การโคลนและการแสดงออกของยีน
แอนติไลโพพอลิแซคคาไรด์แฟคเตอร์จากกุ้งกุลาดำ *Penaeus monodon*

นางสาววิไลรัตน์ อนุรักษ์โอฬาร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2544

ISBN 974-03-0496-6

CLONING AND EXPRESSION OF GENE ENCODING ANTI-LIPOPOLYSACCHARIDE FACTOR FROM BLACK TIGER SHRIMP *Penaeus monodon*

Miss Wilairat Anurakolan

A Thesis Submitted in Partial Fulfillment of Requirements
for the Degree of Master of Science in Biochemistry
Department of Biochemistry
Faculty of Science
Chulalongkorn University
Academic Year 2001

ISBN 974-03-0496-6

Thesis Title	CLONING AND EXPRESSION OF GENE ENCODING ANTI-LIPOPOLYSACCHARIDE FACTOR FROM BLACK TIGER SHRIMP *Penaeus monodon*
By	Miss Wilairat Anurakolan
Field of Study	Biochemistry
Thesis Advisor	Associate Professor Anchalee Tassanakajon, Ph.D.
Thesis Co-advisor	Rath Pichyangkura, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

.....Dean of Faculty of Science
(Associate Professor Wanchai Phothiphichitr, Ph.D.)

Thesis Committee

.....Chairman
(Associate Professor Piamsook Pongsawasdi, Ph.D.)

.....Thesis Advisor
(Associate Professor Anchalee Tassanakajon, Ph.D.)

.....Thesis Co-advisor
(Rath Pichyangkura, Ph.D.)

.....Member
(Sirawut Klinbunga, Ph.D.)

วิไลรัตน์ อนุรักษ์โอฬาร : การโคลนและการแสดงออกของยีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์จากกุ้งกุลาดำ *Penaeus monodon*. (CLONING AND EXPRESSION OF GENE ENCODING ANTI-LIPOPOLYSACCHARIDE FACTOR FROM BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษา : รศ. ดร. อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม ดร. รัฐ พิชญางกูร, 107 หน้า. ISBN 974-03-0496-6

แอนติไลโพพอลิแซคคาไรด์แฟคเตอร์ (Anti-LPS fcator) เป็นโปรตีนที่สำคัญชนิดหนึ่งในระบบภูมิคุ้มกันของครัสเตเชียน ในแมงดาทะเลพบว่าโปรตีนชนิดนี้มีสมบัติในการยับยั้งการเจริญของเชื้อแบคทีเรียแกรมลบ โดยสามารถจับกับเอนโดท็อกซินที่เป็นส่วนประกอบของไลโพพอลิแซคคาไรด์ (LPS) แล้วขจัดพิษของเอนโดท็อกซินได้ จากการแยกและวิเคราะห์ยีนที่เกี่ยวข้องกับระบบภูมิค้มกันของกุ้งกุลาดำ (*Penaeus monodon*) พบยีน anti-LPS factor จากห้องสมุด cDNA ที่เตรียมจากเซลล์เม็ดเลือดกุ้ง โดยพบบริเวณ open reading frame ที่มีขนาด 372 bp ซึ่งแปลรหัสให้โปรตีนที่มีกรดอะมิโน 123 ตัว มีมวลโมเลกุลเป็น 13.7 กิโลดาลตัน ในการศึกษานี้เราสนใจที่จะโคลนยีนที่ให้โปรตีนสายสมบูรณ์ (full-length) และโปรตีนอนุพันธ์ที่ขาดส่วนปลาย N (NH_2-terminal truncated derivative) มาแสดงออกในเซลล์แมลงโดยใช้บาคูโลไวรัสเป็นดีเอ็นเอพาหะในการแสดงออก (Baculovirus Expression System) เพื่อผลิตโปรตีนที่มีโครงสร้างและสมบัติไม่ต่างจากโปรตีนในแหล่งต้นกำเนิด ซึ่งทำได้โดยการนำชิ้นดีเอ็นเอที่ให้โปรตีนสายสมบูรณ์ (full-length) และโปรตีนอนุพันธ์ที่ขาดส่วนปลายด้านอะมิโนมาโคลนเข้าดีเอ็นเอพาหะ (pBacPAK8) และนำไปส่งถ่าย (co-transfected) เข้าสู่ดีเอ็นเอของบาคูโลไวรัส โดยการแลกเปลี่ยนชิ้นดีเอ็นเอที่ต้องการแสดงออกเข้าสู่ดีเอ็นเอของบาคูโลไวรัสเพื่อผลิตโปรตีนในเซลล์แมลง (*Spodoptera frugiperda* Sf9 cells) ทำการวิเคราะห์รีคอมบิแนนท์โปรตีนจากเซลล์ที่ติดเชื้อไวรัสโดยวิธี SDS-PAGE พบว่าโปรตีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์ที่ได้จากการแสดงออกของยีนทั้ง 2 ชนิด ทั้งที่เป็นสายสมบูรณ์ และอนุพันธ์ จะมีขนาดประมาณ 15 กิโลดาลตัน ซึ่งใกล้เคียงกับขนาดของโปรตีนสายสมบูรณ์ และอนุพันธ์ นำโปรตีนหยาบทั้งคู่ที่ได้ไปตรวจสอบสมบัติในการยับยั้งการเจริญของเชื้อแบคทีเรียแกรมลบ *Vibrio harveyi* 1526 และ *Escherichia coli* DH5α และเชื้อแบคทีเรียแกรมบวก *Staphylococcus aureus* พบว่าอัตราการเจริญของเชื้อเมื่อตรวจสอบโดยโปรตีนหยาบทั้งคู่ไม่มีความแตกต่าง ซึ่งอาจจะเกิดจากโปรตีนที่ได้อยู่ในรูปที่ไม่ละลาย (inclusion body) เนื่องจากมีการแสดงออกของโปรตีนมากเกินไป ดังนั้นการตรวจสอบสมบัติในการยับยั้งการเจริญของโปรตีนที่ได้จำเป็นต้องใช้โปรตีนที่ละลายและบริสุทธิ์เพิ่มขึ้น

ได้ทำการศึกษาการแสดงออกของเอ็มอาร์เอ็นเอของโปรตีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์ในกุ้งกุลาดำปกติโดยเทคนิค Northern blot และ RT-PCR analysis พบว่าเซลล์เม็ดเลือดของกุ้งเป็นแหล่งผลิตเอ็มอาร์เอ็นเอของโปรตีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์ที่สำคัญ จากการตรวจสอบการแสดงออกโดยวิธี RT-PCR analysis พบว่าเซลล์เม็ดเลือดจะเป็นแหล่งที่มีการแสดงออกของโปรตีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์มากที่สุด นอกจากนี้ยังพบการแสดงออกที่หัวใจ เหงือก ลำไส้ และในต่อมน้ำเหลือง แต่ไม่พบการแสดงออกในตับ ในการตรวจสอบกุ้งที่ติดเชื้อแบคทีเรีย จะพบปริมาณเอ็มอาร์เอ็นเอของโปรตีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์เพิ่มมากขึ้นหลังจากทำการฉีดเชื้อแล้ว 3 ชั่วโมง แล้วปริมาณเอ็มอาร์เอ็นเอของโปรตีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์ก็จะลดลงมาอยู่ในระดับปกติ แสดงว่าการแสดงออกของโปรตีนแอนติไลโพพอลิแซคคาไรด์แฟคเตอร์จะถูกกระตุ้นได้ในระยะแรกของการติดเชื้อแบคทีเรีย

ภาควิชา	ชีวเคมี	ลายมือชื่อนิสิต.....
สาขาวิชา	ชีวเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2544	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4172445723 : MAJOR BIOCHEMISTRY

KEY WORD : *P. monodon*/ Anti-lipopolysaccharide factor/ hemocytes/ baculovirus expression system/ Co-transfected/ antimicrobial activity

WILAIRAT ANURAKOLAN : CLONING AND EXPRESSION OF GENE ENCODING ANTI-LIPOPOLYSACCHARIDE FACTOR FROM BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : ASSOC. PROF. Anchalee Tassanakajon, Ph.D., THESIS CO-ADVISOR : Rath Pichyangkura, Ph.D., 107 pp. ISBN 974-03-0496-6.

Anti-lipopolysaccharide factor (Anti-LPS factor) is the major protein of crustacean immune system. In horseshoe crabs, this protein has an antibacterial effect on the growth of Gram-negative bacteria by binding and neutralizing bacterial endotoxin, lipopolysaccharide (LPS). In the black tiger shrimp (*Penaeus monodon*), a cDNA encoding anti-LPS factor has been isolated from the hemocytes cDNA library. It contains an open reading frame of 372 bp coding for 123 aa residues with a predicted molecular weight of 13.7 kDa. In this study, two versions of anti-LPS factor, full-length and NH_2-terminal truncated derivative, were cloned and expressed in insect cells by using baculovirus expression system to obtained large amount of recombinant proteins with similar in structure and biological activity to the naturally occurring protein. The DNA fragment of full-length and NH_2-terminal truncated derivative were cloned into transfer vector (pBacPAK8) and co-transfected to baculovirus genome by homologous recombinant inside the insect host cell (*Spodoptera frugiperda* Sf9 cells). Analysis of the recombinant proteins from infected cell lysate by SDS-PAGE revealed over expression, both of the full-length and NH_2-terminal truncated derivative. Protein bands of approximately 15 kDa were observed in the infected cells but not in uninfected cell lysate. The recombinant proteins have appropriate size corresponding to the full-length and N-terminal truncated derivative anti-LPS factor protein. The crude recombinant proteins of both of the anti-LPS factors were tested for their antibacterial effect on the growth of bacteria by measuring bacterial growth rate of Gram-negative *Vibrio harveyi* 1526 and *Escherichia coli* DH5α and Gram-positive *Staphylococcus aureus*. No significant antibacterial activity was found with crude recombinant proteins both of full-length and NH_2-terminal truncated, which may be due to the recombinant proteins formed insoluble inclusion bodies when they were over expressed. Thus, purified protein is required for further antibacterial activity test.

We examined the tissue expression of anti-LPS factor mRNA in normal shrimp by Northern blot and RT-PCR analysis. The result demonstrated that hemocytes were the main site of anti-LPS factor synthesis. From RT-PCR analysis, anti-LPS factor showed strong expression in hemocytes and significant amount of mRNA concentration were observed in heart, gills, intestine and lymphoid organ. No mRNA of anti-LPS factor was detected in hepatopancreas. In microbial challenge, anti-LPS factor was found increase in mRNA concentration level at 3 hours post injection and then return to initial level, indicated that anti-LPS factor is stimulated in hemocytes at early stage of infection in response to bacterial challenge.

