the same gene as all the bases are identical except one additional base at position 274 in ALF*Pm*5 (Figure 3.1). This additional base might be derived from an error during the cDNA synthesis. Clustering analysis also suggested that the ALF*Pm*3 (GenBank accession no. BI018071) and 5 (GenBank accession no. CF415871) were identical (http://pmonodon.biotec.or.th).

A. ALF group A



B. ALF group B

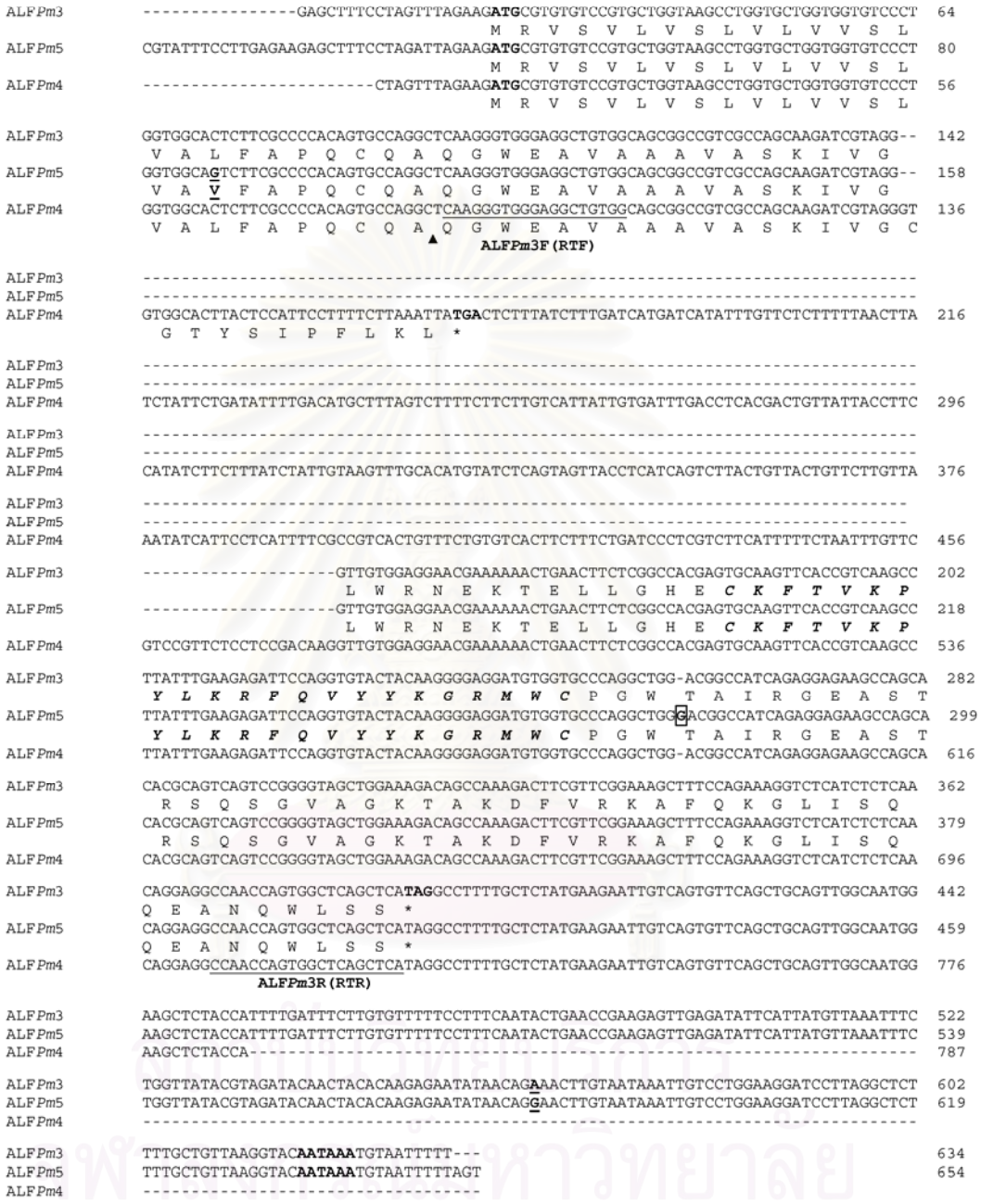


Figure 3.1 Alignment of the nucleotide sequences of ALF groups A (A.) and B (B.). The encoded amino acid sequences are underneath the nucleotide sequences. The start and stop codons and the poly(A) signal sequences are bold-faced. The LPS-binding sites are bold-italicized. The triangles indicate the signal cleavage sites. The annealing sites of the PCR primers are underlined. The bold-underlined letters are different bases between the ALF*Pm*3 and 5. The boxed bold letter is the extra base found only in ALF*Pm*5.

3.1.2 Determination of exon and intron of *P. monodon* ALF genes

It has been hypothesized previously that *P. monodon* ALFs were possibly derived from two different loci (Supungul et al., 2004). In fact, the nucleotide sequence analysis above also supported the hypothesis. It was, therefore, interesting to unveil their gene organization at the genomic level. For organization study of ALF*Pm* genes, the primers designed from the cDNA sequences were used for genomic amplification.

The ALF*Pm*2 gene represented as group A was amplified to determined exon and intron sequences. The nested PCR primers were designed from the cDNA sequences (ALF*Pm*2F1, R1 and ALF*Pm*F2, R2) for amplification of ALF*Pm*2 gene (Table 2.1). The difference in size between primary and secondary PCR products should be about 150 bp according to distance between the primary and nested primers. From Figure 3.2, bands A and B from the first and secondary PCRs were approximately 150 bp difference in size. Thus, the band B from the secondary PCR was chosen for cloning and sequencing.

The ALF*Pm*3 gene was also PCR amplified to determine the exon and intron sequences. By designing the primers at the beginning (ALF*Pm*3F) and at the end of the gene (ALF*Pm*3R) (Table 2.1), the PCR products were obtained (Figure 3.3). The PCR product whose size was about 1.6 kb was cloned and subsequent sequencing.

The genomic sequences were analyzed as compared to the corresponding cDNA sequences. The results showed that the ALF*Pm*2 represented as ALF*Pm* group A consisted of three exons interrupted by two introns (Figure 3.4). Likewise, ALF*Pm*3 genomic sequence was analyzed with corresponding cDNA sequences. For the ALF*Pm*3 (ALF group B gene), there were three exons interrupted by two introns (Figure 3.5).

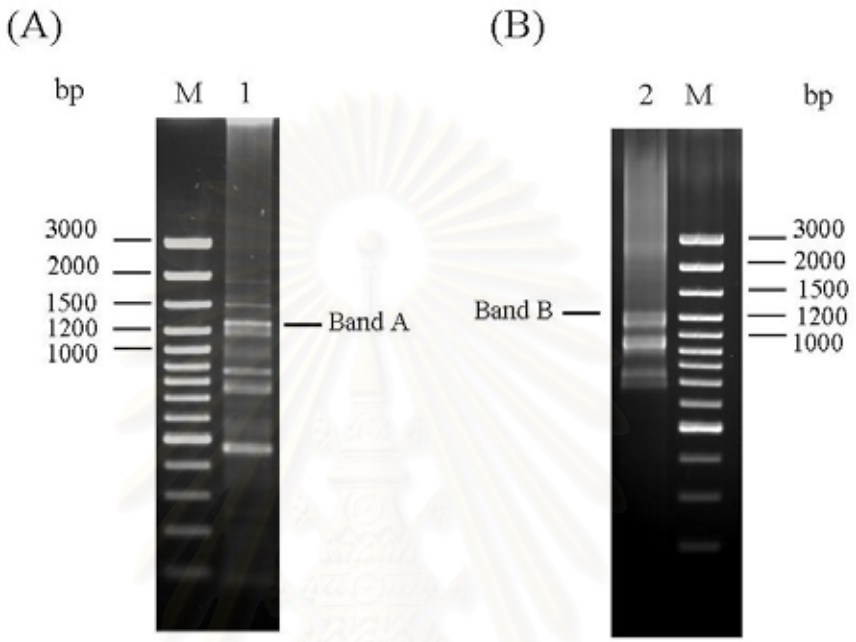


Figure 3.2 Agarose gel electrophoresis of primary (A) and secondary (B) PCR products of ALF*Pm*2 gene amplified from the genomic DNA. The PCR products were run on 1.5% agarose gels at 100 volts for 1 hour.

(A) Lane M: Standard DNA ladder (100 bp marker)
Lane 1: The primary PCR product

(B) Lane M: Standard DNA ladder (100 bp marker)
Lane 2: The secondary PCR product

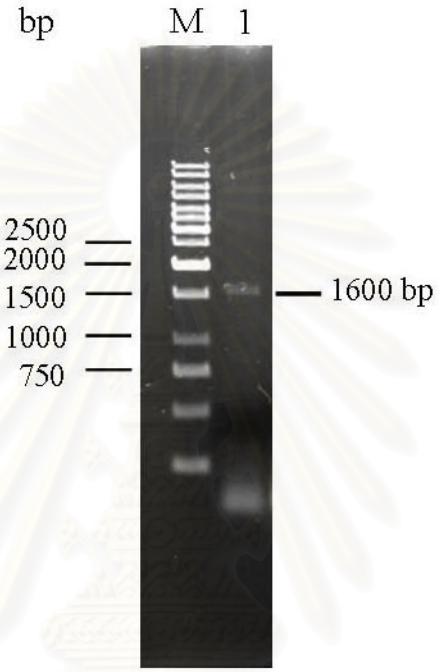


Figure 3.3 Agarose gel electrophoresis of PCR product of ALF*Pm*3 gene amplified from the genomic DNA. The PCR product was run on 1.5% agarose gel at 100 volts for 1 hour.

Lane M: Standard DNA ladder (1 kb marker)

Lane 1: The PCR product of ALF*Pm*3 gene

3.1.3 Determination of promoter and regulatory elements of *P. monodon* ALF genes

After the exon-intron finding, the 5′ upstream sequences were determined to localize the promoter and regulatory elements. The ALF*Pm* gene-specific primers were designed from the known gene sequences close to the 5’ end (Table 2.1). The nested PCR technique was used for the amplification of DNA libraries (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries). The primary and secondary PCR products of ALF*Pm*2 and 3 were analyzed by agarose gel electrophoresis (Figure 3.6-3.8).

Figure 3.6 showed that the secondary PCR product of ALF*Pm*2 genome walking in three libraries (*Dra*I, *Pvu*II and *Stu*I libraries) were approximately 600, 900 and 800 bp, respectively. To show that the PCR products were related, the PCR products from *Pvu*II library was digested with *Dra*I and analyzed. One of the digested products was 600 bp corresponding to the size of PCR product from *Dra*I library. The PCR product of *Pvu*II library was then cloned and subsequently sequenced.

The ALF*Pm*3 genome walking was performed twice as shown in Figures 3.7 and 3.8. In the first ALF*Pm*3 genome walking, the primary PCR products were not detected from all four libraries but the secondary PCR products were sucessfully amplified from the unseen primary PCR products from all four libraries (Figure 3.7). The secondary PCR product from the *Pvu*II library was digested with *Dra*I and analyzed to ensure that the products from both libraries were related to the same gene. The former was cloned and sequenced. The genome walking was repeated to determine the sequence further upstream by designing appropriate primers from the DNA sequence determined from the first walk. This time, the nested secondary PCR product from *Dra*I library was sequenced (Figure 3.8). Analyzing the genome sequences obtained provided information of the regulatory sequences.

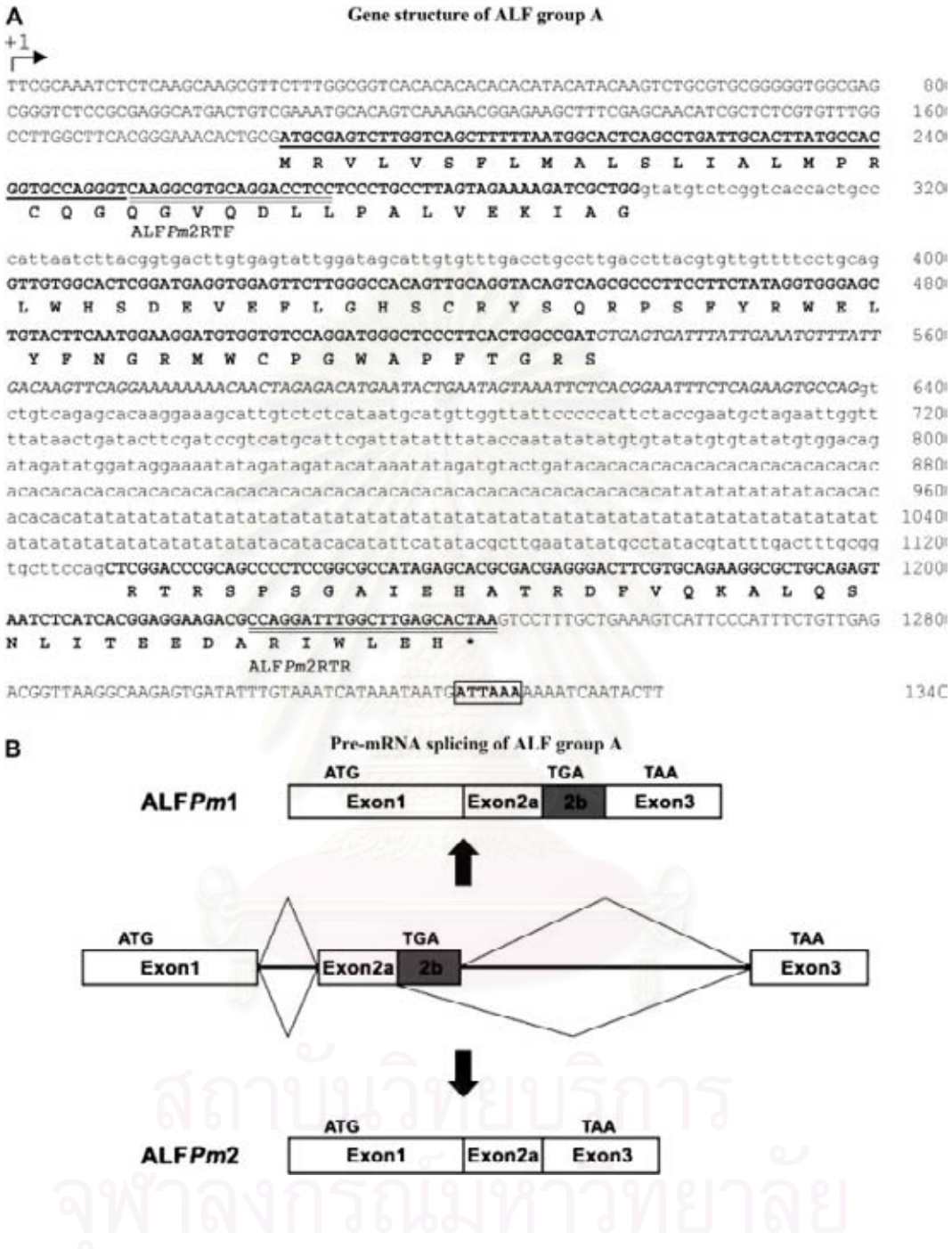


Figure 3.4 Genomic nucleotide and deduced amino acid sequences of ALF group A (A.) and their pre-mRNA splicing (B.). The sequence reads from the putative pre-mRNA start site (+1) with exons in capital letters, coding sequence of ALF*Pm*2 in boldfaced capital letters, the additional intron sequence in ALF*Pm*1 in italicized letters and introns in lowercase letters. The encoding amino acid sequence is bold-faced with the signal peptide underlined. Poly(A) signal sequence is boxed. The annealing site for PCR primer indicated is underlined.

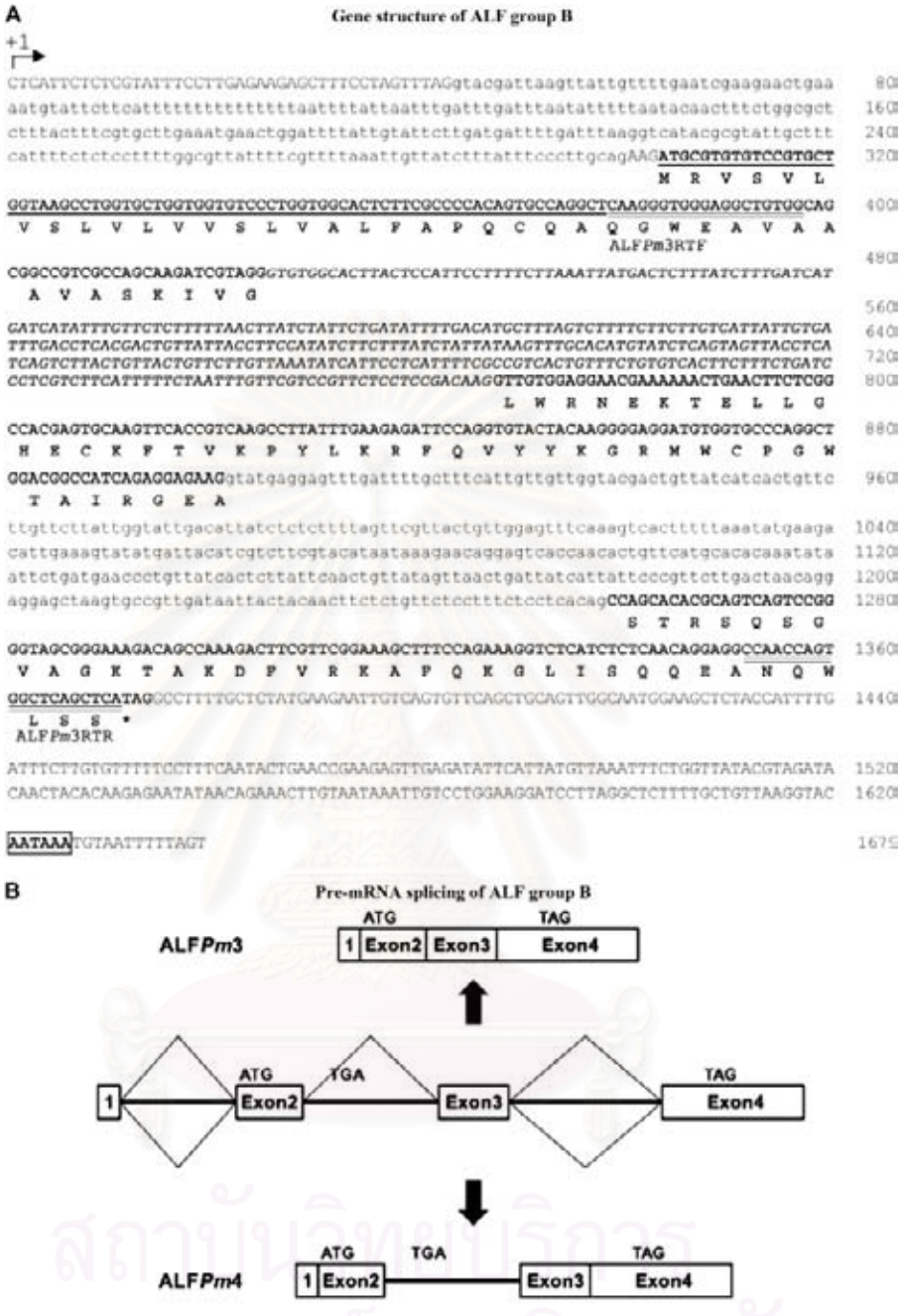


Figure 3.5 Genomic nucleotide and deduced amino acid sequences of ALFs group B (A.) and their pre-mRNA splicing (B.). The sequence reads from the putative pre-mRNA start site (+1) with exons in capital letters, coding sequence of ALF*Pm*2 in boldfaced capital letters and introns in lowercase letters. The encoding amino acid sequence is bold-faced with the signal peptide underlined. Poly(A) signal sequence is boxed. The annealing site for PCR primer indicated is underlined.