Department	Biochemistry	Student 's signature.....……
Field of study	Biochemistry	Advisor 's signature.....……
Academic year	2001	Co-advisor 's signature.....……..

ACKNOWLEDGEMENT

This thesis would not have been completed without a good advise from many person. I am indebted to my advisor, Assoc. Prof. Dr. Anchalee Tassanakajon, for her valuable advice, encouragement and kindness throughout the course of this study. I would also like to express my appreciation to my co-advisor, Dr. Rath Pichyangkura, for his great help on expression technique, guidance, suggestions in laboratory, and comment on my thesis.

I would also like to thank to Assoc. Prof. Dr. Piamsook Pongsawat and Dr. Sirawut Klinbunga for serving as thesis committee member and their recommendations. I am also thankful to Department of Microbiology and Charoenpokphand Group of companies for supporting the bacterial samples.

Special thanks are given to all members in 604, 617, 707, 708, and 709 laboratory rooms, Department of Biochemistry, Chulalongkorn University, for their helps and friendship during my study.

Finally, I would like to express my deepest appreciation and gratefulness to my parents, my sisters and my brother for their warmest love, care, understanding and encouragement extended throughout my study.

Wilairat Anurakolan

CONTENTS

Page

Page

LIST OF FIGURES

FIGURE **Page**

FIGURE **Page**

FIGURE **Page**

LIST OF ABBREVIATIONS

A, T, G, C	=	nucleotides containing the bases adenine, thymine, guanine and cytosine, respectively
bp	=	base pair
BSA	=	bovine serum albumin
°C	=	degree Celcius
cfu	=	colony forming units
cm	=	centimetre
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
EDTA	=	ethylene diamine tetraacetic acid
ESTs	=	expressed sequence tags
FBS	=	foetal bovine serum
FL	=	full-length
hpi	=	hours post infection
kb	=	kilobase
kDa	=	kilodalton
LPS	=	lipopolysaccharides
M	=	molar
MCS	=	multiple cloning sites
ml	=	millilitre
mm	=	millimetre
mM	=	millimolar
MOI	=	multiplicity of infection
ng	=	nanogram
nm	=	nanometre
ΔNAL	=	NH_2-terminal truncated anti-lipopolysaccharide factor
ORF	=	open reading frame
PCR	=	polymerase chain reaction
pfu	=	plaque forming units

PH	=	polyhedrin
RNA	=	ribonucleic acid
rpm	=	revolution per minute
rRNA	=	ribosomal RNA
RT	=	reverse transcription
SDS	=	sodium dodecyl sulfate
TC	=	tissue culture
TEMED	=	*N, N, N', N'*-tetramethylethylenediamine
Tris	=	Tris-(hydroxy methyl)-aminomethane
μg	=	microgram
μl	=	microlitre
μM	=	micromolar
UV	=	ultraviolet
v	=	volume
w	=	weight

CHAPTER I

INTRODUCTIONS

The black tiger shrimp (*Penaeus monodon*, Fabricius 1798) is one of the most important cultured shrimp species among those reported by the food and aquaculture organization of the United Nation (FAO). It has presently cultured worldwide covering a vast geographic area including Australia, Bangladesh, China, India, Indonesia, Philippine, Thailand, and Vietnam (Bailey-Brock and Moss, 1992).

Shrimp farming has had an impressive growth in many developing countries, where this activity has attained a great economic and social importance. Farming of the black tiger shrimp (*P. monodon*) is one of the common aquacultural industry in many tropical countries. The world ’s leading importing countries of shrimp are the United States of America and Japan. Approximately two-third of *P. monodon* exported from Thailand is imported to these countries, the remaining market are Europe, Asian countries, Australia and others.

In Thailand, shrimp is the one of most important export products and constitutes a significant source of revenue and employment. Intensive cultivation of the black tiger shrimp, *P. monodon*, began in Thailand in the mid 1980 ‘s (Flegel et al., 1995) and the shrimp culture production rose rapidly to reach a record export quantity of 300,000 metric tons in 2000, the highest production of Thailand ever, and estimated to be worth around 80,000 million baht. Most of the shrimp productions are cultured shrimp from farming, number of shrimp farms have increased since 1985. Thailand is now one of the top shrimp producing countries, and highly developed culturing systems have been established and expanded in many shrimp farming areas.

The farming of *P. monodon* in Thailand has rapidly increased reflecting large annual production. The increase in shrimp farming and production was enhanced by several factors including the appropriate farming areas without the serious disturbing from typhoons or cyclone, small variable of seawater during seasons, and ideal soils and terrain for pond construction. Culture of *P. monodon* causes increasing national revenue, therefore, this penaeid shrimp species is economically important species in Thailand.

The general characteristic of the black tiger shrimp can be briefly summerized as below:

1.1 Taxanomy of *Penaeus monodon*

The taxonomic definition of the black tiger shrimp, *P. monodon* is as follows (Bailey-Brook and Moss, 1992):

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

Scientific name: *Penaeus monodon* Fabricius, 1798
Common name: giant tiger shrimp or black tiger shrimp
FAO name: giant tiger shrimp

1.2 Morphology

Externally, shrimp can be basically divided into thorax and abdomen (Figure 1.1). The thorax is covered by single immobile carapace, which protects internal organs and supports muscle origins. The internal organs in this part consist of eyes and eyestalks, sensory antennules and antennae arise rostrally. The walking legs (pereiopods) are the thoracic appendages. Gills are formed sac-like outgrowths of the base of the walking legs and sit in branchial chambers on either side of the thorax. The carapace extends laterally to cover the gills completely. The abdomen has segmentation commonly observed in arthropods. It consists of swimming legs (pleopods), which arise from each of six abdominal segments, and a tail. The tail fan comprises a telson, which bears the anus, and two uropods attach to the last abdominal segment. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawns.

Shrimps grow by periodically releasing their cuticle, secreted by the epidermal cell layer, consisting of chitin and proteins. Molting starts when the epidermis detaches from the cuticle layer and begins to secrete a new cuticle. The new cuticle is soft and stretched to accommodate the increased size of the prawn immediately after molting (Anderson, 1993).

A live black tiger shrimp has the following characteristic coloration : generally dark colored with carapace and abdomen transversely banded with black and white, the rest of body variable from light brown to blue or red, the antennae are greyish brown, and the pereiopods and pleopods are brown with crimson fringing setae. In shallow brackish waters or when cultured in ponds, its color darkens and, often, found to be blackish brown (Motoh, 1981 : cited in Solis, 1988).

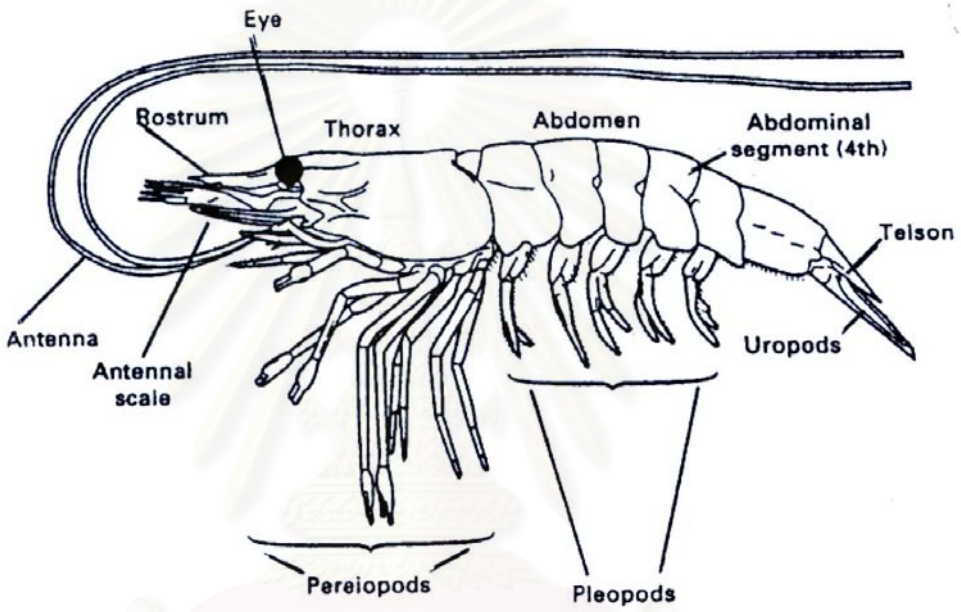


Figure 1.1 Lateral view of *P. monodon* showing important parts (Anderson, 1993)

1.3 Life cycle

Development of penaeid shrimp is complex. It begins with a larvae hatching from the fertilized egg to the first stage, nauplius, followed by protozoea, mysis, and post larval stages (Figure 1.2). These require the developmental period of about 1-5 days, 5 days, 4-5 days, and 6-15 days, respectively (Solis, 1988). Larvae exhibit planktonic behavior with antennal propulsion for swimming nauplius, antennal and thoracic propulsion in mysis, and abdominal propulsion in megalopa. Nauplii utilize yolk granules within their body while the feeding starts in protozoea and mysis. At mysis stage, larvae has five pairs of functioning pereiopods. The carapace now covers all the thoracic segments. The mysis swims like adults. After this stage, larvae metamorphoses to the post-larvae with a full complement of functioning appendages. The post-larvae continue to molt as they grow. They migrate shoreward and settle in nursery areas close to shore or estuaries, before developing into juvenile and sub-adults, which more tolerate to variety of environmental factors. Sub-adults migrate back to the sea where they finally mature and have the first corpulation and spawn. The life span of penaeid shrimp are approximately 2 years (Solis, 1988 ; Anderson, 1993).