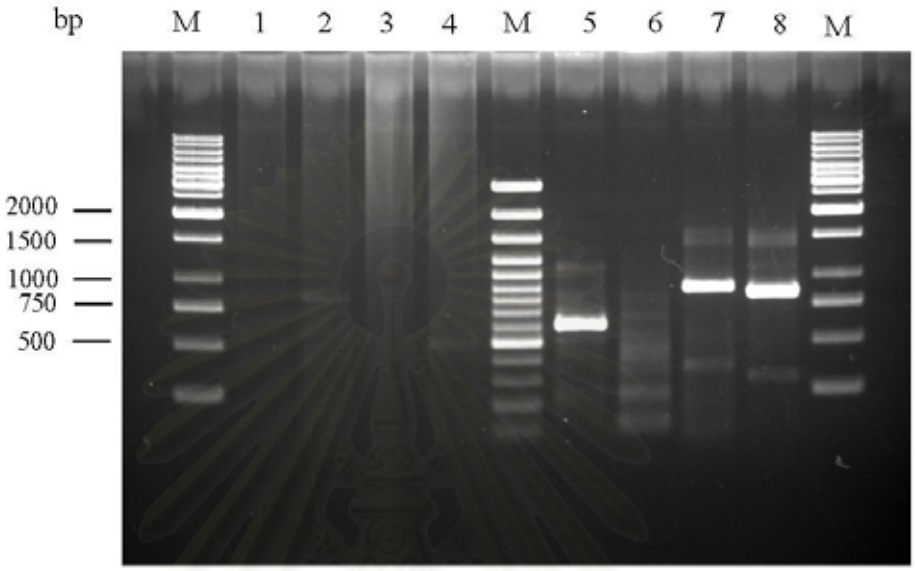


Figure 3.6 Agarose gel electrophoresis of the primary and secondary PCR products of ALF*Pm*2 genome walking amplified from the four genomic libraries (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries). The PCR products were run on 1.5% agarose gel at 100 volts for 1 hour.

Lane M: Standard DNA ladder
Lane 1: The primary PCR product from *Dra*I library
Lane 2: The primary PCR product from *Eco*RV library
Lane 3: The primary PCR product from *Pvu*II library
Lane 4: The primary PCR product from *Stu*I library
Lane 5: The secondary PCR product from *Dra*I library
Lane 6: The secondary PCR product from *Eco*RV library
Lane 7: The secondary PCR product from *Pvu*II library
Lane 8: The secondary PCR product from *Stu*I library

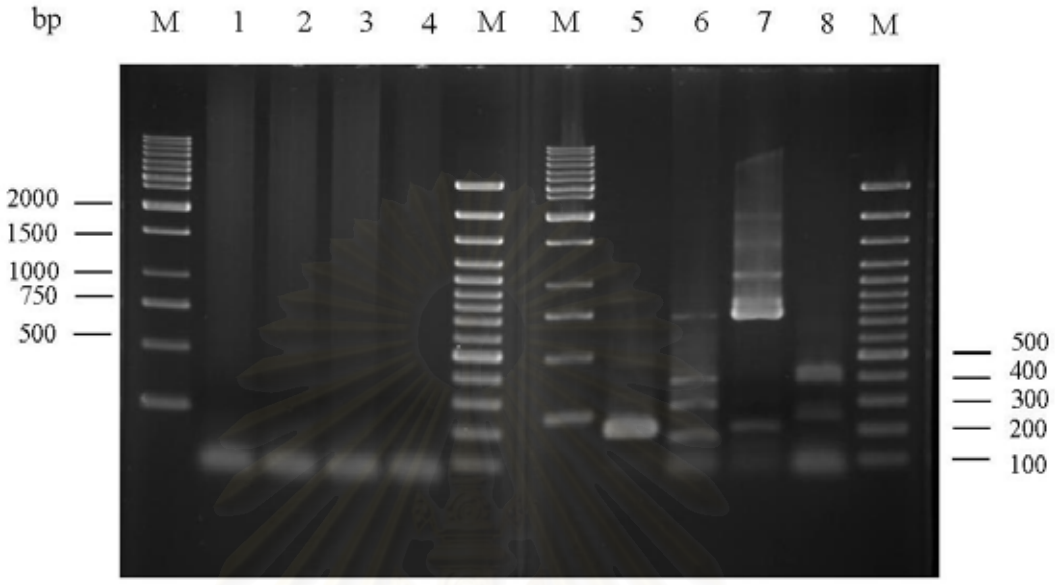


Figure 3.7 Agarose gel electrophoresis of the primary and secondary PCR products of ALF*Pm*3 genome walking amplified from the four genomic libraries (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries). The PCR products were run on 1.5% agarose gel at 100 volts for 1 hour.

Lane M: Standard DNA ladder
Lane 1: The primary PCR product from *Dra*I library
Lane 2: The primary PCR product from *Eco*RV library
Lane 3: The primary PCR product from *Pvu*II library
Lane 4: The primary PCR product from *Stu*I library
Lane 5: The secondary PCR product from *Dra*I library
Lane 6: The secondary PCR product from *Eco*RV library
Lane 7: The secondary PCR product from *PvuII* library
Lane 8: The secondary PCR product from *Stu*I library

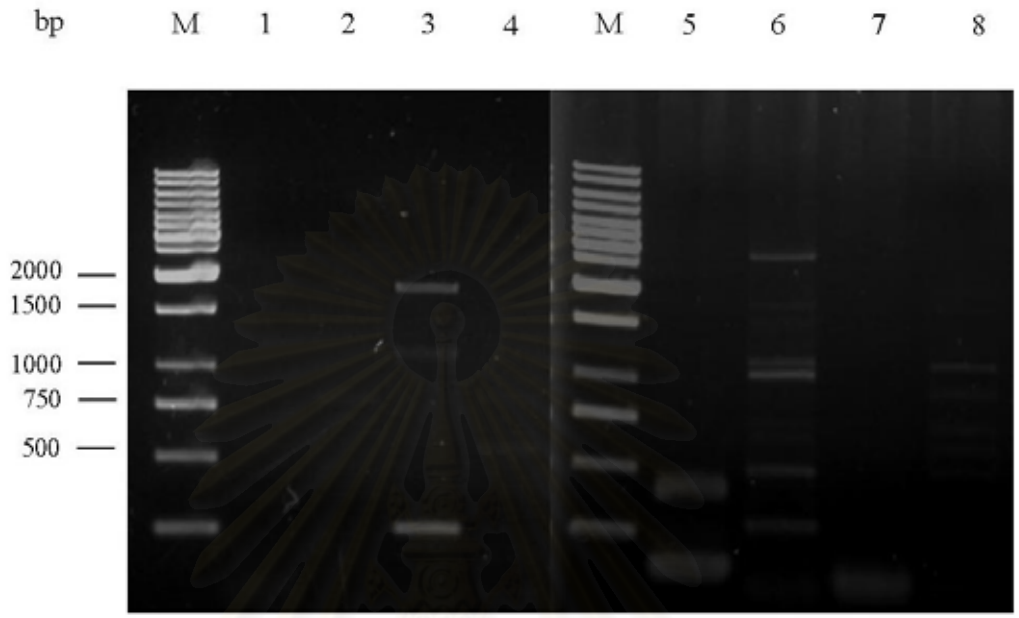


Figure 3.8 Agarose gel electrophoresis of the primary and secondary PCR products of ALF*Pm*3 genome walking amplified from the four genomic libraries (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I libraries). The PCR products were run on 1.5% agarose gel at 100 volts for 1 hour.

Lane M: Standard DNA ladder
Lane 1: The primary PCR product from *Dra*I library
Lane 2: The primary PCR product from *Eco*RV library
Lane 3: The primary PCR product from *Pvu*II library
Lane 4: The primary PCR product from *Stu*I library
Lane 5: The secondary PCR product from *Dra*I library
Lane 6: The secondary PCR product from *Eco*RV library
Lane 7: The secondary PCR product from *PvuII* library
Lane 8: The secondary PCR product from *Stu*I library

The genomic sequences of group A were confirmed by amplification of the whole genomic segments using primers ALF*Pm*2AF and ALF*Pm*2AR (Table 2.1) and sequencing. The same strategy could not be done with the ALF*Pm* group B probably because of the presence of microsatellite GA repeat sequences at positions −287 to −438. The gene organization of *P. monodon* ALF genes starting from the putative pre-mRNA start sites was predicted using the Neural Network Promoter Prediction (Reese, 2001). The genomic structure of ALF group A consisted of three exons interrupted by two introns (Figure 3.4). For the ALF*Pm*1 gene, the exons were 298, 238 and 211 bp in length, and the introns were 102 and 491 bp in length. The ALF*Pm*2 had slightly different gene structure; its exons were 298, 134 and 211 bp in length with the two introns of 102 and 595 bp in length. Figure 3.4B clearly shows that there were two 5′ spliced sites between the exons 2 and 3 that were used to produce the ALF group A transcripts. With different 5′ spliced sites, the ALF*Pm*1, then, contained the additional sequence not found in the ALF*Pm*2.

For the ALF*Pm*3 (ALF group B gene), there were four exons interrupted by three introns (Figure 3.5). The four exons were 43, 125, 154 and 361 bp in length, and the three introns were 257, 342 and 357 bp in length (Figure 3.5B). The ALF*Pm*3 had all three introns completely spliced out. The additional sequence in the ALF*Pm*4 was, in fact, the sequence of intron 2 that was selectively retained upon pre-mRNA splicing. Interestingly, the exon 1 was short and did not encode any amino acid; in other words, the mature peptide was coded for by the exons 2–4. All exon–intron boundaries of the ALF genes in both groups conformed to the consensus GT at the 5′ spliced sites and the AG at the 3′ spliced sites (Shapiro and Senapathy, 1987).

The 5′ upstream sequences were analyzed for the putative *cis*-regulatory elements by using the MATCH™ analysis program searching the sequences against the TRANSFAC database (Heinemeyer et al., 1998) with the cut-offs for both core and matrix similarities of 0.9. The putative promoters of the *P. monodon* ALF groups A and B were identified at positions −31 and −29, respectively. Several transcription factor-binding sites, such as Octamer (Oct-1) (consensus ATGCAAAT), GATA (consensus WGATAR), CCAAT box (consensus GGNCAATCT) and GAAA motifs (consensus GAAANN), were predicted in the 5′ upstream sequences of both the ALF

group A and B genes. A putative activator protein 1 (AP-1) (consensus TGASTCA), nuclear factor (NF)-kappaB binding sites (consensus GGGAMTNYCC), however, were only predicted at the 5′ upstream of the ALF group A genes (Figure 3.9). The genomic sequences including the 5′ upstream and the genes were deposited into the GenBank (accession nos. EF523560, EF523561, EF523562 and EF523563 for ALF*Pm*1, 2, 3 and 4, respectively).

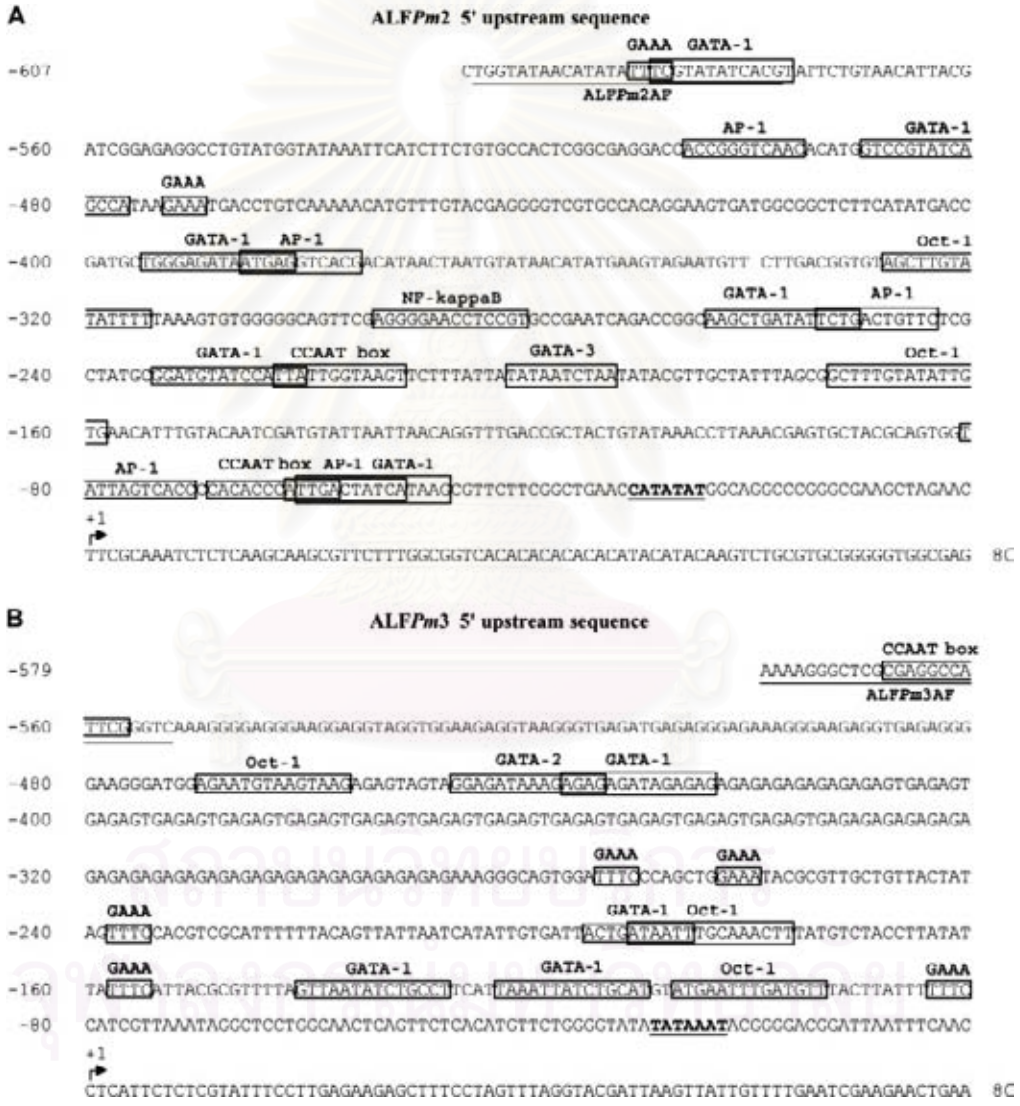


Figure 3.9 The 5' upstream sequences of ALF*Pm*2 (A.) and 3 (B.) containing the putative *cis*-regulatory elements in boxes. The TATA boxes are bold-faced and underlined. The putative start sites are +1. The annealing sites for the PCR primers indicated are underlined.

3.2 Differential expression of ALF genes

To test whether different isoforms of *P. monodon* ALFs were expressed in shrimp hemocytes, the RT-PCR analysis was employed. The shrimp juveniles were injected with 10^3 CFU of *V. harveyi* whereas the control group was injected with 100 µl of 0.85% (w/v) NaCl solution. According to the expression of ALF*Pm*3 up-regulated upon injection of *V. harveyi* with the highest expression at 6 h post-injection, the hemolymph was collected from saline and *V. harveyi* injected shrimp at 6 hpi. Total RNA was prepared and used for cDNA synthesis. Since the ALF mRNAs in each group had different lengths due to the additional sequences, they could be distinguished by using the same set of PCR primers. Using the primers ALF*Pm*2RTF and ALF*Pm*2RTR for ALF group A and ALF*Pm*3RTF and ALF*Pm*3RTR for ALF group B (Table 2.1), all ALF transcripts were identified as PCR products of different lengths. The sizes of RT-PCR products are 403, 297, 286 and 628 for ALF*Pm*1, 2, 3 and 4, respectively. Both unchallenged and *Vibrio harveyi* challenged hemocytes of individual shrimp expressed ALF*Pm*1–4 transcripts (Figure 3.10). The ALF*Pm*3 transcript was the predominant species followed by the ALF*Pm*2 transcript. However, ALF*Pm*1 transcript was barely detectable, and could be observed clearly only after the RT-PCR reactions were re-amplified. The ALF*Pm*4 was seen as a faint band. To make sure that the faint bands in Figure 3.10 were actually the ALF*Pm*1 and 4 transcripts, the expect ALF*Pm*1 and 4 bands were cut and performed nested PCR by ALF*Pm*1 and 4 gene specific primers. The nested PCR products were sequenced and alignment with ALF cDNA sequences. The results showed that both of the faint bands were ALF*Pm*1 and 4 genes. Low expression of ALF*Pm*1 and 4 suggested that they might probably be aberrant splicing products of the ALF*Pm*2 and 3, respectively. It should be noted also that ALF*Pm*1 and 4 seemed to be produced in a constant ratio to their normal counterparts as seen in Figure 3.10, which may indicate that the aberrant incompletely processed transcripts are produced stoichiometrically by a malfunctioning splicing apparatus. The result indicated that the major ALF transcripts were those of ALF*Pm*2 and 3.