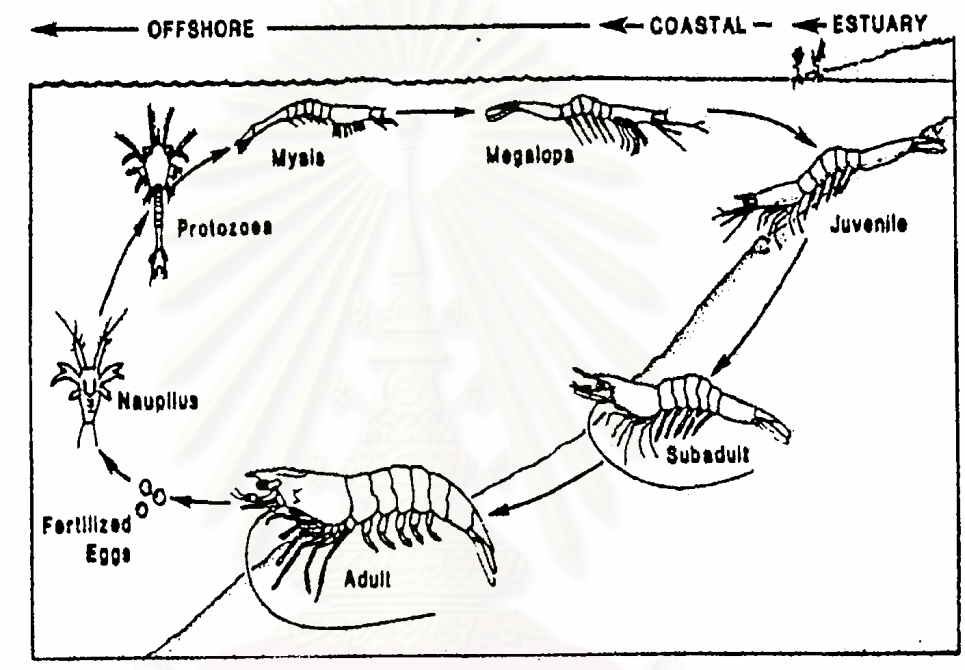


Figure 1.2 Life cycle of penaeid shrimps (Bailey-Brock and Moss, 1992)

1.4 Disease of shrimp

The problem of disease became serious in the shrimp industry because of increasing shrimp farming and lack of proper knowledge involving shrimp biology, farm management and disease. The infectious diseases of shrimp are frenquently accompanied by elevated mortality and great economic loss (Roch, 1999). The causative agents of infectious diseases in shrimp are mainly viruses and bacteria, belonging to *Vibrionacea* (Lightner et al., 1983; Kroll et al., 1991; Mohney et al., 1994; Hasson et al., 1995; Flegel, 1997). These pathogens particularly hamper larval production and lead to profitability problems due to stock mortalities. They also lead to the over-fishing of wild shrimp larvae and an overexploitation of broodstock.

Consequently, the control of disease became a priority at the world level, if shrimp production is to be ecologically and economically sustainable. To a greater extent, the durability of the production is dependent on the equilibrium between the environment quality, the prevention of diseases by diagnosis and epidemiological surveys of the pathogens, and the health status of the shrimp. Finally, shrimp aquaculture is also dependent on the selection of the disease resistant animals. Therefore, the prevention and the control of shrimp diseases need an integrated approach in which basic knowledge of shrimp immunity must be improved. Consideration, should also be given to other research areas related to shrimp pathology and physiology, in close connection to research in genetics.

1.4.1 Viral disease

Disastrous failures have occurred in the shrimp farming industry in Thailand in the part decade mostly due to virus infection. The different species of shrimp viruses, yellow-head virus (YHV) and white spot syndrome virus (WSSV) have been reported to cause yellow-head disease (YH) and white spot

syndrome disease (WSS) in *P. monodon*, respectively (Boonyaratpalin et al., 1993; Wongteerasupaya et al., 1995), which are currently the cause of a very serious and widespread losses in the shrimp industry in Thailand and in Asia. Outbreaks of YH disease was the most serious problem in central and southern Thailand during 1993-1994, while WSS disease has been the most serious problem from 1994-1996.

1.4.1.1 White spot syndrome (WSS) disease

WSSV (known in Thailand as systemic ectodermal and mesodermal baculovirus or SEMBV) is one of the most important shrimp disease, which affects most of the commercially cultivated marine shrimp species globally (Chou et al., 1995; Lightner, 1996; Flegel, 1997; Lotz, 1997; Span and Lester, 1997). In 1993, WSS disease was first discovered in cultured penaeid shrimp in Taiwan (Chou et al., 1995). Since then, WSS disease has become widespread and the most economically damaging disease of shrimp culture in Taiwan.

White spots on the exoskeleton and epidermis are the most commonly observed clinical sign of WSS disease in disease shrimp. However, the presence of white spots does not always mean that the condition is terminal. For instance, under non-stressful conditions, infected shrimp that have white spots may survive indefinitely. However, if the shrimp also appear : lethargic, if their color changes to pink or reddish-brown, if they gather around the edges of ponds at the surface during the day, or if there is a rapid reduction in food consumption, then a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of the signs.

WSS disease can cause up to 100% mortality, which a correspondingly devastating economic impact. In 1996, Lightner pointed out that no significant resistance to this disease had been reported for any species of shrimp, and this

still remains true today. The causative agent of WSS disease, WSSV is extremely virulent and has a wide host range (LO et al., 1996)

1.4.1.2 Yellow-head (YH) disease

YHV of the black tiger shrimp *P. monodon* was first discovered in Thailand in 1992, although it is now known to have caused extensive losses on the eastern coast of the Gulf of Thailand as early as 1991 (Flegel et al., 1995). YH disease occurs in the juvenile to sub-adult stages of shrimp 5 to 15 grams in size, especially at 50-70 days of grow-out (Lightner, 1996).

Early signs of disease include lack of appetite and lethargy; appear weak several hours before death (sink to the bottom). At the onset of YH disease shrimp have been observed consuming feed at an abnormally high rate for several days. Feeding abrubtly ceases and within 1 day, a few moribund shrimp appear swimming slowly near the surface at the pond edges. After infection, mortality may reach as high as 100% of affected poppulations within 3-5 days from one set of disease. Affected shrimp exhibit light yellow coloration of the dorsal cephalothorax area and generally pale or bleached appearance (Limsuwan, 1991). In the black tiger shrimp, typical signs of YH disease include characteristic yellowing of the hepatopancreas and gill.

1.4.2 Bacterial disease

Vibriosis is a serious problem in the majority of penaeid shrimp culture operations, *Vibrio* species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). However, somemore recently occurring disease syndromes of penaeid shrimp have been caused by *Vibrio* species which behave more like true pathogens than opportunistic invader (Lightner et al., 1992).

In Thailand, vibriosis is the main cause of production loss due to bacterial disease in penaeid shrimp farms (Nash et al., 1992). The major vibrio species isolates from diseased shrimp are *Vibrio parahaemolyticus, V. harveyi, V. alginolyticus,* and *V. vulnificus,* while *V. damsela, V. anguillarum,* and *V. fluvia* have been reported less frequently (Leangphibul et al., 1985; Lightner, 1988; Ruangpan and Kitao, 1991; Nash et al., 1992; Jiravanichpaisal et al., 1994). Vibriosis caused mortality in larvae, post-larvae, juveniles, sub-adults and adults. At times, outbreaks cause mortality up to nearly 100% of affected populations (Lightner, 1983). The gross signs of localized infection in the cuticle or sub-cuticle are called shell disease or black or brown spot disease and these superficial infections can develop into systemic infections under some circumstances. It is the systemic infections that cause mortality.

Luminescent vibriosis bacteria, *V. harveyi*, was claimed to be the most causative agent associated with shrimp mortality. The disease is widely known as luminous disease or Kung-ruangsang in Thai. The first reported on shrimp luminous disease in Thailand was published in 1987 by Sae-oui and collaegues. The report indicated an outbreak of the disease in shrimp hatcheries in the central part of country, which used to be the largest productive area for marine shrimp. The bacterial pathogenesis resulted in mortality up to 100% for nauplius to zoea stages of *Penaeus merguiensis*. Living and dead shrimp larvae and even the seawater in disease outbreak areas were luminescent in dim light. Other gross features of the diseased shrimp were milky white bodies, weakness, swimming disorders and loss of appetite, eventually leading to death.

In *P. monodon,* luminous disease was also reported initially from larvae at nauplius to mysis stages (Ruangpan, 1987). Since then, a rising incidence of luminous disease that can cause 80-100% mortality to the larval stocks has occurred in hatchery areas all over the country. The luminous disease in *P. monodon* larvae was also caused by *V. harveyi* (Songserm et al., 1990).

1.5 Defense system in invertebrates

Invertebrates have characteristic host defense systems different to mammalian immune systems. Invertebrates lack antibodies, they do not possess proteins with domains belonging to the immunoglobulins, nor memory following the first encounter with a pathogen. Instead, they have developed innate immune systems to defend themselves against invading foreign materials.

In crustaceans, this innate defense system includes hemolymph coagulation, melanization, cytolysis, cell agglutination, antimicrobial actions, phagocytosis, and encapsulation against pathogens. Their defense systems are activated by the recognition of common epitopes on the surface of pathogens, such as bacterial lipopolysaccharide (LPS), peptidoglycan, and β-1,3-glucans.

Crustacean immune response to pathogens is based on both cellular and humoral component of the circulatory system, which cooperate to eliminate potentially infectious pathogens. The humoral factors comprise molecules that act in the defense without direct involvement of cells, humoral response, although many of the factors are originally synthesized and stored in the blood cells. Consequently, the actions with direct participation of blood cells are understood by the term cellular response.

1.5.1 Blood cells

The major defense systems of crustaceans are carried in the hemolymph, which contains a cells called hemocyte. The recognition molecules may interact with and activate the hemocytes, which play an important and central role in host defense. Hemocytes are the effectors of the cellular immune response and they are also involved in the synthesis of the majority of humoral effectors.

In crustaceans, according to numerous works dealing with the identification of hemocyte cell types, a classification scheme has been commonly adopted with three types of circulating hemocytes : hyaline cells, semigranular cells, and granular cells (Bauchau, 1981; Martin and Graves, 1985; Tsing et al., 1989; Rodriguez et al., 1995; Van de Braak et al., 1996).

In shrimp, hyaline cells are the smallest of the hemocytes, lack cytoplasmic granules, and compose only 5-10% of the circulating hemocytes. Semigranular cells are the most abundant type of hemocyte (75% of all hemocytes) and contain a variable number (1-40) of small (S) granules (0.4 μm diameter). Granular cells compose 10-20% of the hemocytes and contain a large number of secretory large (L) granules (0.8 μm diameter). Based on morphological and cytochemical characterization, some functions and involvement in different defense reactions have been attributed to the different cell types. Hyaline cells response by phagocytosis (Söderhall et al., 1986; Thornqvist et al., 1994) and coagulation (Omori et al., 1989). Semigranular cells, which contain S-granules and display some phagocytic capacities, (Gargioni and Barracco, 1998) would be specialized in particle encapsulation (Persson et al., 1987). Semigranular cells appear to be the most sensitive ones and react first during an immune response, by degranulation. This release of vesicle contents can stimulate granular cells to degranulate as well. These cells also seem to take part in encapsulation. Semigranular cells and granular cells participates in prophenoloxidase (proPO) system, which is an important component of the cellular defense reaction (Johansson and Söderhall, 1985).

Hemocytes play a central role in crustacean immune defense system. Firstly, they remove foreign particles in the hemocoel by phagocytosis, encapsulation and nodular aggregation (Söderhall and Cerenius, 1992). Secondly, hemocytes take part in wound healing by cellular clumping and initiation of coagulation processes through the release of factor required for plasma gelation (Johansson and Söderhall, 1989; Omori et al., 1989; Vargas-

Albores et al., 1998), and carriage and release of the proPo system (Johansson and Söderhall, 1989; Hernandez-Lopez et al., 1996). They are also involved in the synthesis and discharge in the hemolymph of important molecules, such as agglutinins (Rodriguez et al., 1995), and antibacterial peptides (Schnapp et al., 1996; Destoumieux et al., 1997; Lester et al., 1997).

1.5.2 Mechanism of defense reaction

1.5.2.1 Recognition proteins

The first immune process is the recognition of abroad spectrum of factors that are released or are present on the surface of invading microorganisms, which is mediated by the hemocytes and by plasmatic proteins. There is little information about the molecular mechanisms that mediate recognition. However, in crustaceans, several types of modulator proteins have been described that recognize cell wall components of pathogens.

In shrimp, two kinds of proteins are involved in the recognition of microbial products. The first group is constitutes multivalent sugar-binding agglutinins, also named hemagglutinins or lectins. Lectins have the ability to bind to specific carbohydrates expressed on different cell surfaces (Chen et al., 1993; Wilson et al., 1999), promote the agglutination of different cells, such as bacteria and other invading pathogens. It is reasonable to assume that these molecules may be regarded as having a potential role in invertebrate non-self recognition reaction. They can agglutinate microorganisms and enhance their phagocytosis by mediating binding between the hemocyte surface and a foreign body (Wilson et al., 1999). Agglutinating activities have been detected in plasma of *P. monodon* (Ratanapo and Chulavatnatol, 1992), *Penaeus stylirostris* (Vargas-Albores et al., 1992), *Penaeus californiensis* (Vargas-Albores et al., 1993a), *Penaeus japonicus* (Bachere et al., 1995), and *Penaeus indicus* (Maheswari et al, 1997).

The agglutinins from *P. monodon* plasma have been purified by affinity chromatography. This lectin, named monodins, which induced the agglutination of the highly pathogenic bacteria *V. vulnificus*, a major infective bacterium for prawns (Ratanapo and Chulavatnatol, 1992).

Similarly, the agglutinin isolated from *P. californiensis* plasma by affinity chromatography can be inhibited by monosaccharides and glycoproteins. Although specificity has not been completely defined, this agglutinin can react with bacterial LPS and is also capable of agglutinating several Vibrios, such as *V. parahaemolyticus*. This shrimp LPS-binding agglutinin has an apparent molecular weight of 170-180 kDa, has been reported as a multivalent carbohydrate-binding agglutinin that, besides its bacterial agglutination ability, it can enhance the phagocytic rate (Vargas-Albores, 1995).

The second surface recognizing protein detected in shrimp plasma has the capability to react with β-1,3-glucans, and therefore, it is named beta glucan binding protein or BGBP. The first crustacean BGBP was reported in freshwater crayfish, *Pacifastacus leniusculus* (Davic and Söderhall, 1990), and two marine shrimp species, *P. californiensis* (Vargas-Albores et al., 1996) and *Penaeus vannamei* (Vargas-Albores et al., 1997). Shrimp BGBP appears to be a constitutive plasma protein that after binding to β-1,3-glucans reacts with hemocyte surface and stimulates the release of hemocytic granules. The contents of the granules become activated in presence of proPO system (Barracco et al., 1991; Johansson and Söderhall, 1992; Vargas-Albores, 1995; Vargas-Albores et al., 1996).

Another group of recognition protein are the peptidoglycan-recognition proteins (PGRP) have been characterized and cloned from *Trichoplusia ni* (Kang et al., 1998) and *Bombyx mori* (Ochiai and Ashida, 1999) and since peptidoglycan can induce activation of the prophenoloxidase (proPO) system in

shrimps, it many indicate that PGRP may be present in shrimp (Sritunyalucksana et al., 1999).

Thus, these recognition proteins are capable of activating cellular activities only after reaction with the microbial carbohydrates: LPS, peptidoglycans or β-1,3-glucans.

1.5.2.2 The prophenoloxidase (proPO) system

In shrimp, like in all crustaceans, a dark pigmented spot in the cuticle appears after an animal is injured. This is due to the action of phenoloxidase (PO), which promotes hydroxylation of phenols and oxidation of *o*-phenols to quinones, necessary for the melanization process observed in response to foreign matter invading the hemocoele and during wound healing (Johansson and Söderhall, 1989; Ashida and Yamazaki, 1990; Söderhall, 1992; Söderhall et al., 1994). Quinones are subsequently polymerized into melanin by non-enzymatic reaction. Although a direct antimicrobial activity has been described for melanin and its precursors (Nappi and Vass, 1993), the production of reactive oxygen species such as superoxide anions and hydroxyl radicals during the generation of quinoids (Nappi et al., 1995; Song and Hsieh, 1994) also has an important antimicrobial role. In addition, biological reactions such as phagocytosis, encapsulation and nodulation are also activated.

Crustacean PO is located inside hemocytic granules as an inactive proenzyme called proPO and its transformation from proPO to PO involved several reactions known as the proPO activating system. In the penaeid shrimp, enzymes of the proPO system are localized in the semigranular and granular cells (Vargas-Albores et al., 1993; Perazzolo and Barracco, 1997). This is in agreement with a recent study showing that *P. monodon* proPO mRNA is expressed only in the hemocytes (Sritunyalucksana et al., 2000). In shrimp, proPO system is specifically activated by microbial cell wall components, such

as β-1,3-glucans (Vargas-Albores, 1995; Vargas-Albores et al., 1996; Vargas-Albores et al., 1997), LPS (Gollas-Galvan et al., 1997; Hernandez-Lopez et al., 1996), and peptidoglycan at extreamely low quantities.

The proposed proPO activation model for crustaceans (Johansson and Söderhall, 1989; Söderhall, 1992; Söderhall et al., 1994) involves a proteolytic cleavage mediated by a serine proteinase (Aspan et al., 1990a, Aspan et al., 1990b), namely proPO activating enzyme (PPAE). In shrimp, there are two steps involved in the activation of proPO. The first one is the degranulation that occurs when hemocytes are stimulated with bacteria, LPS or beta glucans, and inactive forms of both proPO and PPAE are released. The second one requires the participation of Ca^{2+} for the conversion of inactive PPAE to an active proteinase that, in turn, transforms proPO to active PO (Aspan and Soderhall, 1991; Gallas-Galvan et al., 1997). PO has the ability to adhere to surfaces, and this leads to the formation of melanin on the surface of the pathogen.

1.5.2.3 The coagulation system / the clotting system

Another system activated by microbial products is coagulation. Hemolymph coagulation defense response of crustaceans that prevents both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al., 1991). The clotting in crustaceans involves plasma proteins and cellular components (Durliat, 1985) identified as transglutaminase (TGase) from the hemocytic granules (Durliat, 1985; Martin et al., 1991). The key plasma protein which constitute the clot has been named clotting protein or CP (Kopacek et al., 1993), although it is sometimes called coagulogen or fibrinogen (Fuller and Doolittle, 1971; Madaras et al., 1981; Martin et al., 1991). It appears to be present in relatively high concentrations in hemolymph.

In crustaceans, CP were found in several species, including the spiny lobster, *Panulirus interruptus* (Fuller and Doolittle, 1971), the freshwater crayfish, *P. leniusculus* (Kopacek et al., 1993), the sand crayfish, *Ibacus ciliatus* (Komatsu and Ando, 1998), the white shrimp, *P. vannamei* (Montano-Perez et al., 1998) and in the black tiger shrimp, *P. monodon* (Yeh et al., 1998). In the spiny lobster, *Panulirus japonicus*, TGase was localized in the hemocytes, especially in hyaline and semigranular cells, and was shown to be involved in the clotting process (Aono and Mori, 1996). In all cases, CP was reported as a lipoglycoprotein with a molecular mass of approximately 420 kDa, composed of two identical subunits that are bound by disulfide bridges, and appeared to have similar amino acid compositions and NH_2-terminal sequences.

The basis coagulation system is well known in the freshwater crayfish, *P. leniusculus* (Kopacek et al., 1993; Hall et al., 1999). The crayfish CP, a dimeric protein consisting of 210 kDa subunits, is a VHDL (Hall et al., 1995, Kopacek et al., 1993) and each of 210 kDa subunits has both free lysine and glutamine, which are recognized and become covalently linked to each other by TGases. The TGase- dependent clotting reaction in crayfish is induced when a TGase is released from hemocytes or tissues, becomes activated by the Ca^{2+} in plasma and starts to crosslink the CP molecules into large aggregates. TGases are Ca^{2+}-dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on certain proteins.

However, the clotting reaction has only been fully characterized in crayfish (Hall et al., 1999), the mechanism in other crustaceans have to be elucidated in more detail for comparative studies of the clotting reaction in crustaceans.

1.5.2.4 Antimicrobial peptides or proteins

Antimicrobial peptides are important in the first line of the host defense system of many animal species. Their value in innate immunity lies in their ability to function without either high specificity or memory. The production of antimicrobial peptides is a widespread mechanism of host defense in the living kingdom, present from bacteria, protozoans, invertebrates to vertebrates and in plants. These effectors of innate immunity were initially characterized in insects. The most of antimicrobial substances are small cationic and amphipathic peptides. Their small size makes them easy to synthesize without dedicated cells or tissues and they rapidly diffuse to the point of infection.

Over the past 20 years, several of these antimicrobial peptides were isolated and characterized in arthropods, mainly in insects and chelicerates (horseshoe crabs) (Hetru et al., 1998; Iwanaga et al., 1998). Their involvement in the defense reaction is quite different in these two groups. In horseshoe crabs, they are mainly synthesized in hemocytes where they are stored after processing within their cytoplasmic granules (Iwanaga and Kawabata, 1998). They are believed to be release into hemolymph through regulated exocytosis upon microbial stimulation. This system differs from that described in insects, where the fat body is the main site for the antimicrobial peptide synthesis (Hoffmann and Reichhart, 1997; Engstrom, 1998), and upon injury antimicrobial peptide gene transcription is induced, resulting in their immediate synthesis and subsequent secretion into the blood.