The differential expression experiment of ALF*Pm*1-4 in the hemocytes of normal saline control and *V. harveyi*-challenged shrimp were also determined. The primers ALF*Pm*1RTF, ALF*Pm*1RTR, ALF*Pm*2RTF, ALF*Pm*2RTR, ALF*Pm*3RTF,

ALF*Pm*3RTR, ALF*Pm*4RTF, ALF*Pm*4RTR, EF-1 alphaF and EF-1 alphaR were used (Table 2.2). The PCR products were analyzed on 1.5% agarose gel electrophoresis. The sizes of RT-PCR products are 196, 297, 286, 339 and 150 bp for ALF*Pm*1, 2, 3, 4 and EF-1 alpha, respectively. The band intensities were determined using Genetools analysis software (Syngene) and the ratios of band intensity between ALF*Pm* genes and the house keeping gene, elongation factor-1 alpha, were calculated. Figure 3.11 shows that the transcription levels of ALF*Pm*2, 3 and 4 were significantly increased in the hemocytes of *V. harveyi*-challenged shrimp. However, the expression of ALF*Pm*1 was barely detectable. The second round re-amplification was performed on the PCR product, and still the product was barely detectable. Thus, the differential expression of ALF*Pm*1 transcript could not be determined.

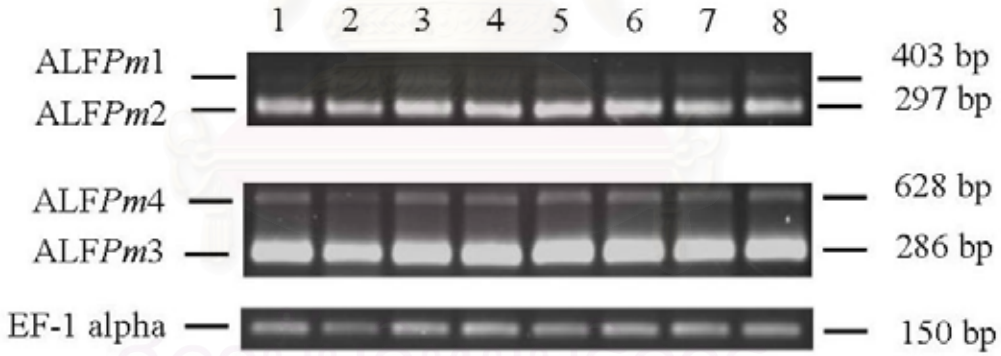


Figure 3.10 The RT-PCR analysis of ALFs in the unchallenged and *V. harveyi*-challenged hemocytes of individual shrimp. The PCR products were run on 1.2% agarose gel. The sizes of RT-PCR products are 403, 297, 286 and 628 for ALF*Pm*1, 2, 3 and 4, respectively.
Lanes 1 to 4: ALF transcripts from unchallenged individual shrimps.
Lanes 5 to 8: ALF transcripts from *V. harveyi*-challenged individual shrimps.

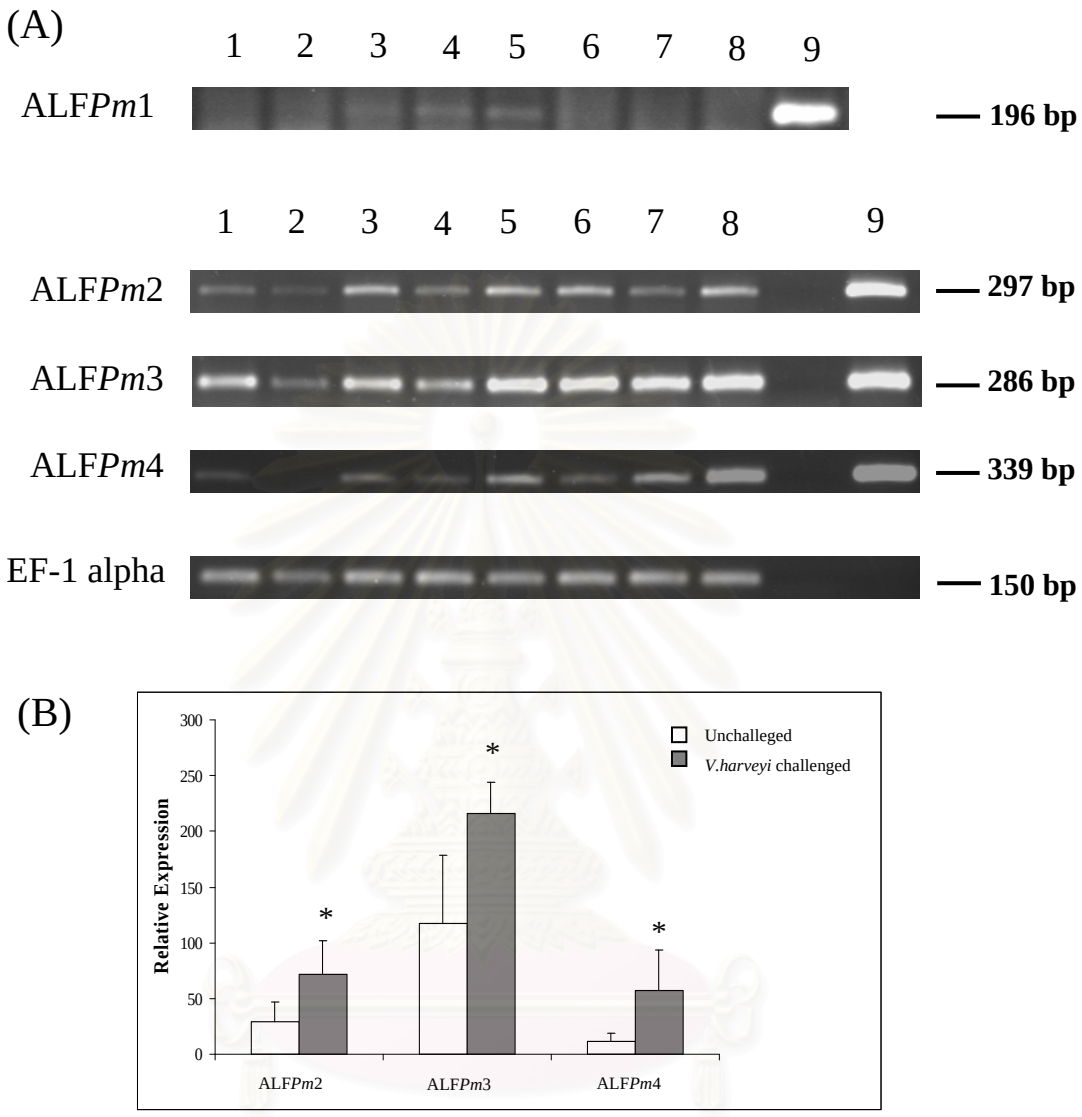


Figure 3.11 The differential expression of ALF transcripts between the unchallenged and challenged shrimp analyzed by RT-PCR. (A) The expression of the transcripts of ALF*Pm*1-4 in unchallenged and challenged individual shrimp. For ALF*Pm*1, the second round PCR products were shown. The sizes of RT-PCR products are 196, 297, 286, 339 and 150 bp for ALF*Pm*1, 2, 3, 4 and EF-1 alpha, respectively. The band intensities were measured, normalized against those of EF-1 alpha, and expressed as relative expression in (B). The values are means ± S.D. (n = 4). The asterisks indicate significant difference between the unchallenged and challenged shrimp ($P < 0.05$).

Lanes 1 to 4: ALF and EF-1 alpha transcripts from unchallenged individual shrimp

Lanes 5 to 8: ALF and EF-1 alpha transcripts from *V. harveyi*-challenged individual shrimp

Lane 9: The equivalent PCR product from each ALF cDNA clone.

3.3 Characterization of anti-lipopolysaccharide factor isoforms 2 and 3

Since the anti-lipopolysaccharide factor isoform 2 was identified in *Vibrio harveyi*-infected hemocyte library of *Penaeus monodon* and was the major form of ALF*Pm* group A, the ALF*Pm*2 at protein level was worth characterization. The recombinant ALF*Pm*2 protein (rALF*Pm*2) was subjected to over-producing in *Pichia pastoris* expression system.

3.3.1 Preparation of a DNA fragment encoded mature ALF*Pm*2

The ALF*Pm*2 clone from *V. harveyi*-infected hemocyte library was amplified using the primers designed from the cDNA sequence that amplified the encoded mature peptide of ALF*Pm*2. The *Xho*I and *Not*I sites were included at the 5' end of the primers. The PCR product was run on 1% agarose gel electrophoresis to isolate the specific fragment of 325 bp (Figure 3.12). The PCR product was purified, digested with *Xho*I and *Not*I, and ligated with an expression vector, pPIC9K.

3.3.2 Preparation of an expression vector, pPIC9K

The expression vector, pPIC9K, was used to carry anti-lipopolysaccharide factor isoform 2 gene. The pPIC9K is known to allow the generation of multicopy gene insert in *Pichia* and secretion of expressed protein product. It contains *kan* gene which confers resistance to geneticin in *Pichia*. This property is used to screen the transformants that harbor multiple copies of the interesting gene. The vector utilizes the *AOX1* promoter for inducible, high-level expression. The ALF*Pm*2 gene was inserted downstream of the 5' *AOX1*. According to *Pichia* expression of ALF*Pm*3, two proteins were detected: major and minor products with molecular weight of 11,314.17 ± 0.34 Da and 11,514.83 ± 0.54 Da, respectively (Somboonwiwat et al., 2005). Edman degradation revealed that the major product was the expected mature rALF*Pm*3 including the additional tyrosine and valine residues. The minor product contained the extra amino acid residues resulted from the inefficient STE13 protease cleavage. To avoid the above problem, the mature ALF*Pm*2 gene was inserted after the Kex2 signal cleavage site. Following the same strategy as Cabral et al. (2003) who expressed *Ps*d1 gene in *Pichia pastoris*, the ALF*Pm*2 gene was inserted into the pPIC9K vector at *Xho*I site upstream of Kex2

signal cleavage site (Figure 3.13). The method could eliminate the STE13 protease site. The outcome was a recombinant protein that did not contain the extra amino acid residues.

The pPIC9K was digested with *Xho*I and *Not*I restriction enzymes. Since there were two sites of *Xho*I (Figure 3.14), the pPIC9K was first partially digested with *Xho*I and then digested with *Not*I. The digest was analyzed on 0.7% agarose gel to isolate the 9,234 bp pPIC9K fragment (Figure 3.15). The vector fragment was excised from the agarose gel purified.

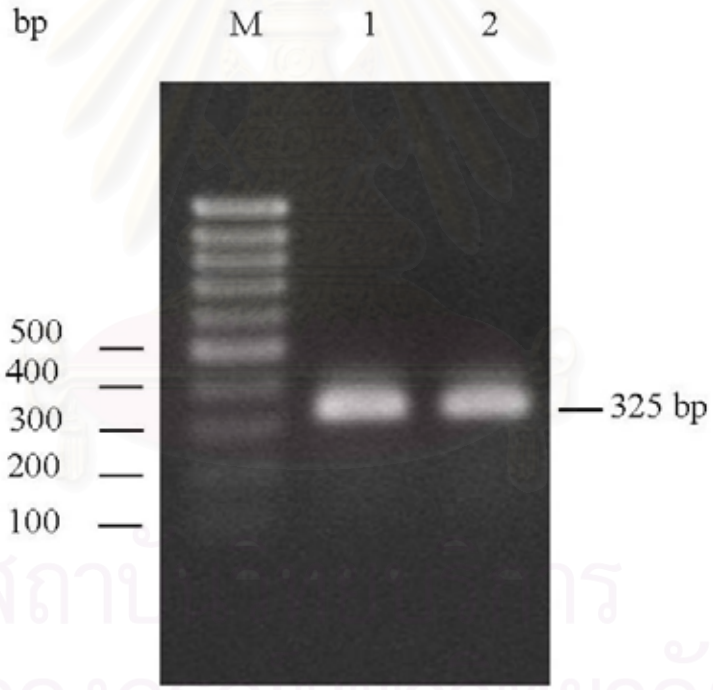


Figure 3.12 Agarose gel electrophoresis of ALF*Pm*2 gene amplified by PCR. The PCR product was run on 1.5% agarose gel at 100 volts for 1 h.
Lane M: Standard 100 bp DNA ladder
Lanes 1 and 2: The PCR product of ALF*Pm*2 gene.

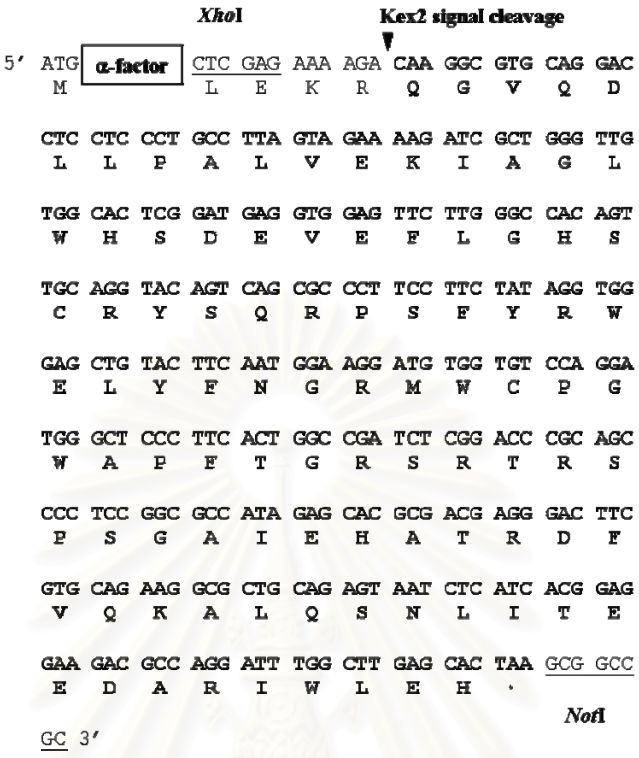


Figure 3.13 The nucleotide and deduced amino acid sequences of cloned DNA sequence of ALF*Pm*2 in the expression vector pPIC9K (pALF*Pm*2). The ALF*Pm*2 gene encoding the mature peptide, shown in bold, was inserted in-frame with the α-factor secretion signal sequence between *Xho*I and *Not*I sites (underlined). Arrow above the nucleotide sequence indicates the Kex2 clevage site necessary for proteolytic processing of the fusion protein between the α-factor secretion signal and ALF*Pm*2.

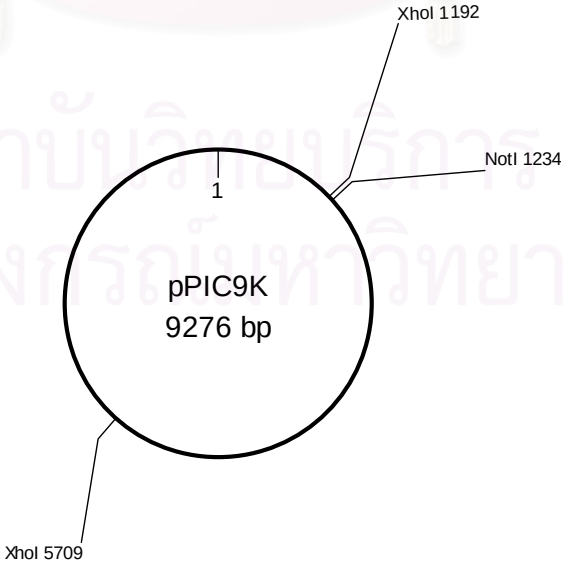


Figure 3.14 The circular map of pPIC9K vector showing the *Xho*I and *Not*I restriction sites.

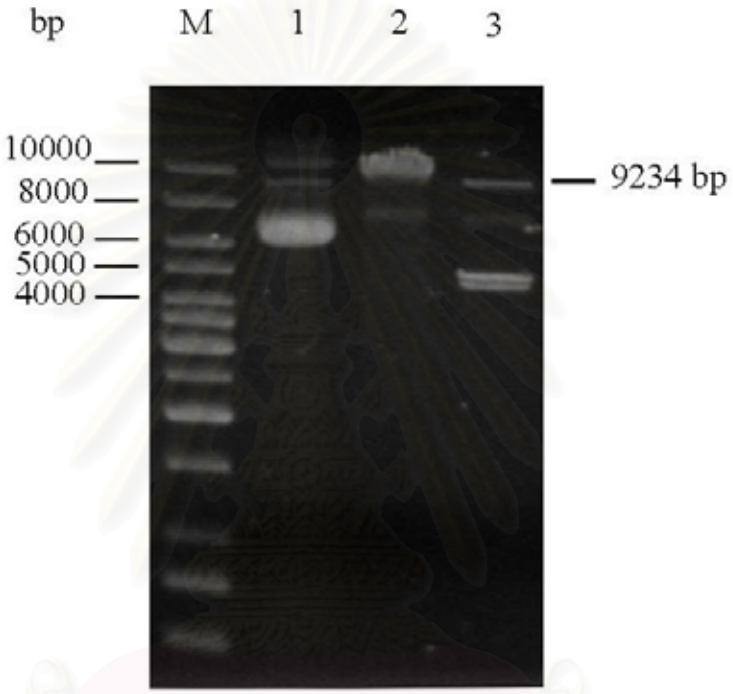


Figure 3.15 Agarose gel electrophoresis of pPIC9K expression vector digested with *Not*I and *Xho*I. The digests were run on a 1.2% agarose gel at 100 volts for 1h.

Lane M: Standard DNA ladder (100 bp marker)

Lane 1: pPIC9K

Lane 2: pPIC9K completely digested with *Not*I

Lane 3: pPIC9K completely digested with *Not*I and partially digested with *Xho*I.