For convenience, these antimicrobial peptides are tentatively classified into four distinct groups based on amino acid sequences, secondary structures, and functional similarities : (i) linear basic peptides forming amphipathic α-helices which are devoid of cysteine residues including the cecropins, the first antimicrobial peptide isolated from insect hemolymph; (ii) peptides with one to six intramolecular disulfide bridges including the arthropod defensins,

antifungal peptides from *Drosophila*, drosomycin and metchnikowin, thanatin from *Podisus*, anti-LPS factor, tachyplesin, big defensin and tachycitin from *Limulus*; (iii) the proline-rich peptides such as the apidaecins or drosocin; (iv) the glycine-rich peptides or polypeptides such as attacins, diptericin, and sarcotoxins.

There are few reports on antimicrobial factors in crustaceans. Only a few antimicrobial factors have been purified mainly from horseshoe crab. However some studies have shown the ability of crustacean hemolymph to inhibit bacterial growth (Adams, 1991; Chisholm and Smith, 1992; Noga et al., 1994, 1996). Several antimicrobial peptides that are active, *in vitro*, against Gram-positive and Gram-negative bacteria, were found in the granular hemocytes of the shore crab, *Carcinus maenas* (Schnapp et al., 1996; Smith, 1997). In 1997, a small peptide named callinectin was reported to be responsible for the majority of antibacterial activity observed in the hemolymph of blue crab *Callinectes sapidus* (Lester et al., 1997). At the same time, research on the penaeid shrimp *P. vannamei*, allowed full characterization of three members of a new family of antimicrobial peptides (Destoumieux et al., 1997). These peptides, named penaeidins, are the first antimicrobial molecules found in penaeid shrimp.

Anti-lipopolysaccharide (anti-LPS) factor

Anti-LPS factor, initially characterized in horseshoe crab. During the purification of proteins in coagulation system, a protein component that inhibits the coagulation cascade was found in the hemocyte lysate from Japanese (*Tachypleus tridentatus*) and American (*Limulus polyphemus*) horseshoe crabs (Iwanaga et al., 1985). This protein, anti-LPS factor specifically inhibits the LPS-mediated activation of clotting system.

Anti-LPS factor is a small basic protein, which binds and neutralizes bacterial endotoxin (LPS) and has a strong antibacterial effect especially on the growth of Gram-negative R-type bacteria (Morita et al., 1985), and a hemolytic activity on the red blood cells sensitized with LPS. In hemocytes, this protein is located in L-granules with several clotting factors (Toh et al., 1991; Iwanaga et al., 1998).

The primary structure of anti-LPS factor isolated from the Japanese horseshoe crab was first determined in 1986 (Aketagawa et al., 1986); that of the American horseshoe crab was established in the following year (Muta et al., 1987) (Figure 1.3). Whereas the Japanese horseshoe crab anti-LPS factor has its NH_2-terminus masked with pyroglutamic acid, the American horseshoe crab anti-LPS factor has its NH_2-terminus masked with an aspartyl residue. Both anti-LPS factors are single chain polypeptides composed of 101 or 102 amino acid residues, which contain two cysteine residues linked with an intramolecular disulfide bridge, and has a relative molecular mass of 12000. No glycosylation was found. The sequence of *L. polyphemus* anti-LPS factor obtained here shows 83% sequence identity with that of *T. tridentatus*.

The hydropathic profile indicates that the NH_2-terminal region of anti-LPS factor is highly hydrophobic and the remaining region contains positively charged residues, mainly found within the disulfide loop. The region from Arg-41 to Lys-49 has basic amino acids at every second residues, and the region from Arg-61 to Arg-76 is also rich in basic residues. In this region (Arg-61 to Arg-76), there are positive charges at almost every third residues. The clusterings of charged groups and high hydrophobicity of the NH_2-terminal region suggest that the molecule of anti-LPS factor is amphipathic.

The three-dimensional structure of *Limulus* anti-LPS factor (LALF) was reported in 1993. The crystal structure of anti-LPS factor reveals a simple tertiary fold with a striking charge distribution and amphipathicity. The

molecule has a single domain consisting of three α-helices packed against a four-stranded β-sheet. The molecule is wedge shaped, about 40 A tall and 28 A wide at its base (Figure1.4) (Hoess et al., 1993). The binding site for LPS probably involves the extend amphipathic loop of anti-LPS factor, which binds the phosphoglucosamine portion of lipid A. An analogous LPS-binding loop also exists for two human proteins that bind LPS, LPS-binding protein (LBP) (Schumann et al., 1990) and bactericidal/permeability-incressing protein (BPI) (Marra et al., 1992).

As mentioned earlier, anti-LPS factor contains two positively charged regions and an NH_2-terminal hydrophobic region. These two positively charged clusters might provide interaction site with phosphate groups in the lipid A portion of LPS. Once anti-LPS factor interacts with LPS on the cell membrane through the LPS-binding loop, the membrane structure seems to be perturbed by insertion of the hydrophobic NH_2-terminal region, including up to about the 27^{th} residue. The region appears to be long enough to cross a lipid bilayer-like transmembrane α-helix of the bacteriorhodopsin molecule.

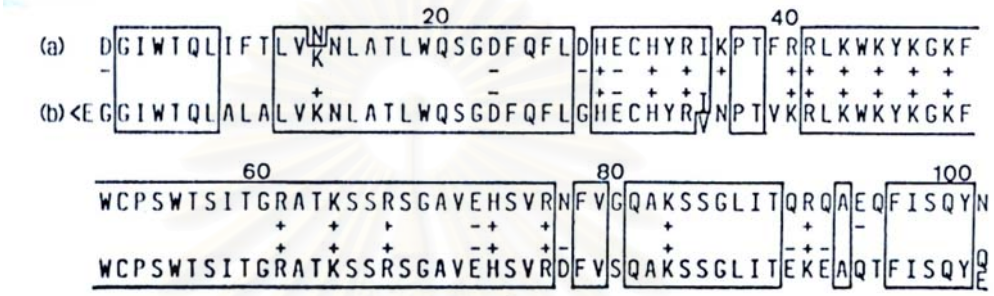


Figure 1.3 Alignment of the sequences of anti-LPS factors from *L. polyphemus* (a) and *T. tridentatus* (b). Identical residues are boxed and the charged residues are indicated by + or - between the two sequences (Aketagawa et al., 1986; Muta et al., 1987).

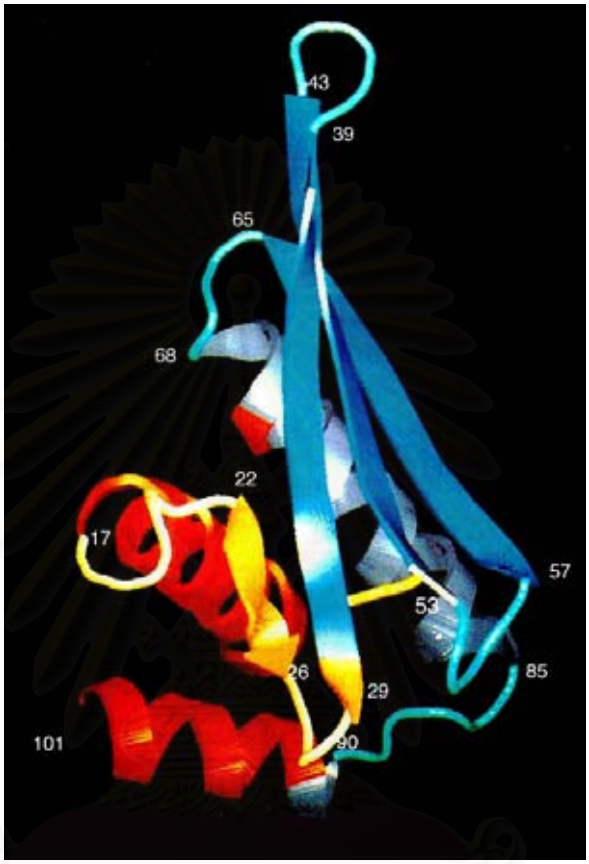


Figure 1.4 Schematic ribbons representation of LALF with the ends of secondary structure elements numbered (Hoess et al., 1993).

In a previous study, expressed sequence tags (ESTs) from the hemocyte of the black tiger shrimp, *P. monodon*, were generated in order to identify gene associated with shrimp immunity. A hemocyte cDNA library was constructed. Randomly selected cDNA clones with insert over 500 bp in length were sequenced. The partial nucleotide sequences of cDNA clones were compared with sequences in the GenBank database using the BLAST program. From these ESTs, 9% matched with genes involved in defense reaction molecules, such as the components of the proPO system including PO, proPO activating enzymes; the components of the clotting system including glutamine gamma-glutamyl transferase, hemocyte protease; the antioxidative enzymes, peroxidase and catalase; antimicrobialpeptides including anti-LPS factor, penaeidins, 11.5 kDa antibacterial peptides, lysozyme, and serine proteinase inhibitors.

Three full-length cDNA clones were found to encode a homologue of anti-LPS factor (Sh13, Sh20 and Sh71). The insert fragment of the cDNA clone was 517 bp in length, which contains an open reading frame (ORF) of 372 bp encoding 123 amino acids. The deduced amino acid shows 57% homology to the anti-LPS factor from Atlantic horseshoe crab.

The amino acid sequences alignment of anti-LPS factor purified from the horseshoe crabs, *L. polyphemus*, *T. tridentatus*, and the black tiger shrimp *P. monodon* shows the two conserved cysteine residues, and highly positive charged residues within the disulfide loop, which are necessary for disulfide bridge formation in three dimensional structure and the binding to the lipid A moiety of LPS, respectively. The NH_2-terminal region is highly hydrophobic and anti-LPS factor purified from horseshoe crabs were found to lack the NH_2-terminal portion. The extra 26 amino acid residues at the NH_2-terminus of the *P. monodon* anti-LPS factor was proposed to be a signal peptide for protein transport.