3.3.3 Construction of expression vector

The purified *Xho*I-*Not*I ALF*Pm*2 fragment was ligated into the *Xho*I/*No*tI-digested pPIC9K. Then, the ligation mixture was transformed into the *E.coli* JM109. The colony PCR technique was used for screening of the desired recombinant expression plasmid. The plasmid was extracted from the positive colony and subjected to sequencing to confirm the correct construction. The plasmid was named the pALF*Pm*2.

3.3.4 *Pichia* transformation

To transform the yeast cells, the pALF*Pm*2 was linearized with *Sac*I and transformed into *Pichia pastoris* by electroporation. The transformants were spread on the MD plates before screening for G418 resistance. The transformants were screened on YPD plates containing 0.25, 0.75 and 1 mg/ml G418. The resistant clones that grew on the YPD plates containing G418 were tested to determine the presence of integrated ALF*Pm*2 expression cassette in *Pichia* genome by PCR amplification (Figure 3.16). The α-signal peptide and the 3'-AOX primers were used for the amplification of *Pichia* genome. The parental plasmid pPIC9K provided 197-bp PCR product while the pALF*Pm*2 gave 464-bp PCR product as expected. All selected transformants contained the integrated ALF*Pm*2 gene in *Pichia* genome (Figure 3.16). Because the G418 hyper-resistance clones inferred the multiple copies of ALF*Pm*2 gene in *Pichia* genome, the hyper-resistance clone which could grown on YPD plates containing 1 mg/ml G418 were selected for expression.

3.3.5 Expression of recombinant clones

The hyper-resistance clones were selected for the expression. Each clone was grown in YPD broth at 30 ºC for overnight. The starter was inoculated in BMGY media to increase the number of *Pichia* cells. Then, the *Pichia* cells were harvested and resuspended in BMMY media. In the BMMY media, the *Pichia* cells were treated with 0.5% methanol to induce the expression of ALF*Pm*2. Methanol was added to a final concentration of 0.5% every 24 h to maintain the induction. After 6

days, the culture was collected and centrifuged to separate the supernatant. The supernatant was analyzed using 16.5% Tricine SDS-PAGE and the proteins were detected by silver staining (Figure 3.17). The crude recombinant ALF*Pm*2 protein was then analyzed by MALDI-TOF mass spectrometer to determine its molecular mass (Figure 3.18). The size of ALF*Pm*2, the major protein, was 11,367.216 Da closed to the estimated molecular weight. The supernatants were also assayed for antimicrobial activity by liquid growth inhibition assay. The highest rALF*Pm*2 expressed clone was further scaled up for protein production and purification.

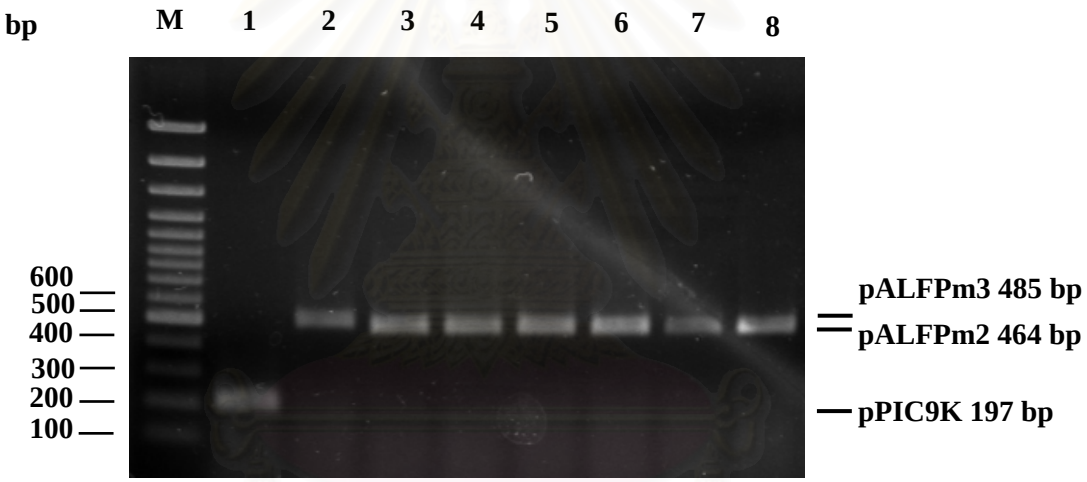


Figure 3.16 Agarose gel electrophoresis of the direct PCR screening of *Pichia* clones containing ALF*Pm*2 gene. The PCR products were run on 1.2% agarose gel at 100 volts for 1h

Lane M: Standard 100 bp DNA ladder

Lane 1: PCR product of *Pichia* clone transformed with parental plasmid pPIC9K.

Lane 2: PCR product of 1 mg/ml G418 resistant *Pichia* transformant containing the integrated pALF*Pm*3.

Lane 3-8: PCR product of 1 mg/ml G418 resistant *Pichia* transformant containing the integrated pALF*Pm*2.

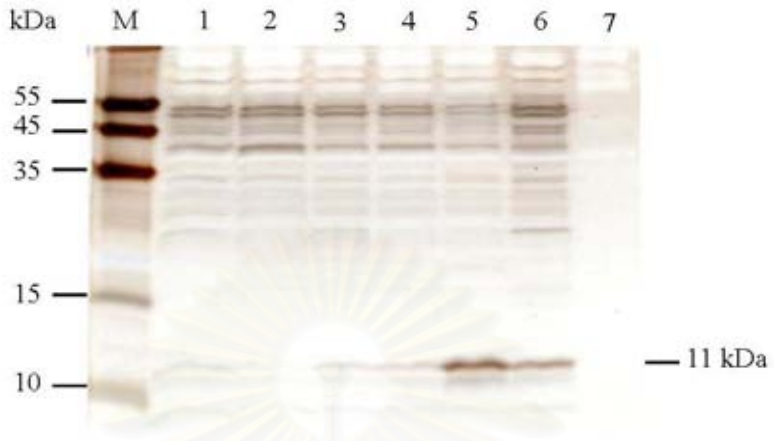


Figure 3.17 Silver-stained 16.5% Tricine SDS-PAGE analysis of the expressed rALF*Pm*2 protein from *P. pastoris* transformants.

Lane M: Protein marker

Lanes 1 to 6: The culture supernatants of different *P. pastoris* transformants containing rALF*Pm*2

Lane 7: The culture supernatants of *P. pastoris* transformants containing parent vector

Figure 3.18 MALDI-TOF mass spectrometric determination of the molecular weight of the rALF*Pm*2.

3.3.6 Purification of anti-lipopolysaccharide factor isoform 2

The crude recombinant ALF*Pm*2 protein was further purified by cation exchange chromatography using Hitrap SP column. The crude rALF*Pm*3 in 50 mM MES, pH 5.6, was loaded into the Hitrap SP column. Then, the rALF*Pm*2 was eluted with 1 M NaCl in 50 mM MES buffer pH 5.6. The protein fractions were collected and the A_{280} was determined. Coomassie blue-stained SDS-PAGE analysis revealed only one band of rALF*Pm*2 protein in the eluted fraction. The fractions also exhibited antimicrobial activity against *Escherichia coli* 363 (Gram-negative bacterium) and *Bacillus megaterium* (Gram-positive bacterium) (Figure 3.19)

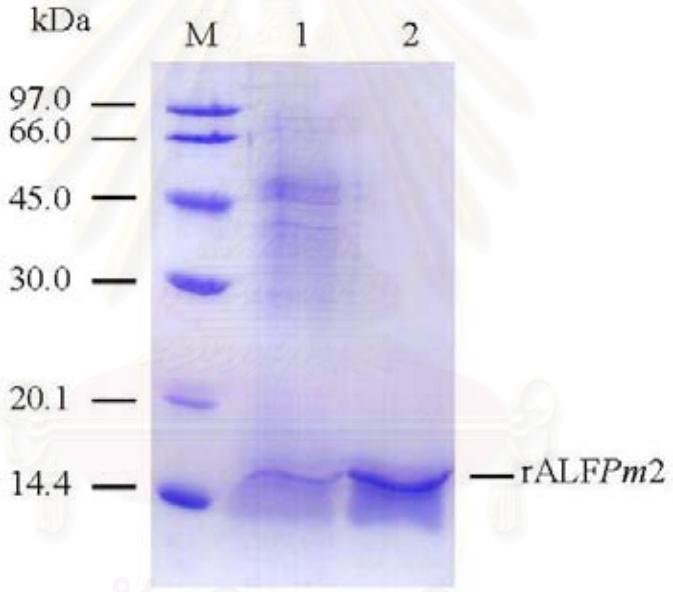


Figure 3.19 The 15% SDS-PAGE of rALF*Pm*2 and purified rALF*Pm*2. The crude rALF*Pm*2 from the culture medium and the purified rALF*Pm*2 were analyzed by 15% SDS-PAGE electrophoresis and stained with Commassie blue.

Lane M: Protein marker

Lane 1: The crude rALF*Pm*2 protein

Lane 2: The purified rALF*Pm*2 protein

3.3.7 Antimicrobial activity of anti-lipopolysaccharide factor isoform 2

The purified rALF*Pm*2 protein was further tested for its antimicrobial activity against Gram-positive and Gram-negative bacteria. The minimum inhibitory concentration (MIC) values were determined by the liquid growth inhibition assay (Shafer, 1997). The yield of purified rALF*Pm*2 protein was low because the protein tended to precipitate during storage at 4 ºC. Thus, the maximum concentration of purified rALF*Pm*2 for MIC available for the test was only 9.5 μM as compared to 100 μM, the starting concentration, used in the MIC test. The rALF*Pm*2 exhibited antimicrobial activity against *E. coli* 363 (MIC 1.56-3.12) and *B. megaterium* (MIC 1.56-3.12). There are no antimicrobial activities at 9.5 μM against the other bacteria: Gram-positive bacteria: *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus hamolyticus*, and Gram-negative bacteria: *Enterobacter clocae*, *Erwinia carotovora*, *Klebsiella pneumoniae*, *Salmonella thyphimurium*, *V. anguillarum and V. harveyi*. Obviously, the MIC values of ALF*Pm*2 against all tested bacteria were lower than those of ALF*Pm*3 (Table 3.1) except that against *S. aureus* which could not be compared because the maximum concentration of ALF*Pm*2 was already lower than the MIC value of ALF*Pm*3.

Table 3.1 Concentration range of rALF*Pm*2 and 3 against various strains of microorganisms in liquid growth inhibition assay.

Microorganisms	MIC value (μM)	
	rALF*Pm*2	rALF*Pm*3
Gram (+) bacteria		
Bacillus megaterium	1.56-3.12	0.19–0.39
Micrococcus luteus	> 9.5	1.56–3.12
Staphylococcus aureus	> 9.5	50–100
Gram (-) bacteria		
Enterobacter cloacae	> 9.5	3.12–6.25
Erwinia carotovora	> 9.5	1.56–3.12
Escherichia coli 363	1.56-3.12	0.095–0.19
Klebsiella pneumoniae	> 9.5	3.12–6.25
Salmonella thyphimurium	> 9.5	6.25–12.5
Vibrio anguillarum	> 9.5	0.78–1.56
Vibrio harveyi	> 9.5	0.78–1.56

* MIC are expressed as the interval *a*-*b*, where *a* is the highest concentration tested at which microorganisms are growing and *b* the lowest concentration that causes 100% growth inhibition.

3.3.8 Antiviral activity of anti-lipopolysaccharide factor

The hematopoietic cell culture from the signal crayfish, *Pacifastacus leniusculus*, shown to be susceptible to the white spot syndrome virus (WSSV) was used for antiviral study. The WSSV propagated in the hematopoietic cells (hpt cells) was detected by RT-PCR using specific primers for WSSV VP28 mRNA. The mixtures of WSSV with either rALF*Pm*2 or 3 were incubated with hematopoietic cell cultures at 20 ºC. After 2 h, the medium was removed and replaced by fresh medium. The cultures were incubated at 20 ºC for 36 h. The infection of WSSV was then determined by RT-PCR of the VP28 gene. The 40S ribosomal, crayfish housekeeping gene, was used as an internal control. The results showed that the 20 μM of rALF*Pm*3 but not rALF*Pm*2 exhibited the antiviral activity against the white spot syndrome virus (WSSV) by inhibiting the infection of the hematopoietic cell by WSSV (Figure 3.20 and 3.21).

To assess the inhibitory activity of ALF*Pm*3 on WSSV propagation, the purified ALF*Pm*3 was tested. The purified ALF*Pm*3 were serially diluted two-fold (20, 10, 5 and 2.5 μM) and incubated with crayfish cell culture in the presence or absence of WSSV. The cell culture infected with WSSV was used as negative control. After incubated at 20 ºC for 2 h, the medium was removed and replaced by fresh medium. The cultures were further incubated at 20 ºC for 36 h. Total RNA was extracted and used for cDNA synthesis. The infection of WSSV was then determined by quantitative RT-PCR of the VP28 gene. The 40S ribosomal gene, crayfish housekeeping gene, normalized the expression of the mRNA encoding the VP28 gene. The experiment was repeated three times. Quantitative RT-PCR data were analyzed by comparative quantification. Dissociation curve of VP28 and 40S ribosomal gene showed a single peak at expected melting temperature indicating that the intended gene was amplified and there is no other non-specific amplification or primer-dimer (Figure 3.22). Figure 3.23 shows that ALF*Pm*3 has an effect on WSSV propagation in crayfish cell culture. At high concentration of ALF*Pm*3, WSSV propagation was lower. The WSSV propagation was considered lowest after 10 μM of ALF*Pm*3. The concentration of ALF*Pm*3 required for 50% inhibition of viral propagation *in vitro* (IC50) was less than 2.5 μM (Figure 3.23).

To determine the cytotoxicity of ALF*Pm*3 on hpt cells, the trypan blue exclusion test was performed on hpt cultures that were incubated with ALF*Pm*3. Viable cells with intact cell membranes were observed as clear white cells while the dead cells were stained with trypan blue. Because only viable cells can select the compounds that pass through the membrane, trypan blue was selectively excluded from the viable cells. Incubation of hpt cultures with 5, 10 and 20 μM of ALF*Pm*3 for 30 min at 20 ºC showed that the viable cells were slightly decreased after the cultures were incubated with higher concentration of ALF*Pm*3 (Figure 3.24). For each experiment, the number of viable cells was counted in three areas of one well. The viable cells in hpt cultures incubated with 20 μM of ALF*Pm*3 were 96% of those without ALF*Pm*3. Thus, the concentration required to reduce the viable cells to 50% of control, CC_{50}, of ALF*Pm*3 was more than 20 μM.

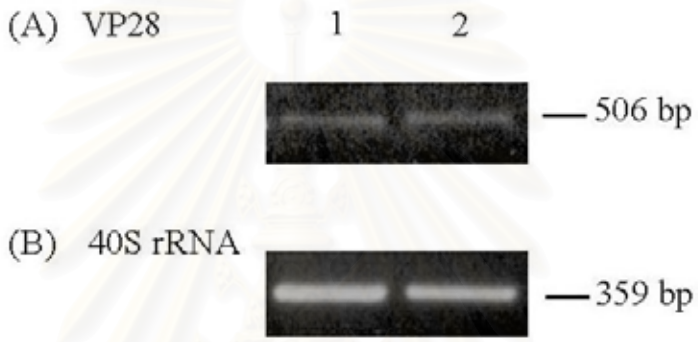


Figure 3.20 Effect of purified anti-lipopolysaccharide factor isoform 2 on WSSV in hpt cell culture. The RT-PCR analyses of VP28 (A) and 40S ribosomal RNA (B) expression were carried out.

Lane 1: Hpt incubated with WSSV and 20 μM of purified ALF*Pm*2

Lane 2: Hpt incubated with WSSV

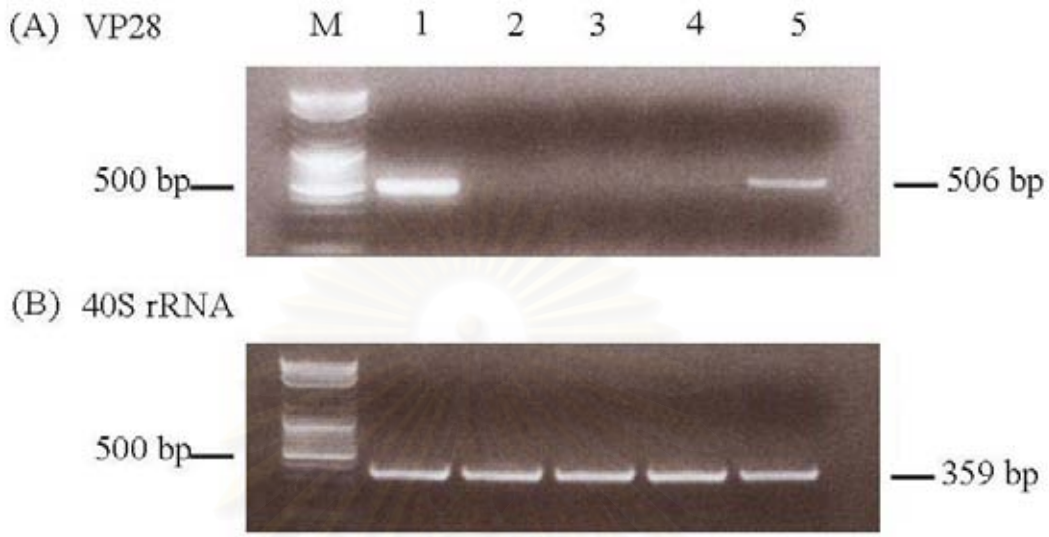


Figure 3.21 Effect of purified anti-lipopolysaccharide factor isoform 3 on WSSV in hpt cell culture. RT-PCR analyses of VP28 (A) and 40S ribosomal RNA (B) expression were carried out.

Lane 1: Hpt incubated with WSSV

Lane 2-5: Hpt incubated with WSSV and 20, 10, 5, 2.5 μM of purified ALF*Pm*3, respectively.