In this study, we aim to characterize the anti-LPS factor of *P. monodon* by cloning and expression of the cDNA clone (Sh71) in the baculovirus expression system. Two versions of the gene expressing full-length and NH_2-terminal truncated derivative of the protein were constructed. A full-length version of anti-LPS factor with a signal peptide was constructed to direct the expressed protein out of the cells for the ease of protein purification, while NH_2-terminal deletion version was constructed to eliminate the signal peptide to obtain a mature protein. The gene product will be characterized to confirm its identity, and investigate the activity of both derivatives of the protein.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave Model # LS-2D (Rexall industries Co. Ltd., Taiwan)

A –20 °C Freezer

A –80 °C Freezer (Revco)

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

Balance : Satorius 1702 (Scientific Promotion Co.)

Biochiller 2000 (Fotodyne, USA)

Flexi-dry FTS system (USA)

Fraction collector : FRAC-200 (Pharmacia)

Gene Pulser (Bio-RAD)

GS Gene Linker^{TM} : UV Chamber (Bio-RAD Laboratories, USA)

Gyrotory water bath shaker Model # G76 (New Brunswick Scientific, USA)

Hoefer Mini VE Vertical Electrophoresis System (Amersham Pharmacia)

Hybridization oven (Hybrid, USA)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Larminar flow : Dwyer Mark II Model # 25 (Dwyer instruments, USA)

Light microscope (Olympus, Japan)

Microcentrifuge tube 0.5 and 1.5 ml (Bio-RAD Laboratories, USA)

Minicentrifuge (Costar, USA)

Nipro disposable syringe (Nissho)

Orbital Shaker (Gallenkamp)

PCR thermal cycler : Gene Amp PCR System 2400 (Perkin Elmer)

PCR thin wall microcentrifuge tube 0.2 ml (Perkin Elmer)

PCR workstation Model # P-036 (Scientific Co., USA)

Pharmacia LKB-Pump P-1 (Pharmacia)

pH meter Model # SA720 (Orion)

Pipette boy ACU (Integra biosciences)

Pipette tips 10, 20, 200, and 1000 µl (Bio-RAD Laboratories, USA)

Power supply : Power PAC 3000 (Bio-RAD Laboratories, USA)

Refrigerated centrifuge Model # J2-21 (Beckman, USA)

Refrigerated microcentrifuge Kubota 1300 (Kubota, Japan)

Rocker platform (Bellco, USA)

Spectrophotometer : Spectronic 2000 (Bausch & Lomb)

Sterile disposable plastic pipettes 1, 5, and 10 ml (Sterilin)

Stirring hot plate (Fisher Scientific)

Tissue culture dish 35 mm (Iwaki)

Tissue culture flask 25, 150, and 225 cm^2 (Iwaki)

Touch mixer Model # 232 (Fisher Scientific, USA)

Transilluminator 2011 Macrovue (LKB)

Vacuum blotter Model # 785 (Bio-RAD Laboratories, USA)

Vacuum pump (Bio-RAD Laboratories, USA)

Water bath MT / 2 (Lauda)

White/ UV transilluminator : UVP ImageStore 7500 (Mitsubishi Electric Corporation,Japan)

2.2 Chemical reagents

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

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

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bromophenol blue (BDH)

Chloroform, $CHCl_3$ (Merck)

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

Dextran sulphate (Sigma)

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

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

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

Ethidium bromide (Sigma)

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

Ficoll^{TM}-400 (Amersham)

Foetal bovine serum (Gibco BRL)

Formaldehyde, CH_2O (BDH)

GeneAmp PCR core reagent (Perkin Elmer)

: 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl)

: 25 mM $MgCl_2$

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine NH_2CH_2COOH (Scharlau)

Grace insect medium (Gibco BRL)

Hydrochloric acid, HCl (Merck)

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

Isopropanol, C_2H_5COOH (Merck)

Kodak tri-X pan400 film

Liquid nitrogen (TIG)

Lipofectin reagent (Gibco BRL)

Lysozyme (Sigma)

Methanol, CH_3OH (Merck)

2-mercaptoethanol, C_2H_6OS (Fluka)

0.22 μM millipore membrane filter (Millipore)

N, *N*-dimethyl formamide, $HCONH_2$ (Fluka)

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

N, *N*, *N'*, *N'*-tetramethylethylenediamine (TEMED) (BDH)

Neutral red (Fluka)

Nytrans® super charge nylon membrane (Schleicher & Schuell)

Phenol crystals, C_6H_5OH (Carlo Erba)

Potassium chloride, KCl (Merck)

SeaPlaque GTG agarose (FMC Bioproducts)

Sephadex G-50 (Pharmacia)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

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

Sodium hydrogen carbonate, $NaHCO_3$ (BDH)

Sodium hydroxide, NaOH (Eka Nobel)

TC-100 insect medium (Gibco BRL)

Tris-(hydroxy methyl)-aminomethane, $NH_2C(CH_2OH)_3$ (USB)

Tryptic soy broth (Difco)

Trizol reagent (Gibco BRL)

Tween^{TM}-20 (Fluka)

Unstained molecular weight marker (Owl separation systems)

Whatman 3 MM^{TM} filter paper (Whatman)

Xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$ (Sigma)

2.3 Enzymes

Eco RI (Biolabs)

Exonuclease free *Klenow* (Amersham)

Hind III (Biolabs)

Not I (Biolabs)

Pst I (Biolabs)

RNase A (Sigma)

Shrimp alkaline phosphatase (USB)

Sma I (Biolabs)

Taq DNA polymerase (Pharmacia)

T_4 DNA ligase (Gibco BRL)

Xho I (Biolabs)

2.4 Bacterial strains

Escherichia coli strain DH5α

E. coli strain XL-1blue

Staphylococcus aureus

Vibrio harveyi 1526

2.5 Animals

The black tiger shrimps, *P. monodon*, were obtained from local shrimp farms. They were maintained in an aquarium (24 ± 4 °C, salinity: 20 ppt) at least 2 days before the experiment.

2.6 Hemocytes and tissues collection

The shrimp hemolymph was collected from the ventral sinus located at the base of the first abdominal segment, using a 26G needle syringe containing 0.2 ml anticoagulant [10 % (w/v) sodium citrate]. The hemolymph was immediately centrifuged at 2000 rpm at 4 °C for 10 minutes to seperate the hemocytes from plasma. The hemocyte pellets were resuspended in Trizol reagent (Gibco BRL) and total RNA was extracted. After hemolymph collection, shrimp tissues were harvested by disection. The tissues were collected from the shrimp *P. monodon* including lymphoid organ, intestine, hepatopancreas, heart, gills, muscle and eyes stalk. The disected tissues were

immediately dropped in liquid nitrogen and ground to fine powder in liquid nitrogen by means of mortar and pestle.

2.7 Total RNA preparation

Total RNA was extracted from *P. monodon* tissues and hemocytes using Trizol reagent (Gibco BRL). Homocyte pellets and tissues powder were homogenized in 1 ml Trizol reagent (Gibco BRL) and stored the homogenated at room temperature for 5 minutes. The samples were added 0.2 ml chloroform, mixed by vigorously vortex for 15 minutes and stored at room temperature for 2-15 minutes. The mixture was centrifuged at 12000 rpm at 4 °C for 20 minutes and then transferred the aqueous phase into a new microcentrifuge tube. RNA from the aqueous solution was precipitated by mixing with 0.5 ml isopropanol and stored at –20 °C overnight. Total RNA was isolated by centrifuged at 12000 rpm at 4 °C for 15 minutes, removed the supernatant and washed RNA pellet with 75 % ethanol and subsequent centrifugation at 12000 rpm at 4 °C for 10 minutes. The RNA pellet was kept in 75 % ethanol until used. The RNA pellet was air-dried and dissolved in RNase free water by incubating at 55-60 °C for 10 minutes for Northern analysis.

The concentration of total RNA can be determined by measuring the OD at 260 nm. A 1 OD unit at 260 nm corresponds to approximately 40 μg/ml (Sambrook et al., 1989). The RNA concentration is estimated in μg/ml by the following equation,

$$[\text{RNA}] = \text{OD}_{260} \times \text{Dilution factor} \times 40$$

2.8 Preparation of *Vibrio harveyi* infected shrimp (modified from Roque et al., 1998)

V. harveyi 1526 (kindly provided by Charoenpokphand Group of companies) was cultured in tryptic soy broth (TSB) (DIFCO, Becton Dickinson France S.A.) with 1 % (w/v) sodium chloride (NaCl) at 30 °C for 8-10 hours and then diluted 1 : 100 with steriled normal saline solution [0.85 % (w/v) NaCl. Before being injected, the inoculum was monitored the density by the plate count method in tryptic soy agar (TSA) with 1 % (w/v) NaCl (modified from Austin, 1988). A 0.1 ml of inoculum with a density of 10^7 CFU/ml was injected into the muscle of the fourth abdominal segment. The control group or unchallenged animal was prepared for injected with 0.1 ml sterile normal saline solution. After 48 hours post injection, the shrimps were tested infection by streaking suspensions of hepatopancreas or hemolymph onto TSA with 1 % (w/v) NaCl. It was incubated at 30 °C overnight and resulted the colonies of *V. harveyi* 1526 from infected shrimps showed strong luminescence or emitted light in the dark.

2.9 Recombinant expression of anti-LPS factor in baculovirus expression system

2.9.1 Construction of transfer vector

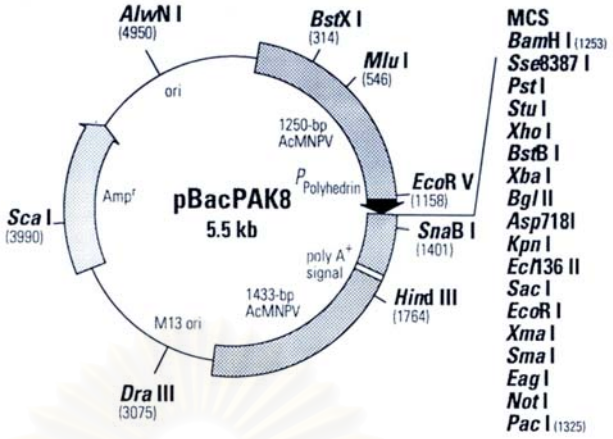
2.9.1.1 Preparation of anti-LPS factor gene

A full-length anti-LPS factor (FL-ALF) gene was excised from pSh71 with restriction enzyme *Eco* RI then the gene fragment was purified by using QIAquick gel extraction kit (QIAGEN). The FL-ALF gene was subcloned into the *Eco* RI site of baculovirus transfer vector, pBacPAK8 (Figure 2.1), yielding a recombinant plasmid, ptr13.