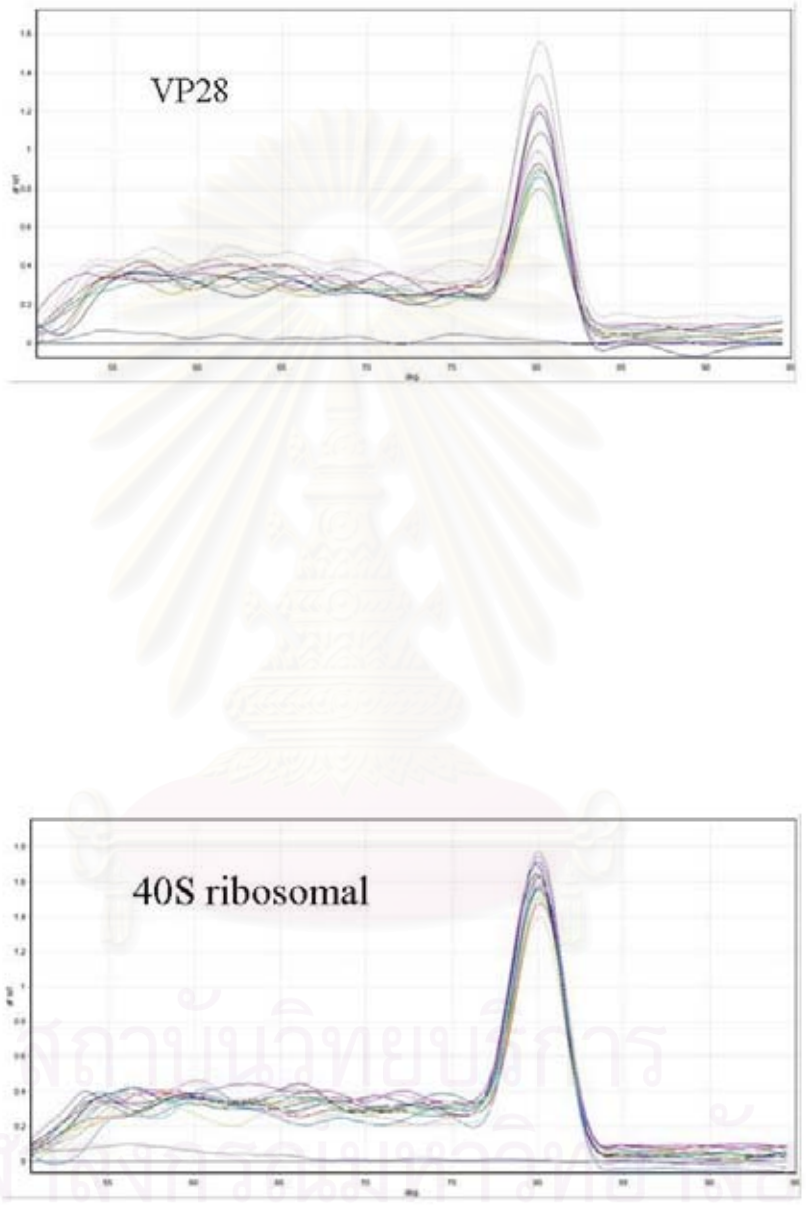


Figure 3.22 The dissociation curves of VP28 gene and 40S ribosomal RNA gene as internal control.

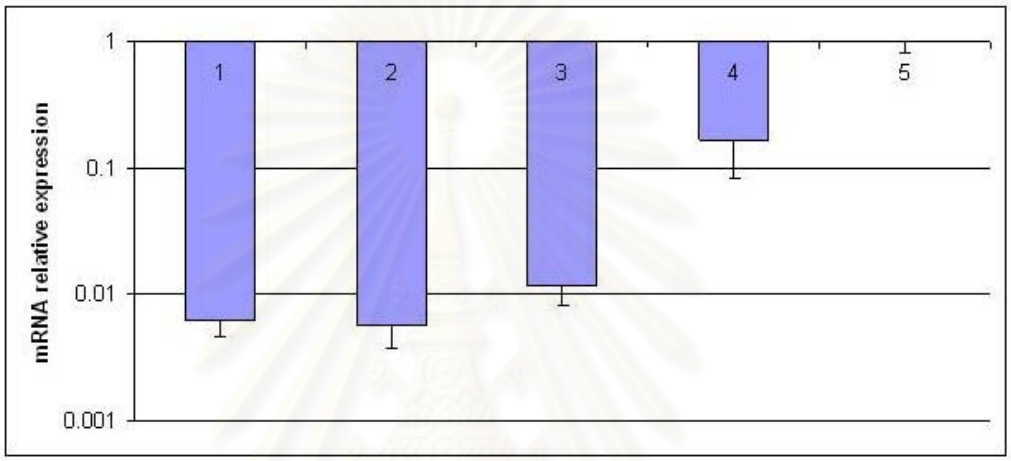


Figure 3.23 Effect of purified anti-lipopolysaccharide factor isoform 3 on WSSV in hpt cell culture. The quantitative PCR analysis of WSSV VP28 expression was carried out in hpt cell culture using 40S ribosomal RNA as an internal control.

Columns 1 to 4: Hpt incubated with WSSV and 20, 10, 5, 2.5 μM of purified ALF*Pm*3, respectively.

Column 5: Hpt incubated with WSSV only.

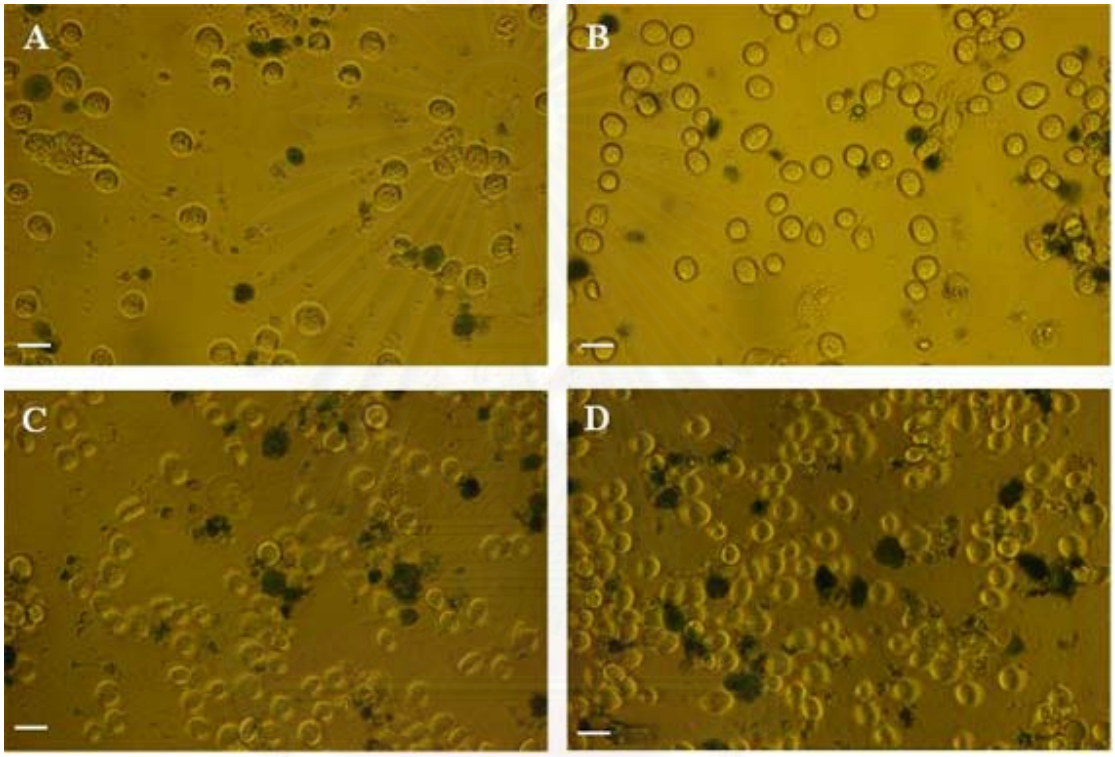


Figure 3.24 Trypan blue exclusion test of hpt cultures treated with ALF*Pm*3 or ALF*Pm*3 and WSSV. A, B, C and D: Hpt cultures incubated with water, 5, 10, and 20 μM of purified ALF*Pm*3, respectively.
Scale bars = 10 μm

3.3.9 Antiviral mechanism of ALF*Pm*3

To further investigate the antiviral mechanism of ALF*Pm*3, the effect of ALF*Pm*3 on WSSV attachment to the cell culture was studied. A condition that slowed down the infection, i.e. most WSSV particles possibly attached to the hpt cells but had not entered the cells, was selected for this experiment. Under this condition, if the virus is blocked from binding to the cells, low propagation would be observed. According to the previous report, the mortality of WSSV-injected crayfish and WSSV propagation in crayfish cell culture were reduced at low temperature. For example, at 4 ± 2 ºC and 12 ± 2 ºC, no mortality was observed in crayfish while crayfish had 100% mortality at 20 ± 2 ºC at 12 day post-injection of WSSV. It was concluded that the water temperature influenced the development of the virus (Jiravanichpaisal et al., 2004). Time-course study of WSSV propagation in hpt cell culture revealed that the WSSV propagation in the hpt cells were lower at low temperature (below 16 ºC) (Jiravanichpaisal et al., 2006). It also showed that only the bound viral fraction gave rise to viral propagation, i.e. the hpt cells were incubated with WSSV at low propagation condition and washed to remove non-bound virus, the level of VP28 expression is measurable.

In this experiment, a mixture of WSSV and 5 μM of ALF*Pm*3 was incubated with hematopoietic cell cultures at 11 ºC and 20 ºC. For a control, only WSSV was incubated with hpt cell culture. After 2 h of incubation, the medium was removed completely and washed 2 times with CPBS. The CPBS buffer was replaced completely with fresh medium. The incubation was continued at 20 ºC for 36 hours. The infection of WSSV was then determined by RT-PCR as described above. At 11 ºC and 20 ºC, no propagation of WSSV was observed in the presence of ALF*Pm*3 while WSSV replicated normally in the control culture (Figure 3.25). It was, therefore, likely that the ALF*Pm*3 prevented the entry of WSSV into the hpt cells.

To test whether the ALF*Pm*3 had any influence on the cells that prevent the viral infection, the cells were treated first with ALF*Pm*3 followed by WSSV after excess ALF*Pm*3 was removed. In the experiment, the ALF*Pm*3 was incubated with hpt cell cultures at 20 ºC for 30 min. The cultures were either washed or not washed with CPBS buffer. The WSSV in fresh medium was added after the old medium or the CPBS buffer was removed. After incubated at 20 ºC for 2 h, the

medium was removed and replaced by fresh medium. The cultures were incubated further at 20 ºC for 36 h. The infection of WSSV was then determined by RT-PCR as described above. Figure 3.26 shows that the effect of ALF*Pm*3 on WSSV propagation was not observed after it was removed. The WSSV replicated normally after the ALF*Pm*3 was removed.

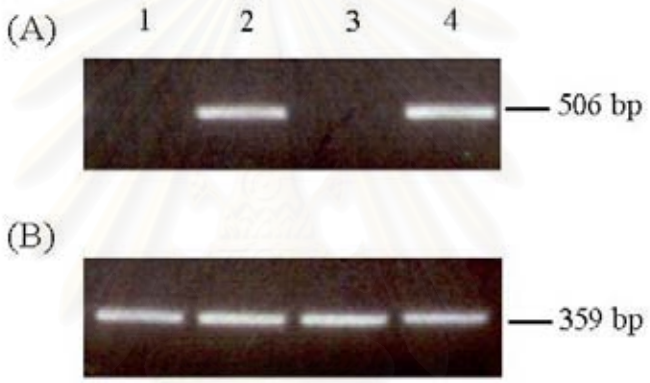


Figure 3.25 Effect of purified anti-lipopolysaccharide factor isoform 3 on WSSV in hpt cell culture at 11 ºC and 20 ºC. RT-PCR analysis of VP28 (A) and 40S ribosomal RNA (B) expression was carried out.

Lanes 1 and 3: Hpt incubated with WSSV and 5 μM of purified ALF*Pm*3 at 20 ºC and 11 ºC, respectively;

Lanes 2and 4: Hpt incubated with WSSV at 20 ºC and 11 ºC, respectively.

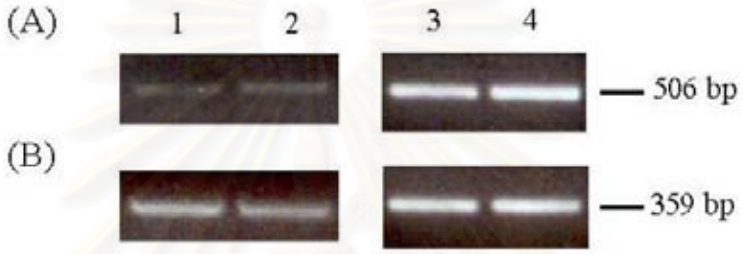


Figure 3.26 Effect of purified anti-lipopolysaccharide factor isoform 3 on WSSV in hpt cell culture. RT-PCR analysis of VP28 (A) and 40S ribosomal RNA (B) expression was carried out.

Lane 1: Hpt incubated with water for 30 min, washed with C-PBS buffer and added WSSV

Lane 2: Hpt incubated with 5 μM of purified ALF*Pm*3 for 30 min, washed with C-PBS buffer and added WSSV

Lane 3: Hpt incubated with water for 30 min and then added WSSV

Lane 4: Hpt incubated with 5 μM of purified ALF*Pm*3 for 30 min and added WSSV.

CHAPTER IV

DISCUSSION

Occasionally, the aquaculture of shrimp is threatened by the severe outbreak of pathogenic microorganisms. The diseases have caused a decrease in shrimp production. The causative agents of such infectious diseases in shrimp are mainly viruses and bacteria belonging to the *Vibrionacea*. To alleviate the problem, the farmers are apt to use the antibiotics though there are negative consequences of antibiotic contamination in the shrimp produces. Alternatively and beneficially, the problem can be controlled by using the knowledge and immune agents from the study of the shrimp innate immune system. The antimicrobial agents are such immune agents found in the innate immune system of shrimp and potentially useful for aquaculture.

Recently, five isoforms of anti-lipopolysaccharide factor (ALF) were identified from the hemocyte cDNA libraries of the *P. monodon* EST database, namely ALF*Pm*1-5. They were predominantly found in the hemocyte libraries of *V. harveyi*-infected shrimp suggesting that they might be involved in the defense mechanism against the pathogenic bacteria. The ALF*Pm*3 was the predominant isoform among the *P. monodon* ALFs and had been characterized by Somboonwiwat et al.(2005). In their study, a cDNA encoding ALF*Pm*3 was cloned into a yeast expression vector and expressed in the yeast *Pichia pastoris* expression system. The protein was produced and assayed for antimicrobial activity against several strains of bacteria and fungi. The purified recombinant ALF*Pm*3 protein (rALF*Pm*3) exhibited broad antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria and fungi.

In this study, the nucleotide and amino acid sequences of the five isoforms of ALF were subjected to further analyses. Upon sequence alignment, the similarity had undoubtedly grouped the ALFs into two groups, ALF*Pm*1-2 and ALF*Pm*3-5 designated as group A and B, respectively. The two groups possessed different putative lipopolysaccharide binding sequences from which different antimicrobial

activities might be inferred. The presence of the two groups of ALF cDNAs indicated that they were encoded from different loci in the genome as suggested by Supungul et al.(2004)

To study the genome arrangement of the two ALF genes, genome walking technique was used for approaching the unknown DNA sequences adjacent to a known DNA sequences. Amplification of the genomic ALF genes was also performed to unveil the intron-exon structures. In the genome walking technique, the four DNA libraries of shrimp genomic DNA were constructed and used for the nested PCR reactions using the gene specific nested PCR primers. Although there were more than one PCR products after the second PCR reaction, the specific products were identified by restriction enzyme digestion. The PCR products were cloned and subjected to nucleotide sequencing.

The results from genomic amplification of ALF genes and genome walking to identify the 5' upstream regions, the genomic DNA sequences of two separate ALF loci were obtained. The analysis illustrated that either the ALF*Pm*1 and 2 or ALF*Pm*3, 4 and 5 were, indeed, derived from the same genomic genes. The isoforms were generated by alternative splicing of the same pre-mRNAs. The mRNAs of ALF*Pm*1 and 2 contained the second exons of different lengths owing to the presence of two 5' spliced sites. The second intron of ALF*Pm*4 mRNA was retained rather than spliced out as in the ALF*Pm*3 mRNA.

With additional sequences in the mRNAs, the reading frames of ALF*Pm*1 and 4 were shorter. The ALF*Pm*1 contained the amino terminal part of the protein and the lipopolysaccharide binding sequence. Whether the ALF*Pm*1 was active was not experimentally confirmed. The ALF*Pm*4 was obviously inactive for it contained only the amino terminal part of the protein without the lipopolysaccharide binding sequence. The results suggested that the ALF*Pm*1 and 4 were aberrant products of the pre-mRNA splicing. Supporting the suggestion came from the RT-PCR results that the amounts of ALF*Pm*1 and 4 mRNAs were considerably lower than those of ALF*Pm*2 and 3. Therefore, the ALF*Pm*2 and 3 were the major ALFs in the shrimp hemocytes.

As far as the regulation of gene expression is concerned, there are no report about the promoter and regulatory sequences involved in the ALF gene expression. It

was then interesting to analyze the 5' upstream sequences of the ALF genes. By sequence searching against the TRANFAC regulatory sequence database, the 5' upstream sequences of ALF groups A and B were marked with a variety of putative *cis*-regulatory elements. The *cis*-regulatory elements, known to be involved in immune response and/or regulate the expression of antimicrobial peptides, such as nuclear factor (NF)-kappaB, GATA, activator protein 1 (AP-1), GAAA and Octamer (Oct-1) motifs, were particularly interesting as they were identified in the upstream regions of the *P. monodon* ALF genes.

The NF-kappaB motif is important for inducible production of antimicrobial peptide in mammals, amphibian and insect (Beinke and Ley, 2004; Engstrom, 1999; Miele et al., 2001; Mineshiba et al., 2005; Tsutsumi-Ishii and Nagaoka, 2002). In *Drosophila*, the kappaB-like site is necessary for tissue-specific and LPS-inducible expression of cecropin A1 and diptericin genes (Engstrom et al., 1993; Kappler et al., 1993; Meister et al., 1994; Reichhart et al., 1992). Despite the transcription level of ALF group B is higher than that of ALF group A upon *V. harveyi* challenge, a putative NF-kappaB motif was only found in the upstream sequence of ALF group A.