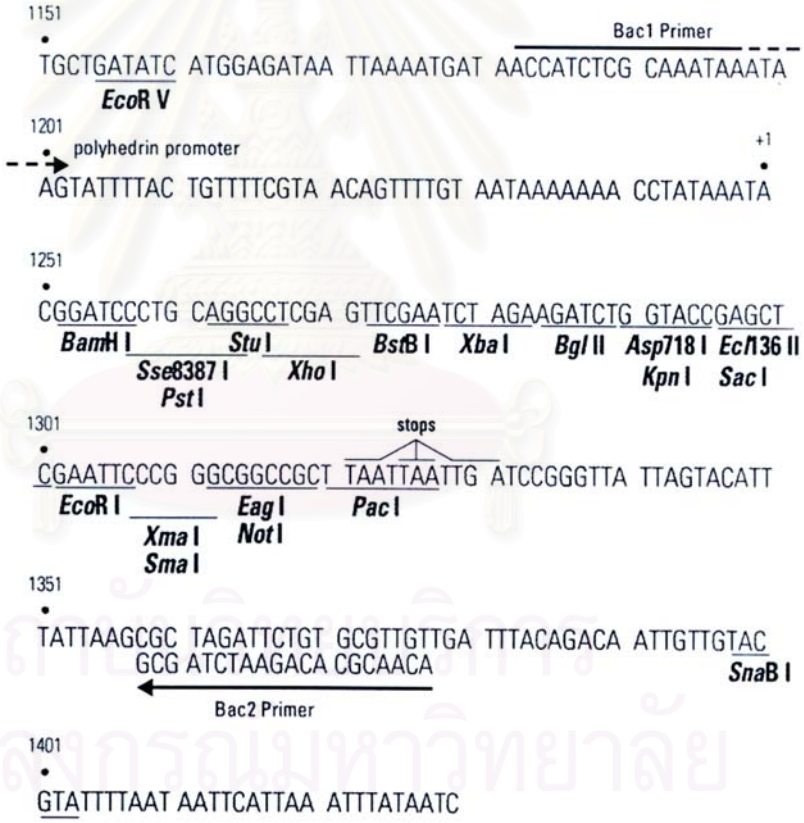
5'-terminal truncated (ΔNAL) gene was constructed by polymerase chain reaction (PCR) mutagenesis, using pSh71 as a template and oligonucleotide primers incorporating 5' *Xho* I (5'- CCGCTCGAGCGGATGTGGGAGGCTGT G –3') and 3' *Eco* RI (5'- GGAATTCCTCATAGAGCAAAAGG –3') cleavage sites. The amplification reaction was prepared in a 25 μl reaction volume containing 50 ng of DNA template, 1.25 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1x PCR buffer, 1.2 mM $MgCl_2$, 5 pmole of each primers, and 1 unit of *Taq* DNA polymerase (5 units/μl). Sterile distilled water was added to make the final volume to 25 μl. The amplification program of ΔNAL gene was initially denatured at 94 °C for 3 minutes followed by 35 cycles of 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute. The final extension was also carried out at 72 °C for 10 minutes. The resulting PCR product was run in 1.5 % agarose gel to determined whether the reaction was successfully amplified and the resulting in the amplification of a 330 bp fragment. The ΔNAL fragment gene was digested with restriction enzymes *Xho* I and *Eco* RI then purified with QIAquick gel extraction kit (QIAGEN) and subcloned into *Xho* I and *Eco* RI sites of pBacPAK8 (Figure 2.1) to yield pΔNAL7.

2.9.1.2 Ligation

The mixture of sticky-end ligation must contained a suitable molecular ratio between vector and DNA insert which is usually 1 : 3. The 15 μl of ligation reaction was composed of 1.5 μl of 10x T4 DNA ligase buffer, 1 μl of 10 mM ATP, 1 μl of T4 DNA ligase (3 units/μl), 150 ng of DNA insert, and 50 ng of pBacPAK8/*Eco* RI with dephosphorylated for FL-ALF gene or 50 ng of pBacPAK8/*Xho* I/*Eco* RI for ΔNAL gene. Steriled distilled water was added to make the final volume to 15 μl. The mixture was mixed, quick spun for 30 seconds and incubated at 14-16 °C overnight.



[a]



[b]

Figure 2.1 Transfer vector maps and MCS sequences

[a] Map of pBacPAK8 transfer vector

[b] Sequences in and around the pBacPAK8 multiple cloning sites

2.9.1.3 Competent cells preparation

A single colony of *E. coli* XL-1blue was cultured as the starter 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 for overnight. One percent of the microbial starter was inoculated into 1000 ml of L broth [1 % (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 0.5 % (w/v) NaCl] and the culture was incubated for cell grow at 37 °C with vigorous shaking for 3-5 hours until the optical density at 600 nm (OD_{600}) of the cells reached 0.5-0.8. Cells were then chilled on ice for 15-30 minutes and harvested by centrifugation at 5000 rpm for 10 minutes at 4 °C. The supernatant was removed as possible. The cell pellet was washed by resuspending in a total of 1000 ml of cold steriled water, gently mixing and centrifugation. The pellet was washed further with different kinds of solution, first with 500 ml of cold steriled water, followed with 20 ml of ice cold steriled 10 % (v/v) glycerol, and resuspended to a final volume of 2-3 ml ice cold steriled 10 % (v/v) glycerol. This cell suspension was divided into 40 µl aliquots and stored at –80 °C until used. The cells were good for at least 6 months under these conditions.

2.9.1.4 Electrotransformation

The competent cells were gently thawed at room temperature and then immediately placed on ice. Fourty microlitres of the cell suspension was mixed with 1 to 2 µl of ligation mixture, mixed well and placed on ice for approximately 1 minute. The mixture of cell and DNA was transformed by electroporation in a cold 0.2 cm cuvette with setting the apparatus as follows; 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, the cells were immediately resuspended with 1 ml 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] and

transferred to test tube. The cell suspension was incubated at 37 °C with shaking at 250 rpm for 1 hour. The content was spread on the LB agar plates, which contained 50 mg/ml ampicillin, and incubated at 37 °C overnight. After incubation, colonies were selected randomly for plasmid DNA isolation.

2.9.1.5 Plasmid DNA preparation

The colonies were inoculated into 1.5 ml of LB broth containing ampicillin (50 mg/ml) (one colony per tube) and incubated at 37 °C with shaking for overnight. The cultures were transferred into 1.5 ml microcentrifuge tube and spun at 8000 rpm for 1 minute. The supernatant was discarded, 100 μl of solution I [25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose, and 0.5 % (w/v) lysozyme] was added, mixed by vigorous vortexing and placed on ice for 30-60 minutes. 200 μl of freshly prepared solution II [0.2 N NaOH and 1 % (w/v) SDS] was added for cell lysis and DNA denaturation and mixed gently. After incubating on ice for 10 minutes, the mixture was added with 150 μl of solution III (3 M sodium acetate, pH 4.8) for renaturation, mixed gently and placed on ice for 30 minutes. The tube was spun at 10000 rpm for 10 minutes to separate cell debris. The supernatant was transferred into a new microcentrifuge tube, added with equal volume of the solution containing Phenol : Chloroform : Isoamyl alcohol (25 : 24 : 1), mixed and spun at 10000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube. The plasmid DNA was precipitated by adding 2 volumes of absolute ethanol then mixed well and kept at –80 °C for at least 1 hour. The mixture was centrifuged at 10000 rpm for 10 minutes. The plasmid DNA was washed with 70 % (v/v) 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).

2.9.1.6 Detection of the desired recombinant plasmid

Transfer vector of FL-ALF gene was detected with restriction enzyme mapping to confirm the correct orientation of the gene. The recombinant plasmid was digested with restriction enzymes *Eco* RI, *Hind* III, *Pst* I, *Not* I, *Sma* I, and *Xho* I and analyzed by agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (100 bp marker, Biolabs) and the restriction enzyme mapping was constructed.

Transfer vector of ΔNAL gene was detected by polymerase chain reaction (PCR), using recombinant plasmid as a template and oligonucleotide Bac1 (5'- ACCATCTCGCAAATAAATAAG –3') and Bac2 (5'- ACAACGCA CAGAATCTAGCG –3'). The amplification reaction was prepared in a 25 μl reaction volume containing 50 ng of DNA template, 1.25 mM of each dNTPs (dATP, dCTP, dGTP, dTTP), 1x PCR buffer, 1.2 mM $MgCl_2$, 5 pmole of each primers, and 1 unit of *Taq* DNA polymerase. Sterile distilled water was added to make the final volume to 25 μl. The PCR cycles was initially denatured at 94 °C for 3 minutes followed by 35 cycles of 94 °C for 1 minute, 52 °C for 1 minute, and 72 °C for 1 minute. The final extension was also carried out at 72 °C for 10 minutes. The resulting in the amplification of a 486 bp fragments.

The recombinant transfer vectors containing FL-ALF gene (ptr13) and ΔNAL gene (pΔNAL7) were amplified and purified by using QIAprep plasmid purification kit (QIAGEN). 1.5 ml overnight cultured of ptr13 and pΔNAL7 in LB broth with containing ampicillin (50 mg/ml) was transferred into 1.5 ml microcentrifuge tube and spun at 8000 rpm for 1 minute. The bacterial cell pellet was resuspended in 250 μl of buffer P1 and mixed by vortexing. 250 μl of buffer P2 was added and mixed gently. Then 350 μl of buffer N3 was added, mixed gently by immediately inverted the tube for 4-6 times, and centrifuged at 12000 rpm for 10 minutes. During centrifugation, QIAprep spin column was

placed in a 2 ml collection tube. The supernatant was transferred into QIAprep spin column, centrifuged at 10000 rpm for 1 minute, and discarded the flow-through. QIAprep spin column was washed with different buffer containing 500 μl of buffer PB and 750 μl of buffer PE, respectively, centrifuged at 10000 rpm for 1 minute and discarded the flow-through. QIAprep spin column was removed residual washed buffer by additional centrifuged at 12000 rpm for 1 minute and then placed the QIAprep spin column into a new 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 μl of buffer EB (10 mM Tris-Cl. PH 8.5) or steriled distilled water to the center of QIAprep column, incubated at room temperature for 5 minutes, and centrifuged at 12000 rpm for 1 minute.