The GATA site is required for the activity of penaeidin 2 promoter of *Litopenaeus vannamei* (O'Leary and Gross, 2006). Seven and five putative GATA sites were found in the ALF group A and B genes, respectively. The activator protein 1 (AP-1) factor is involved in the cell proliferation and survival (Karin et al., 1997), and also produced in response to viral infection (Douglas et al., 2003). The GAAA motif is recognized by interferon regulatory factor I (IRF-1) upon transcriptional activation of interferon-stimulated genes in virus-infected mammal cells (Au et al., 1993). The presence of AP-1 and GAAA motifs in the upstream sequences perhaps regulated the transcription of the *P. monodon* ALF in response to viral infection. Recent study indicated that ALFs could interfere with the propagation of the major viral pathogen, white spot syndrome virus (Liu et al., 2006). The GAAA motif had also been shown to cooperate with the NF-kappaB motif in the LPS-induced expression of diptericin (Georgel et al., 1995). The Octamer-binding site 1 (Oct-1) is often found in the enhancer, and promotes the transcription of immune-relevant genes (Douglas et al., 2003). The putative Oct-1 sites were found in the *P. monodon* ALF

genes. Nevertheless, whether the above selected motifs were functional and involved in the regulation of the *P. monodon* ALF expression needed further investigation.

The ALF cDNAs were found predominantly in the hemocyte libraries of *V. harveyi*-infected shrimp as reported by Supungul et al. (2004). Subsequently, it was shown by RT-PCR analysis that the expression of ALF*Pm*3 was up-regulated upon injection of *V. harveyi* with the highest expression at 6 hpi (Somboonwiwat, 2004). In this study, the RT-PCR experiment with the RNAs isolated from the hemocytes of normal and six-hour *V. harveyi*-challenged shrimp confirmed the above notion. The transcriptional levels of ALF*Pm*2 and 3 were significantly increased in response to *V. harveyi* injection suggesting an important role of both ALF isoforms in shrimp immune response. The expression of ALF*Pm*4, minor isoform, was also up-regulated in *V.harveyi* infection shrimp. To more understand the role of ALF*Pm*, the ALF*Pm*2 which was a major isoform of ALF group A was over-produced as recombinant protein. Its properties and antimicrobial activities were studied as compared with ALF*Pm*3.

The ALF*Pm*2 was over-produced using the *P. pastoris* expression system. This system was chosen because *P. pastoris* is a eukaryote and possesses many of the advantages of higher eukaryotic expression system such as protein processing, protein folding and posttranslational modification (Cregg, 2007; Cregg et al., 1985). Being a single cell eukaryote, it is easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. The expression vector pPIC9K contains the alcohol oxidase promoter (P_{AOX1}) that drives the heterologous protein expression in *P. pastoris*. Upon transformation into *P. pastoris*, the expression clone is integrated into the host genome as single or multiple copies or “expression cassettes”. The kanamycin gene in the expression cassette is then useful for screening the highest expression clone. Kanamycin gene confers resistance to geneticin or G418 in *P. pastoris*. The level of G418 resistance roughly depends on the number of kanamycin gene. One can infer from G418 hyper-resistance whether the clone in question contains multiple copies of interesting gene. With multiple copies of the gene, protein expression can be increased.

Paus et al. (2002) attempted to express the *Limulus polyphemus* endotoxin neutralizing protein in *E. coli* and the Gram-positive bacterium, *Bacillus* sp. but were unsuccessful. The protein was successfully expressed in *P. pastoris* expression system.

The expression of other antimicrobial peptides were also successful in *P. pastoris* expression system, for example, cecropin (Jin et al., 2006), penaedin (Kang et al., 2007; Li et al., 2005), anti-lipopolysacchairde factor (Somboonwiwat et al., 2005) and defensin1 (Cabral et al., 2003).

In this study, the rALF*Pm*2 gene was cloned downstream of the α-factor in pPIC9K. The α-factor is sometimes removed by the somewhat inefficient STE13 protease cleavage which was observed in *P. pastoris* expression of ALF*Pm*3 (Somboonwiwat et al., 2005). The mature ALF*Pm*2 gene sequence was inserted after the more efficient Kex2 signal cleavage site following the same strategy as Cabral et al. (2003) who expressed *Ps*d1 gene in *Pichia pastoris*. The resulting pALF*Pm*2 was transformed into *P. pastoris*.

The transformant yeast was selected and induced for protein production by adding methanol. A protein corresponding to the expected size of rALF*Pm*2 was detected in the culture supernatant. This crude preparation also exhibited antimicrobial activity against *E. coli* 363 and *B. megeterium*. The MALDI-TOF mass spectrometer indicated that the major protein in expression culture is 11,367.216 Da close to the estimated size of rALF*Pm*2 protein.

The crude rALF*Pm*2 protein was further purified based on charge property using cation exchanger chromatography. The SDS-PAGE analysis showed that more than 90% of the elution fractions were rALF*Pm*2. These fractions also exhibited antimicrobial activity against an *E. coli* 363.

To compare the antimicrobial activity to those of other antimicrobial peptides, the minimum inhibitory concentration (MIC) values of rALF*Pm*2 protein were determined. Since the purified rALF*Pm*2 protein tended to precipitate during storage at 4 ºC, its maximum concentration of purified rALF*Pm*2 for MIC test was only 9.5 μM as compared to 100 μM, the start concentration of MIC test. Nevertheless, this concentration was used for the MIC assay. Compared to the rALF*Pm*3, the rALF*Pm*2 showed lower antimicrobial activity against *E.coli* 363 (MIC 1.56-3.12) and *B. megaterium* (MIC 1.56-3.12). No activity was observed at 9.5 μM with other bacteria: *Micrococcus luteus, Staphylococcus aureus, Staphylococcus hamolyticus Enterobacter clocae, Erwinia carotovora, Klebsiella pneumoniae, Salmonella thyphimurium, Vibrio anguillarum and Vibrio harveyi*. It was possible that the

antimicrobial activity against these microorganisms might be detected at concentration more than 9.5 μM of rALF*Pm*2. Therefore, the rALF*Pm*2 had lower antimicrobial activities than the rALF*Pm*3.

For antiviral activity, the cell culture was employed for viral propagation in the presence of rALF*Pm*2 and 3. So far, there are no continuous crustacean cell lines established, although over the past decade primary cultures from various organ sources have been used and are reported with increasing frequency in the literature. Primary cell cultures are obtained from various tissues and organs of shrimp. For example, the lymphoid (Oka) organ (Chen and Wang, 1999; Hsu et al., 1995; Itami et al., 1999; Kasornchandra et al., 1999; Natividad et al., 2007; Owens and Smith, 1999; Wang et al., 2000; West et al., 1999), the heart (Chen and Wang, 1999; Owens and Smith, 1999), hemocyte (Jiang et al., 2007), nerve cord (Nadala et al., 1993; Owens and Smith, 1999), gut (Nadala et al., 1993), hepatopancreas (Owens and Smith, 1999), and gonads (Chen and Wang, 1999; Fraser and Hall, 1999; Luedeman and Lightner, 1992; Nadala et al., 1993; Owens and Smith, 1999). Some of these has been shown to be susceptible to virus infection, for example, primary cell culture of lymphoid organs from *Penaeus monodon* (Kasornchandra et al., 1999; Wang et al., 2000), the blue shrimp, *Litopenaeus stylirostris* (Tapay et al., 1995; Tapay et al., 1997) and the kuruma shrimp, *Marsupenaeus japonicus* (Itami et al., 1999) as well as ovarian primary cultures from kuruma shrimp (Maeda et al., 2003; Maeda et al., 2004) that are susceptible to WSSV. Moreover, cell culture from freshwater crayfish, *Pacifastacus leniusculus* is also susceptible to WSSV (Jiravanichpaisal et al., 2006). Though the shrimp cell culture was available, the crayfish cell hematopoietic culture was chosen in this study because of its superior advantages for studying the virus-host model. For example, hpt cells are easily available under aseptic conditions, an initial number of cells can be calculated after dissociated from the tissues by enzyme treatment and seeded, the cells can survive in a wide range of temperature from 4 to 25 ºC, and pre-screening the animals for WSSV is not needed prior to the experiment since crayfish is not a natural host for WSSV.

The propagation of WSSV in cell culture was observed by RT-PCR using primers for detecting the VP28 gene, a major envelop protein gene of WSSV. The rALF*Pm*2 and 3 proteins were mixed with WSSV and added into the cell culture.

After incubation, the effect of ALF*Pm*2 and 3 on WSSV propagation was investigated. Only the 20 μM of rALF*Pm*3 protein inhibited the propagation of WSSV whereas the rALF*Pm*2 protein could not. This was probably due to the different amino acid sequences of the reactive domains between the ALF*Pm*2 and 3. Quantitatively, the WSSV propagation was considered lowest at ALF*Pm*3 concentration below 10 μM. The concentration of ALF*Pm*3 required for 50% inhibition of viral propagation in vitro (IC50) was less than 2.5 μM.

Since the dead cells were observed in the hpt cultures incubated with ALF*Pm*3, it was possible that the ALF*Pm*3 was toxic to the cells resulting in the decrease of WSSV propagation. To determine the cytotoxicity of ALF*Pm*3 to the hpt cells, the trypan blue exclusion protocol was used for the detection of cell viability. It was found that the concentration of ALF*Pm*3 required to reduce the viable cells to 50% of control (hpt cells without ALF*Pm*3), CC_{50}, was more than 20 μM. In fact, the viable cells in hpt cultures incubated with 20 μM ALF*Pm*3 were 96% of the control. At concentrations less than or equal to 20 μM of ALF*Pm*3, the small amount of dead cells should not have any affect on WSSV propagation.

In another experiment, ALF*Pm*3 and WSSV were mixed, added into the cell culture and incubated at 20 ºC for 2 h. Then, the medium was removed completely, washed twice with CPBS buffer and replaced completely with fresh medium. The culture was incubated further at 20 ºC for 36 h. By RT-PCR, no propagation was detected in the presence of ALF*Pm*3. This implied that the WSSV was unable to attach itself to the cell and was removed during CPBS washing. It was possible that the ALF*Pm*3 interfered with the binding of WSSV to the cells.

To test whether the ALF*Pm*3 had any influence on the cells that prevented the viral infection, the cells were treated first with ALF*Pm*3 followed by WSSV after ALF*Pm*3 was removed. The effect of ALF*Pm*3 on WSSV propagation was not observed. The WSSV replicated normally after the ALF*Pm*3 was removed.

Mechanism of WSSV infection is still unknown. Integrins, transmembrane proteins that recognize a large variety of extracellular and cell surface proteins, have emerged as receptors or co-receptors for a large number of viruses. Several studies have reported that many RGD (Arg-Gly-Asp)-containing viral proteins serve as ligands through which these viruses bind to the integrins on the cell surface and then

gain entry into the cells. Many envelope proteins of WSSV contain RGD motifs including VP281, VP31, VP36A, VP110, VP136A, VP664 and VP187 (Huang et al., 2002; Li et al., 2006; Tsai et al., 2004). Recently, there was a report indicated that shrimp β-integrin could bind to a WSSV envelope protein VP187 and might be partly involved in WSSV infection (Li et al., 2007). This finding suggested that the β-integrin might function as a cellular receptor for WSSV. The PmRab7 might be a receptor for VP28 envelope protein of WSSV in shrimp. It bound directly and had the specific binding to a major viral envelope protein. In addition, the depletion of PmRab7 by using dsRNA injection into the shrimp inhibited viral propagation (WSSV and YHV). These results suggested that the PmRab7 is a common cellular factor required for WSSV or YHV propagation in shrimp. Because the PmRab7 should function in the endosomal trafficking pathway, its silencing prevents successful viral trafficking necessary for propagation (Ongvarrasopone et al., 2008; Sritunyalucksana et al., 2006). The effect of ALF*Pm*3 on WSSV propagation might not involve in the blocking of binding between β-integrin and RGD motif because ALF*Pm*3 did not contain the RGD motif. The ALF*Pm*3 probably bound directly to the WSSV and prevented its entry into the cell. Whether the ALF bound to the WSSV awaited experimentation.

CHAPTER V

CONCLUSIONS

Anti-lipopolysaccharide factors from *Penaeus monodon* are encoded by two genomic loci. The ALF*Pm*1 and 2 or ALF*Pm*3, 4 and 5 were derived from the same genes. The isoforms were generated by alternative splicing of the same pre-mRNAs. ALF*Pm*1 mRNA resulted from the 5´-alternative splicing mode of the primary transcript of ALF*Pm*2 whereas the primary transcript of ALF*Pm*3 transcribed to ALFPm4 mRNA by intron retention mode.

The putative promoters of the *P. monodon* ALF groups A and B were identified at positions −31 and −29, respectively. Several transcription factor-binding sites, including Octamer (Oct-1), GATA, CCAAT box and GAAA motifs, were predicted in the 5′ upstream sequences of both the ALF group A and B genes. A putative activator protein 1 (AP-1), nuclear factor (NF)-kappaB binding sites, however, were only found at the 5′ upstream of the ALF group A gene.

The ALF*Pm*3 transcript was the predominant species followed by the ALF*Pm*2 transcript. Low expression of ALF*Pm*1 and 4 suggested that they might probably be aberrant splicing products of the ALF*Pm*2 and 3, respectively. The transcription levels of ALF*Pm*2, 3 and 4 were significantly increased in the hemocytes of *V. harveyi*-challenged shrimp.

A recombinant protein of anti-lipopolysaccharide factor isoform 2 from the black tiger shrimp (rALF*Pm*2) was successfully produced in the yeast *Pichia pastoris* system. The crude recombinant ALF*Pm*2 protein has the molecular mass of 11,367.216 Da. The purified rALF*Pm*2 protein had antimicrobial effect against the *Escherichia coli* 363 and *Bacillus megaterium*, representatives of Gram-negative and Gram-positive bacteria, respectively. The 20 μM of rALF*Pm*3 but not rALF*Pm*2 exhibited the antiviral activity against the white spot syndrome virus (WSSV).

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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

Tris (hydroxymethyl)-aminomethane	18.17	g

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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2	g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06	g

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

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

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

Solution B (SDS PAGE)

2.0 M Tris-HCl pH 8.8	75	ml
10% SDS	4	ml
Distilled water	21	ml

Solution C (SDS PAGE)

1.0 M Tris-HCl pH 8.8	50	ml
10% SDS	4	ml
Distilled water	46	ml

SDS-PAGE

15 % Seperating gel

30 % Acrylamideml solution	5.0	ml
Solution B	2.5	ml
Distilled water	2.5	ml
10% $(NH_4)_2S_2O_8$	50	µl
TEMED	10	µl

5.0 % Stacking gel

30 % Acrylamideml solution	0.67	ml
Solution C	1.0	ml
Distilled water	2.3	ml
10 % $(NH_4)_2S_2O_8$	30	µl
TEMED	5.0	µl

5X Sample buffer

1 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 min. 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 Tricine-SDS-PAGE electrophoresis

Stock reagents

49.5 % Acrylamide, 3% bis-acrylamide, 100 ml

Acrylamide	48.0	g
N,N′-methylene-bis-acrylamide	1.5	g

Adjust volume to 100 ml with distilled water.

Gel buffer: 3.0 M Tris-HCl, 0.3% SDS pH 8.45

Tris (hydroxymethyl)-aminomethane	36.4	g
SDS	0.3	g

Adjust pH to 8.45 with 1 M HCl and adjust volume to 100 ml with distilled water.

Tricine SDS-PAGE

16.5 % Seperating gel

49.5 % Acrylamide, 3% bis-acrylamide	3.3	ml
Gel buffer	3.3	ml
Glycerol	1.0	ml
Distilled water	2.3	ml
10% $(NH_4)_2S_2O_8$	50	µl
TEMED	10	µl

4.0 % Stacking gel

49.5 % Acrylamide, 3% bis-acrylamide	0.4	ml
Gel buffer	1.2	ml
Distilled water	3.3	ml
10% $(NH_4)_2S_2O_8$	40	µl
TEMED	5	µl

2X Sample buffer

1 M Tris-HCl pH 6.8	1.0	ml
Glycerol	2.4	ml
SDS	0.8	g
2-Mercaptoethanol	0.4	ml

Commasie blue G	2	ml
Phenol red	2	ml
1 % Bromophenol blue	1.0	ml
Distilled water	0.9	ml

Adjust volume to 10 ml with distilled water

One part of sample buffer was added to one part of sample. The mixture was heated 10 min. in boiling water before loading to the gel.

Electrophoresis buffer, 1 litre

Anode buffer: 0.2 M Tris-HCl pH 8.9

Tris (hydroxymethyl)-aminomethane	24.2	g

Adjust pH to 8.9 with 1 M HCl and adjust volume to 100 ml with distilled water.

Cathode buffer: 0.1 M Tris-HCl, 0.1 M Tricine, 0.1%(w/v) SDS

Tris (hydroxymethyl)-aminomethane	12.11	g
Tricine	17.92	g
SDS	1.0	g

adjust volume to 100 ml with distilled water but do not adjust pH

3. Preparation for sliver staining solution

Solution A

Sliver nitrate	0.8	g
Distilled water	4.0	ml

Solution B

0.36% NaOH	21.0	ml
14.8 M Ammonium hydroxide	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 1% citric acid with 50 µl 3.8% formaldehyde, add water to 100 ml. Solution must be fresh

APPENDIX B

1. Independent Samples *t*-test of ALF*Pm*2 expression

Group Statistics

	Shrimp	N	Mean	Std. Deviation	Std. Error Mean
ALFPm2	1.00	4	28.6125	18.19725	9.09862
	2.00	4	72.0300	29.52976	14.76488

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ALFPm2	Equal variances assumed	.757	.418	-2.503	6	.046	-43.41750	17.34320	-85.85479	-.98021
	Equal variances not assumed			-2.503	4.991	.054	-43.41750	17.34320	-88.02298	1.18798

2. Independent Samples *t*-test of ALF*Pm*3 expression

Group Statistics

	Shrimp	N	Mean	Std. Deviation	Std. Error Mean
ALFPm3	1.00	4	117.5150	61.46113	30.73057
	2.00	4	215.8975	27.58461	13.79230

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ALFPm3	Equal variances assumed	4.620	.075	-2.921	6	.027	-98.38250	33.68376	-180.804	15.96132
	Equal variances not assumed			-2.921	4.161	.041	-98.38250	33.68376	-190.490	-6.27531

3. Independent Samples *t*-test of ALF*Pm*4 expression

Group Statistics

	Shrimp	N	Mean	Std. Deviation	Std. Error Mean
ALFPm4	1.00	4	11.4900	7.63959	3.81980
	2.00	4	57.3450	35.86538	17.93269

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
ALFPm4	Equal variances assumed	2.712	.151	-2.501	6	.046	-45.85500	18.33500	90.71912	-.99088
	Equal variances not assumed			-2.501	3.272	.081	-45.85500	18.33500	-101.558	9.84757

APPENDIX C

Fish & Shellfish Immunology (2008) 24, 46–54

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Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci

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Received 8 May 2007; revised 17 July 2007; accepted 29 July 2007
Available online 5 October 2007

KEYWORDS
Penaeus monodon;
Anti-lipopolysaccharide factor;
Genomic sequence;
RNA splicing;
cis-regulatory element

Abstract Different isoforms of the ALF homologues (ALF*Pm*1–5) have been previously identified from *Penaeus monodon* expressed sequence tag (EST) database (http://pmonodon.biotec.or.th). The nucleotide and amino acid sequences of the *P. monodon* ALF homologues were analyzed and categorized into two groups, ALF*Pm*1 and 2 in group A and ALF*Pm*3–5 in group B. The genomic sequences of the two ALF gene groups were obtained by using the PCR and genome walking techniques. The ALF group A gene consisted of three exons interrupted by two introns whereas the ALF group B gene contained four exons interrupted by three introns. The alignment of genomic sequences with the ALF cDNA sequences revealed that different transcripts in both groups were generated by alternative RNA splicing of the pre-mRNA transcripts. The 5′ upstream sequences of the two ALF groups contained the putative *cis*-regulatory elements, including the activator protein 1, the Octamer, the GATA, the nuclear factor-kappaB, and the GAAA motifs, which possibly promoted transcription in response to infection as in other antimicrobial peptide genes. The RT-PCR analysis revealed that although all ALF isoforms were expressed in individual shrimp, the ALF*Pm*2 and 3 were the major or authentic ALFs in the hemocytes. The expression of both ALF*Pm*2 and 3 were increased in response to *Vibrio harveyi* infection indicating the important function of the ALFs against bacterial invasion.

Introduction

Animal and plant organisms possess an innate immune system for immediate protection from invading foreign microorganisms. Besides many other peptides and proteins in the system, antimicrobial peptides (AMPs) are one of the

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doi:10.1016/j.fsi.2007.07.010

key elements [1]. The AMPs have broad spectra of antimicrobial activity, an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi, parasites and viruses [2]. Lacking the adaptive immunity, crustaceans including shrimps rely on only the innate immunity. Therefore, the AMPs are crucial for them to fight the pathogenic invasion. The AMPs reported in shrimp include anti-lipopolysaccharide factor (ALF), crustin and penaeidin. They are produced and stored in the hemocyte, a prime cell in the immune system [3–5].

The ALFs were originally isolated from the hemocytes of horseshoe crabs, *Tachypleus tridentatus* (TALF) and *Limulus polyphemus* (LALF) [6]. Thereafter, the ALF was identified and characterized in many crustacean species including *Penaeus monodon* [7], *Litopenaeus setiferus* [8], *Fenneropenaeus chinensis* [9], *Marsupenaeus japonicus* [10] and *Pacifastacus leniusculus* [11]. Recently, an ALF from *Scylla paramanosian* was characterized and shown to be phylogenetically related to the ALFs from the horseshoe crabs, shrimps and crabs [12].

The ALF is a small basic protein that has the ability to inhibit the endotoxin or lipopolysaccharide (LPS) mediated coagulation system and, thus, exhibits strong anti-bacterial activity against the Gram-negative bacteria in particular [13]. Other ALFs from shrimp (ALF*Pm*3) and mud crab (ALF*Sp*) have much broader antimicrobial activity [4,12]. The ALF*Pm*3 has been expressed in the yeast *Pichia pastoris* expression system [4] and the recombinant ALF*Pm*3 protein exhibits antimicrobial activity against gram-negative and gram-positive bacteria and fungi. Another possible role of the ALF as an antiviral molecule has been shown in *P. leniusculus* as it interferes with the replication of white spot syndrome virus [11].

The function of the ALF lies on the positively charged cluster within a disulfide loop in its primary structure as suggested by Aketagawa et al.[14]. From the three-dimensional structure of LALF, the amphipathic disulfide loop binds lipid A and neutralizes the biological effects of LPS [15,16]. The ALFs from horseshoe crabs, shrimps and crabs also contain the two highly conserved-cysteine residues and the highly conserved cluster of positively charged residues within the disulfide loop. The synthetic disulfide loops from ALF*Pm*3 and ALF*Sp* have been shown unequivocally to be antimicrobially active, though the actual mechanism is not known [4,12].

In *Drosophila*, regulation of AMP gene at the transcriptional level can be revealed by the presence of some specific conserved *cis*-regulatory element [17]. In shrimp, however, the genomic organization of the constitutively and highly expressed AMP gene, penaeidin-3 (*PEN*3), has only been reported to date. No clear immune-regulatory motifs are identified in the upstream regions of either *PEN*2 or *PEN*3 genes [18]. For the *P. monodon*, five different ALF isoforms, designated as ALF*Pm*1–5, have been identified from the *P. monodon* expressed sequence tag (EST) database [19,20]. The *P. monodon* ALF homologues were found in the hemocyte libraries of both normal and *Vibrio harveyi*-infected shrimp. However, the majority of the predominant ALF*Pm*3 cDNA clones were found in the *V. harveyi*-infected hemocyte library, indicating that the ALF*Pm*3 was up-regulated upon stimulation.

In this study, we further examined the genomic organization as well as promoters and the *cis*-regulatory elements of the *P. monodon* ALF isoforms. Nucleotide sequences of the ALF genes were determined and compared with the corresponding cDNA sequences. Expression of different ALF isoforms in the hemocytes of individual shrimp as well as the expression in response to pathogenic bacteria, *V. harveyi*, is also reported herein.

Materials and methods

Animals

P. monodon juveniles (16–20 g body weight) were used for the experiment. The pleopods were used for genomic DNA preparation. The shrimp injected with live 10^3 cfu of *V. harveyi* and normal saline solution (0.85% (w/v) NaCl) as a control at the fourth-ventral abdominal segment, were used for gene expression analysis experiment. Hemolymph from four individual shrimp was collected at 6 h postinjection for total RNA preparation.

Amplification of genomic ALF genes

Genomic DNA was prepared from the pleopods of *P. monodon* by using phenol–chloroform extraction. The primers, ALF*Pm*2F, ALF*Pm*2R, ALF*Pm*3F and ALF*Pm*3R (Table 1), designed from the corresponding cDNA sequences were used for the amplification. The genomic DNA (20 ng) was used for the PCR amplification in 50 μl reaction containing one unit Advantage 2 Polymerase Mix (Clonetech), 1 × Advantage 2 PCR buffer, 200 μM of dNTP, 0.2 μM each of the appropriate forward and reverse primers. The initial denaturation was at 94 °C for 3 min, followed by five cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 45 s, elongation at 72 °C for 2 min; 25 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, elongation at 72 °C for 2 min, and the final extension at 72 °C for 10 min. The PCR products were cloned into the pGEM®-T easy vector (Promega) and subsequently sequenced.

Determination of 5′ upstream sequences of ALF genes

The 5′ upstream sequences of ALF genes were determined by using the BD GenomeWalker™ Universal Kit (Clonetech). The genomic GenomeWalker™ DNA libraries were constructed using restriction enzymes *Dra*I, *EcoR*V, *Pvu*II, and *Stu*I as instructed in the kit manual. The primary PCR used the outer adaptor (AP-1) and the gene-specific primers (ALF*Pm*GSP1) (Table 1). The secondary PCR was performed with the nested adaptor (AP-2) and the nested gene-specific primers (ALF*Pm*GSP2) (Table 1). The secondary PCR products were cloned into the pGEM®-T easy vector (Promega) and subsequently nucleotide sequenced.

The primers ALF*Pm*2AF and ALF*Pm*2AR were designed to amplify the whole genomic DNA segments containing both the 5′ regulatory sequence and the ALF group A gene. The PCR products were cloned, sequenced and analyzed in order to confirm the contiguous nature of the DNA fragments obtained from the genome walking and the genes.

The putative promoters (5′ *cis*-regulatory elements in the 5′ upstream sequences) and the putative start sites of

Table 1 Nucleotide sequences of primers

Primer	Sequence (5′–3′)
Primers for genomic amplification	
ALF*Pm*2F	CTCAGCCTGATTGCACTTATGCCACGGT
ALF*Pm*2R	CTGGCGTCTTCCTCCGTGATGAGATTAC
ALF*Pm*2AF	TGGTATAACATATATTTCGTATATCACG
ALF*Pm*2AR	ATTTATGATTTACAAATATCACTCTTGC
ALF*Pm*3F	CAAGGGTGGGAGGCTGTGG
ALF*Pm*3R	TGAGCTGAGCCACTGGTTGG
Primers for genomic walking	
ALF*Pm*2GSP1R	ACCGTAAGATTAATGGGCAGTGGTGAC
ALF*Pm*2GSP2R	TGGCACCGTGGCATAAGTGCAATCAG
ALF*Pm*3GSP1R	TAGTACACCTGGAATCTCTTCAAATAAGGC
ALF*Pm*3GSP2R	TAAGTGCCACACCCTACGATCTTGCTG
ALF*Pm*3GSP1R2	ACTGAGTTGCCAGGAGCCTATTTAACG
ALF*Pm*3GSP2R2	GACGTGGAAACTATAGTAACAGCAACG
AP-1	GTAATACGACTCACTATAGGGC
AP-2	ACTATAGGGCACGCGTGGT
Primers for RT-PCR	
ALF*Pm*2RTF	CAAGCGGTGCAGGACCTCC
ALF*Pm*2RTR	TTAGTGCTCAAGCCAAATCCTGG
ALF*Pm*3RTF	CAAGGGTGGGAGGCTGTGG
ALF*Pm*3RTR	TGAGCTGAGCCACTGGTTGG
EF-1 alphaF	GGTGCTGGACAAGCTGAAGGC
EF-1 alphaR	CGTTCCGGTGATCATGTTCTTGATG

the ALF genes were predicted by using the MATCH™/TRANFAC program (http://www.gene-regulation.com) (Biobase GmbH) and the Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) [21], respectively.

Differential expression of ALF isoforms by RT-PCR

The mRNA expression of the four ALF isoforms was determined by the RT-PCR analysis. The hemolymph of *V. harveyi*-challenged and the normal saline control shrimp was collected using 10% sodium citrate as an anticoagulant. The total RNA was prepared from the hemocytes using the TRIzol reagent (Invitrogen). The synthesis of first strand cDNA was performed using the ImProm-II™ Reverse Transcription System (Promega). The 2 μg of total RNA were mixed with the oligo$(dT)_{15}$, incubated at 70 °C for 5 min and chilled on ice. Then, the reverse transcription reaction mix (4 μl of 5× reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of 10 mM each of the dNTP, 1 μl of ImProm-II reverse transcriptase and 20 units of RNasin) was added and gently mixed. Then, 0.5 μl of each cDNA preparation was used for PCR amplification in a 15 μl reaction volume containing 0.3 unit DyNAzyme II DNA polymerase (Finnzymes), 1× buffer, 200 μM of dNTP, and 0.2 μM each of the appropriate forward and reverse primers (Figs. 1A and 2A, Table 1). The reactions were carried out using the following conditions: an initial denaturation step of 94 °C for 2 min; followed by 21 cycles for EF-1 alpha, 27 cycles for ALF*Pm*2, 19 cycles for ALF*Pm*3, and 36 cycles for the detection of two ALF isoforms simultaneously (ALF*Pm*1–2 and ALF*Pm*3–4) of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min; and the final extension phase of 72 °C for 10 min. The PCR products of ALF genes were analyzed by electrophoresis using 1.2% agarose gel. The band intensity of the PCR products were recorded and analyzed by using the Genetools analysis software. The expression of ALF isoforms was reported relative to that of the internal control, EF-1 alpha, which was normalized to 100. Statistical analysis of the RT-PCR results was performed using the independent samples *t*-test as indicated in the results. Data differences were considered significant at $P < 0.05$.

Results

Genomic organization of *P. monodon* ALF genes

In our previous studies, the deduced amino acid sequences of *P. monodon* ALFs were compared and the results revealed the existence of at least five different isoforms. Here, we compared and divided the nucleotide sequences into two groups, A and B, and showed that they were encoded from two different loci. Group A consists of ALF*Pm*1 and 2 (GenBank accession nos. BI784448, and BI784449), and group B consists of ALF*Pm*3-5 (GenBank accession nos. BI018071, BI784451, and CF415871).

In group A, the nucleotide sequence of ALF*Pm*2 totally matched that of ALF*Pm*1. The ALF*Pm*1, however, contained an additional sequence of 104 bp at positions 535–638 (Fig. 1A). Due to the stop codon (TGA) at the beginning of the extra sequence, the reading frame of ALF*Pm*1 contained only 84 amino acid residues, compared to the 120 amino acid reading frame of ALF*Pm*2. Nevertheless, both isoforms contained a sequence of the disulfide loop CRYSQRPSFYRWELYFNGRMWC that was proposed to be the LPS binding sequence [19].

Similar to the nucleotide sequence of ALF*Pm*1, the ALF*Pm*4 cDNA contained an additional sequence of 342 bp at positions 426–767 (Fig. 1B). The reading frame of ALF*Pm*4 encountered a stop codon (TGA) when it read not very far into the extra sequence. The encoding protein was only 52 amino acid residues in length. This additional sequence was missing in ALF*Pm*3 and 5; therefore, their reading frames could read through into the putative LPS binding sequence, CKFTVKPYLKRFQVYYKGRMWC, and coded for a protein of 123 amino acid residues. However, comparing the nucleotide sequences of ALF*Pm*3 and 5, it was quite certain that they were from the same gene as all the bases are identical except one additional base between position 882 and 883 in ALF*Pm*5. This additional base might be derived from an error during the cDNA synthesis. Clustering analysis also suggested that the ALF*Pm*3 (GenBank accession no. BI018071) and 5 (GenBank accession no. CF415871) were identical (data not shown).

By designing the primers at the beginning (ALF*Pm*2F and ALF*Pm*3F) and at the end of the gene (ALF*Pm*2R and ALF*Pm*3R) (Table 1), the genomic sequences of these genes were amplified. They were cloned, and their nucleotide sequences were determined and aligned with the corresponding cDNA sequences. The gene organization of *P. monodon* ALF genes starting from the putative pre-mRNA start sites was predicted by using the Neural Network Promoter Prediction [21]. The genomic structure of ALF group A

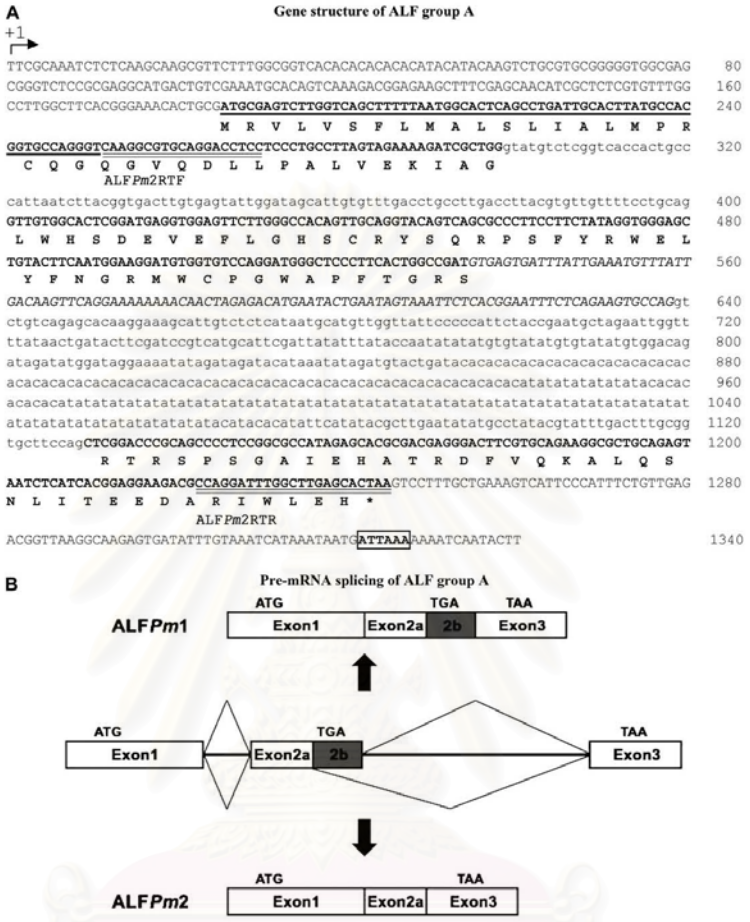

Figure 1 Genomic nucleotide and deduced amino acid sequences of ALF group A (A) and their pre-mRNA splicing (B). The sequence reads from the putative pre-mRNA start site (+1) with exons in capital letters, coding sequence of ALF*Pm*2 in bold-faced capital letters, the additional intron sequence in ALF*Pm*1 in italicized letters and introns in lowercase letters. The encoding amino acid sequence is bold-faced with the signal peptide underlined. Poly(A) signal sequence is boxed. The annealing site for PCR primer indicated is double-underlined.

consisted of three exons interrupted by two introns (Fig. 1B). For the ALF*Pm*1 gene, the exons were 298, 238 and 211 bp in length, and the introns were 102 and 491 bp in length. The ALF*Pm*2 had slightly different gene structure; its exons were 298, 134 and 211 bp in length with the two introns of 102 and 595 bp in length. Fig. 1B clearly shows that there were two 5′ spliced sites between the exons 2 and 3 that were used to produce the ALF group A transcripts. With different 5′ spliced sites, the ALF*Pm*1, then, contained the additional sequence not found in the ALF*Pm*2.

For the ALF group B gene, there were four exons interrupted by three introns. The four exons were 43, 125, 154 and 361 bp in length, and the three introns were 257, 342 and 357 bp in length (Fig. 2B). The ALF*Pm*3 had all three introns completely spliced out. The additional sequence in the ALF*Pm*4 was, in fact, the sequence of intron 2 that was selectively retained upon pre-mRNA splicing.

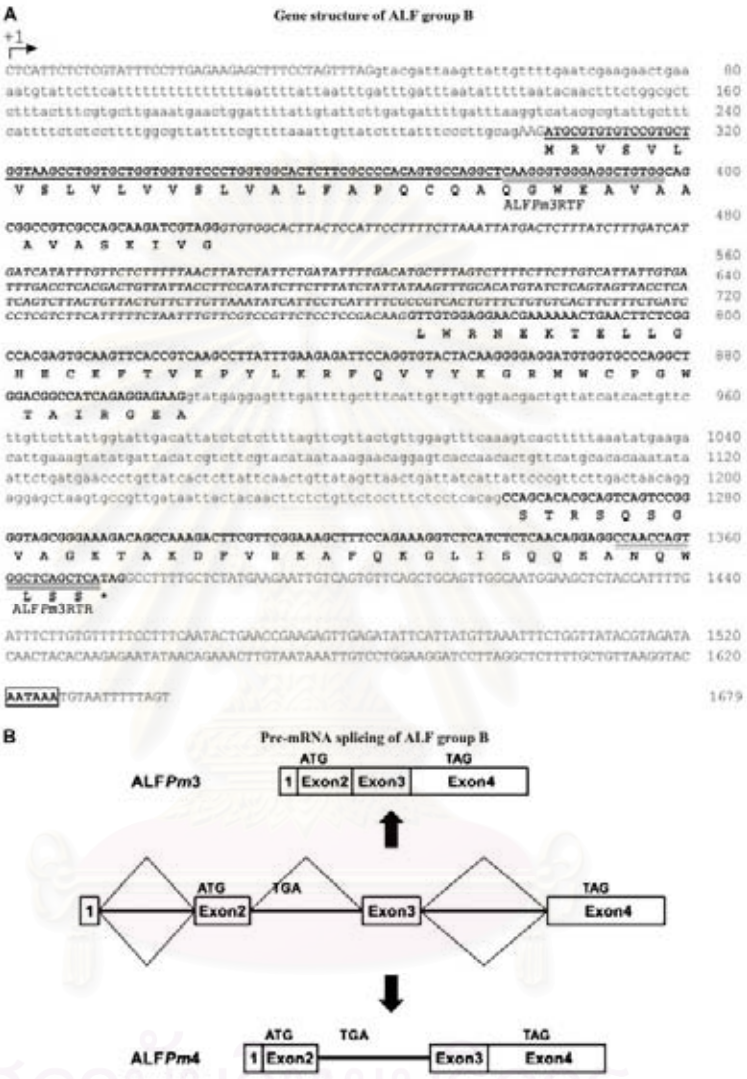

Figure 2 Genomic nucleotide and deduced amino acid sequences of ALFs group B (A) and their pre-mRNA splicing (B). The sequence reads from the putative pre-mRNA start site (+1) with exons in capital letters, coding sequence of ALF*Pm*3 in bold-faced capital letters and introns in lowercase letters. The retention intron in ALF*Pm*4 is in the italicized letters. The encoding amino acid sequence is bold-faced with the signal peptide underlined. Poly(A) signal sequence is boxed. The annealing site for the PCR primer indicated is double-underlined.

Interestingly, the exon 1 was short and did not encode any amino acid; in other words, the mature peptide was coded for by the exons 2–4. All exon–intron boundaries of the ALF genes in both groups conformed to the consensus GT at the 5′ spliced sites and the AG at the 3′ spliced sites [22].

Determination of promoter and regulatory elements of *P. monodon* ALF genes

The 5′ upstream sequences were determined using the genome walking approach starting from the gene-specific primers in the cDNA sequences. Approximately 600 bp upstream from the start site were obtained for both ALF group A and B genes using two and four gene-specific primers, respectively (Table 1). Obviously, it was more difficult to obtain the 5′ upstream sequence of the group B gene. This is probably due to the presence of microsatellite sequences (GA repeats) at positions −287 to −438. The genomic sequences including the 5′ upstream and the genes were deposited into the GenBank (accession nos. EF523560, EF523561, EF523562 and EF523563 for ALF*Pm*1, 2, 3 and 4, respectively). It should be noted that only the genomic sequences of group A were confirmed by amplification of the whole genomic segments using primers ALF*Pm*2AF and ALF*Pm*2AR (Table 1) and sequencing.

The 5′ upstream sequences were analyzed for the putative *cis*-regulatory elements by using the MATCH™ analysis program searching the sequences against the TRANSFAC database [23] with the cut-offs for both core and matrix similarities of 0.9. The putative promoters of the *P. monodon* ALF groups A and B were identified at positions −31 and −29, respectively. Several transcription factor-binding sites, including Octamer (Oct-1), GATA, CCAAT box and GAAA motifs, were predicted in the 5′ upstream sequences of both the ALF group A and B genes. A putative activator protein 1 (AP-1), nuclear factor (NF)-kappaB binding sites, however, were only predicted at the 5′ upstream of the ALF group A genes (Fig. 3).

Differential expression of ALF genes

To test whether different isoforms of *P. monodon* ALFs were expressed in shrimp hemocytes, the RT-PCR analysis was employed. Since the ALF mRNAs in each group had different lengths due to the additional sequences, they could be distinguished by using the same set of PCR primers. Using the primers ALF*Pm*2RTF and ALF*Pm*2RTR for ALF group A and ALF*Pm*3RTF and ALF*Pm*3RTR for ALF group B (Table 1, Figs. 1 and 2), all ALF transcripts were identified as PCR products of different lengths. All individual shrimp expressed ALF*Pm*2–4 transcripts (Fig. 4A). The ALF*Pm*3 transcript was the predominant species followed by the ALF*Pm*2 transcript. However, ALF*Pm*1 transcript was barely or not detectable, and could be observed clearly only after the RT-PCR reactions were re-amplified. The ALF*Pm*4 was seen as a faint band. To make sure that the faint bands in Fig. 4A were actually the ALF*Pm*1 and 4 transcripts, the re-amplified fragment of ALF*Pm*1 and the faint band of ALF*Pm*4 were cloned and sequenced (data not shown). Low expression of ALF*Pm*1 and 4 suggested that they might probably be aberrant splicing products of the ALF*Pm*2 and 3, respectively. It should be noted also that ALF*Pm*1 and 4 seemed to be produced in a constant ratio to their normal counterparts as seen in Fig. 4A, which may indicate that the aberrant incompletely processed transcripts are produced stoichiometrically by a malfunctioning splicing apparatus.

The result above indicated that the major ALF transcripts were those of ALF*Pm*2 and 3. Therefore, the differential expression experiment of ALF*Pm*2 and 3 in the hemocytes of normal saline control and *V. harveyi*-challenged shrimp were performed. The transcription levels were measured against that of the elongation factor-1 alpha transcript. Fig. 4B and C show that the transcription levels of ALF*Pm*2 and 3 were significantly increased in the hemocytes of *V. harveyi*-challenged shrimp.

Discussion

Recently, five isoforms of anti-lipopolysaccharide factor (ALF) have been identified in the hemocyte cDNA libraries of the *P. monodon* EST database, namely ALF*Pm*1–5. They were predominantly found in the hemocyte libraries of *V. harveyi*-infected shrimp suggesting that they might be involved in the defense mechanism against the pathogenic bacteria [19]. Herein, the nucleotide and amino acid sequences of the five isoforms were analyzed. The sequence alignment and similarity undoubtedly grouped the ALF clones into two groups, ALF*Pm*1–2 as group A and ALF*Pm*3–5 as group B. They possessed different putative lipopolysaccharide binding sequences from which different antimicrobial activities might be inferred.

The presence of the two groups of ALF cDNAs indicated that they were encoded from different loci in the genome as suggested by Supungul et al. [19]. By the amplification of *P. monodon* ALF genes and the genome walking to identify the 5′ upstream regions, the genomic DNA sequences of two separate ALF loci were obtained. The analysis illustrated that either the ALF*Pm*1 and 2 or ALF*Pm*3–5 were, indeed, derived from the same genomic genes. Moreover, the nucleotide sequence analysis revealing the identical of ALF*Pm*3 and 5 genes gave rise to the conclusion that *P. monodon* has four different ALF isoforms. The isoforms in each group were generated by alternative splicing of the same pre-mRNAs. The mRNAs of ALF*Pm*1 and 2 contained the second exons of different lengths owing to the presence of two 5′ spliced sites. The second intron of ALF*Pm*4 mRNA was retained rather than spliced out as in the ALF*Pm*3 mRNA.

With additional sequences in the mRNAs, the reading frames of ALF*Pm*1 and 4 were shorter. The ALF*Pm*1 contained the amino terminal part of the protein and the lipopolysaccharide binding sequence. Whether the ALF*Pm*1 was active was not experimentally confirmed. The ALF*Pm*4 was obviously inactive for it contained only the amino terminal part of the protein without the lipopolysaccharide binding sequence. The results suggested that the ALF*Pm*1 and 4 were aberrant products of the pre-mRNA splicing. Supporting the suggestion came from the RT-PCR results that the amounts of ALF*Pm*1 and 4 mRNAs were considerably lower than those of ALF*Pm*2 and 3. Aberrant splicing has been reported in several organisms including human, mouse and the fruit fly *Drosophila melanogaster* [24,25]. Splicing

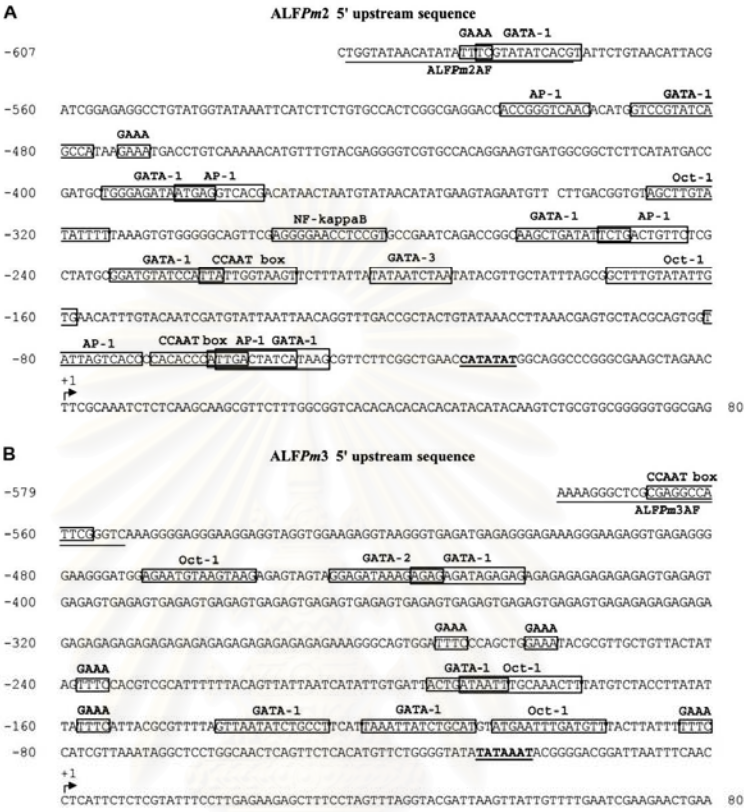

Figure 3 The 5′ upstream sequences of ALF*Pm*2 (A) and 3 (B) containing the putative *cis*-regulatory elements in boxes. The TATA boxes are bold-faced and underlined. The putative start sites are +1. The annealing sites for the PCR primers indicated are underlined.

errors generally occur as a result of mutations at splice sites or surrounding sequences. However, they can be induced by environmental factors without sequence alterations [26].

As far as the regulation of gene expression is concerned, there are no reports about the promoter and regulatory sequences involved in the ALF gene expression. It was then interesting to analyze the 5′ upstream sequences of the ALF genes. By sequence searching against the TRANFAC regulatory sequence database, the 5′ upstream sequences of ALF groups A and B were marked with a variety of putative *cis*-regulatory elements. The *cis*-regulatory elements, known to be involved in immune response and/or regulate the expression of antimicrobial peptides, such as nuclear factor (NF)-kappaB, GATA, activator protein 1 (AP-1), GAAA and Octamer (Oct-1) motifs, were particularly interesting as they were identified in the upstream regions of the *P. monodon* ALF genes.

The NF-kappaB motif is important for inducible production of antimicrobial peptide in mammals, amphibians and insects [17,27–30]. In *Drosophila*, the NF-kappaB-like site is necessary for tissue-specific and LPS-inducible expression of cecropin A1 and diptericin genes [31–34]. Despite the transcription level of ALF group B being higher than that of ALF group A upon *V. harveyi* challenge, a putative NF-kappaB motif is found only in the upstream sequence of ALF group A.

The GATA site is required for the activity of penaeidin 2 promoter of *L. vannamei* [18]. Seven and five putative GATA sites are found in the ALF group A and B genes, respectively. The activator protein 1 (AP-1) factor is involved in the cell proliferation and survival [35], and also produced in response to viral infection [36]. The GAAA motif is recognized by interferon regulatory factor 1 (IRF-1) upon transcriptional activation of interferon-stimulated genes in

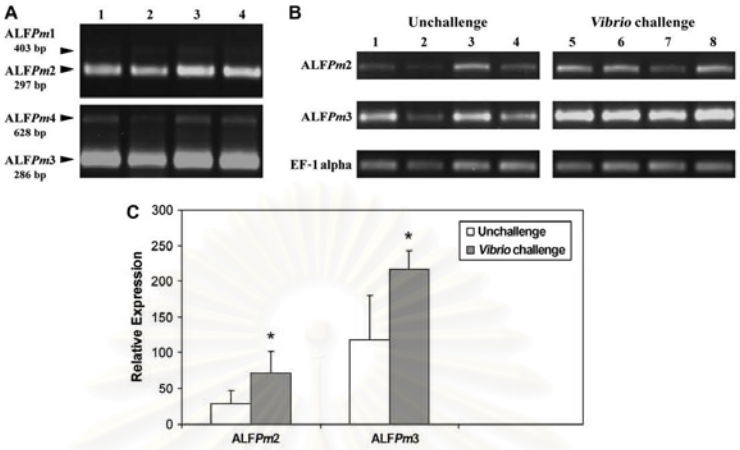

Figure 4 The differential expression of ALF transcripts measured by RT-PCR. The four types of ALF transcripts, ALF*Pm*2–4, were detected in individual shrimp (A). The major ALF*Pm*2 and 3 transcripts were measured in both unchallenged and *Vibrio harveyi*-challenged hemocytes of individual shrimp (B). The PCR products were run on 1.2% agarose gel. The sizes of RT-PCR products are 401, 297, 286, and 628 and 150 bp for ALF*Pm*1, 2, 3, and 4 and EF-1 alpha, respectively. The band intensities of ALF*Pm*2 and 3 in (B) were measured, normalized against those of EF-1 alpha, and expressed as relative expression (C). The values are means ± S.D. ($n = 4$). The asterisks indicate significant difference between the unchallenged and challenged shrimp ($P < 0.05$).

virus-infected mammal cells [37]. The presence of AP-1 and GAAA motifs in the upstream sequences perhaps regulates the transcription of the *P. monodon* ALF in response to viral infection. Recent study indicates that ALF can interfere with the replication of the major viral pathogen, white spot syndrome virus [11]. The GAAA motif has also been shown to cooperate with the NF-kappaB motif in the LPS-induced expression of diptericin [38]. The Octamer-binding site 1 (Oct-1) is often found in the enhancer, and promotes the transcription of immune-relevant genes [36]. The putative Oct-1 sites are found in the *P. monodon* ALF genes. Nevertheless, whether the above selected motifs are functional and involved in the regulation of the *P. monodon* ALF expression needs further investigation.

The ALF cDNAs were found predominantly in the hemocyte libraries of *V. harveyi*-infected shrimp as reported by Supungul et al. [19]. Subsequently, it was shown by RT-PCR analysis that the expression of ALF*Pm*3 was up-regulated upon injection of *V. harveyi* with the highest expression at 6 hpi. In this study, the RT-PCR experiment with the RNAs isolated from the hemocytes of normal and 6-h *V. harveyi*-challenged shrimp confirmed the above notion. The transcriptional levels of ALF*Pm*2 and 3 were significantly increased in response to *V. harveyi* injection suggesting an important role of both the ALF isoforms in shrimp immune response.

The antimicrobial activities of *P. monodon* ALFs are interesting as the ALFs can be applied in the aquaculture as an alternative to antibiotics. The activity of ALF*Pm*3 had already been studied in detail [4]. We found that the crude extract of ALF*Pm*2 had antimicrobial effect against the *Escherichia coli* 363 and *Bacillus megaterium*, representatives of Gram-negative and Gram-positive bacteria, respectively (data not shown). It would be of particular interest to compare the activity of ALF*Pm*2 and 3 against various strains of microorganisms and the work is in progress.

Acknowledgments

This work is supported by research grants from the National Center for Genetic Engineering and Biotechnology (BIOTEC) (Grant no. BT-B-01-SG-09-4803 and the Research Team Strengthening Grant) and from the Commission on Higher Education. A student fellowship granted to Sirinit Tharntada from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant no. PHD/0171/2544) is greatly appreciated. We also thank the support from Chulalongkorn University under the Ratchadaphisek Somphot Endowment to the Shrimp Molecular Biology and Genomics Laboratory.

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Biography

Miss Sirinit Tharntada was born on January 8, 1980 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of Biochemistry, Chulalongkorn University in 2001. She has further studied for the Doctor of philosophy (Ph.D.) degree in Biochemistry Department, Chulalongkorn University.