2.9.2 Co-transfection of anti-LPS factor into the insect Sf9 cells

2.9.2.1 Cells and baculoviruses

Spodoptera frugiperda Sf9 cells were maintained as monolayer at 27 °C in TC-100 insect medium (Gibco BRL) supplemented with 10 % Foetal bovine serum (Gibco BRL) [TC-100/10 % (v/v) FBS]. The recombinant baculoviruses were obtained by *in vivo* homologous recombination in Sf9 cells between the transfer vector carrying the foreign gene and *Bsu* 36I-digested DNA from the AcNPV baculovirus DNA (Pharmigen, USA). The two types of baculovirus transfer vectors, ptr13 and pΔNAL7, used in this study to express genes of interest under the control of the polyhedrin (PH) promoter.

2.9.2.2 DNA co-transfection

Sf9 cells were seeded in 4-5 ml TC-100/10 % (v/v) FBS onto 25 cm^2 tissue culture (TC-25) flask and incubated at 27 °C for 18-24 hours before transfection. The density of the cells should be 30-50 % confluent. Sf9 cells were co-transfected by mixing 2-5 μg of transfer vector containing foreign

gene, 500 ng of linearized baculovirus DNA (Pharmigen, USA), and 15 μl of Lipofectin reagent (Gibco BRL) in a total volume 200 μl. The serum free TC-100 insect medium was added to make the final volume to 200 μl. The mixture was mixed gently and incubated at room temperature for 10-15 minutes. Meanwhile, the Sf9 cell monolayers were removed the medium and washed the cells with 2 ml of serum free insect medium. After incubation, the DNA-Lipofectin mixture was added 1.8 ml of serum free insect medium, mixed gently, and overlaid onto insect cell monolayers. After 24 hours of incubation at 27 °C, the DNA-Lipofectin mixture was removed, added 4 ml of TC-100/10 % (v/v) FBS, and incubated at 27 °C for 48 hours. The recombinant virus were collected from the supernatant by centrifugation at 1000 rpm for 10 minutes to and purified by plaque purification.

2.9.3 Plaque Assay

Sf9 cells were seeded onto the 35 mm tissue culture dish and incubated at 27 °C for 24 hours before infection. The density of the Sf9 cell monolayers should be 70-80 % confluent. The co-transfection supernatant was diluted with serial dilution to give final dilution of 10^{-4}, 10^{5-}, and 10^{-6}. The Sf9 cell monolayers were removed the medium and washed the cells with 1 ml of serum free insect medium. 100 μl of each dilution of the co-transfection supernatant was added to the center of the dish and mixed gently by rocking the dish back every 20 minutes at room temperature for 1 hour. During this incubation, the steriled 2 % (w/v) SeaPlaque agarose (FMC Bioproducts) was melted and cooled to 37 °C. Grace insect medium was pre-warmed to 37 °C. After incubation, virus inoculum was removed from the cell monolayers by tilting the dish and aspirating from the edge. One percent (w/v) SeaPlaque agarose solution was prepared by mixing equal volume of 2 % (w/v) SeaPlaque agarose solution and pre-warmed Grace insect medium. The infected cell monolayers were overlaid with 1.5 ml of 1 % (w/v) SeaPlaque agarose solution by carefully

adding agarose to the side of the tilted dish. When the agarose was set completely, placed the dish in a plastic storage box with a moisted paper towel, and incubated at 27 °C for 4-5 days. After incubation, the dishes were stained with 0.03 % (w/v) neutral red with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) at 27 °C for 2-3 hours then removed the staining solution. The dishes were inverted and kept them in the dark at room temperature overnight to allow the plaques to appear.

The plaques were counted on each dish and calculated the average number of plaques per dish. 0.1 ml of virus inoculum was applied to each dish. The titer of the virus stock in plaque forming units per ml (pfu/ml) was calculated from this equation,

$$\text{Titer of the virus stock (pfu/ml)} = (\text{average plaques per dish}) \times 10 \times (\text{dilution factor})^{-1}$$

2.9.4 Preparative production of recombinant proteins

The 70-80 % confluent monolayers of Sf9 cells in 150 cm^2 tissue culture (TC-150) flask were washed with serum free insect medium and infected with 5 multiplicity of infection (MOI = plaque forming units/cell number) of each the recombinant baculoviruses. Incubation was allowed to proceed for 1 hour, mixed gently by rocking the flask back every 15 minutes, and was followed by addition of 25-30 ml TC-100/10 % (v/v) FBS. The Sf9 cell monolayers was incubated at 27 °C for 60-72 hours. During incubation, the cells were checked for signs of infection 2-3 days after inoculation under the light microscope. The infected Sf9 cells should be enlarged in size (about 2 fold) and large nucleus should be visible. The infected cells and the supernatant were harvested 60-72 hours post infection (hpi) by centrifugation at 3000 rpm for 10 minutes. The cell pellet was stored at –20 °C and the supernatant was collected. The proteins

in the supernatant were precipitated by adding equal volume of cooled acetone, mixed gently, and kept at –20 °C overnight. The mixture was centrifuged at 10000 rpm for 10 minutes, discarded the supernatant, and stored the protein pellet at –20 °C until used. The infected cell pellet and protein pellet were divided and resuspended in 1x sample loading buffer [50 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol, and 14.4 mM 2-mercaptoethanol] to analyze by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.9.5 Analysis of recombinant proteins by SDS-PAGE

The two types of recombinant proteins, full length and NH_2-terminal truncated anti-LPS factor proteins, were separated on 15 % SDS-PAGE gels (Sambrook et al., 1989). The 15 % separating gel (for two 9.5 cm × 10 cm × 1 mm gels, need 15 ml) was prepared by mixing 3.4 ml of deionized water, 7.5 ml of 30 % acrylamide mix solution [29 % (w/v) of acrylamide and 1 % (w/v) *N*, *N'*, methylenebisacrylamide], 3.8 ml of 1.5 M Tris-HCl, pH 8.8, 0.15 ml of 10 % (w/v) SDS, 0.15 ml of 10 % (w/v) ammonium persulfate, and 6 μl of TEMED, respectively. Polymerization will began as soon as the TEMED has been added. Without delay, the mixture was swirled rapidly and poured the separating gel solution into the gap between the glass plates. Deionized water was carefully overlaid on the top of the separating gel solution and placed the gel in a vertical position at room temperature. After polymerization was completed (30-60 minutes), poured off the overlaid and washed the top of the gel with deionized water to remove any unpolymerized acrylamide.

The 5 % stacking gel (for two 9.5 cm × 10 cm × 1 mm gels, need 5 ml) was prepared by mixing 3.4 ml of deionized water, 0.83 ml of 30 % acrylamide mix solution [29 % (w/v) of acrylamide and 1 % (w/v) *N*, *N'*, methylenebisacrylamide], 0.63 ml of 1.0 M Tris-HCl, pH 6.8, 50 μl of 10 %

(w/v) SDS, 50 μl of 10 % (w/v) ammonium persulfate, and 5 μl of TEMED, respectively. Polymerization will began as soon as the TEMED has been added. The mixture was swirled rapidly and poured the stacking gel solution directly onto the surface of polymerized separating gel and immediately inserted comb into the stacking gel solution by carefully to avoided trapping air bubbles. The stacking gel solution was added to fill the spaces of comb completely and placed the gel in a vertical position at room temperature. While the stacking gel was polymerizing, the samples were prepared by heating them in boiling water for 10 minutes in 1x sample loading buffer [50 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, 10 % (v/v) glycerol, and 14.4 mM 2-mercaptoethanol] to denature proteins. After polymerization was completed (30-60 minutes), the comb was removed carefully and washed the wells immediately with deionized water to remove any unpolymerized acrylamide. The gel was placed into the electrophoresis chamber and added 1x Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine, pH 8.3, and 0.1 % (w/v) SDS] to the top and bottom reservoirs. The samples and unstained molecular weight marker (Owl separation systems) were loaded into the bottom of the wells and run the gel at 20 mA/gel until the bromophenol blue reached the bottom of the separating gel (about 4 hours). The gel was stained with Coomassie gel stain 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 4 hours with gently shaking. After staining, the gel was destained by soaking in Coomassie gel destain solution [10 % (v/v) methanol and 10 % (v/v) glacial acetic acid] with gently shaking and changed destain solution three or four times for 24 hours.

2.10 Antibacterial assay

The crude protein samples were tested for their antibacterial activity against bacteria including the Gram-negative *Vibrio harveyi* 1526 and *Escherichia coli* DH5α and the Gram-positive *Staphylococcus aureus*.

Inhibitory activity was determined by the assay on bacterial growth rate. *E. coli* DH5α and *S. aureus* were grown on LB broth medium at 37 °C overnight and *V. harveyi* 1526 was grown on LM broth medium [1 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, and 2 % (w/v) NaCl] at 30 °C overnight. The overnight cultured were diluted with steriled normal saline solution [0.85 % (w/v) NaCl] to a final optical density at 600 nm of 0.01. The 0.5 ml of *E. coli* DH5α and *S. aureus* suspensions were added into LA broth medium, while *V. harveyi* 1526 was added into LM broth medium. Then, the 0.5 ml of crude protein samples were added to a final volume of 5 ml. The LA and LM broth medium were incubated at 37 °C and 30 °C, respectively. During incubation, the bacterial growth rate was determined by measuring the OD at 600 nm every hour and the growth curve was constructed.

2.11 Analysis of mRNA of anti-LPS factor

2.11.1 Northern blot analysis

2.11.1.1 Northern blotting

Total RNA was extracted from *P. monodon* hemocytes and tissues by using Trizol reagent (Gibco BRL). RNA samples were prepared in a solution containing 1x MOPS buffer (50 mM MOPs, 8 mM sodium acetate, 1 mM EDTA), 2.2 M formaldehyde, and 50 % (v/v) formamide. The total RNA of samples were denatured by heating at 60 °C for 10 minutes. Each RNA sample (20-25 μg) was loaded on a 1.4 % agarose-formaldehyde gel with containing 1x MOPS buffer, 0.66 M formaldehyde, and 0.02 μg/ml ethidium bromide. The gel was run in 1x MOPS buffer with 0.02 μg/ml ethidium bromide at 56 volts for 4 hours. Following electrophoresis, the gel was visualized under UV transillumination and photographed through a red filter onto Kodak tri-X pan400 film. Then the gel was equilibrated in excess water for 5-15 minutes

with gentle agitation. Finally, the gel was equilibrated in excess 10x SSC for 15 minutes with gentle agitation. The RNA samples were transferred to Nytrans® super charge nylon membrane (Schleicher & Schuell) by vacuum blotting.

The white porous vacuum plate was placed within the base of the apparatus. Whatman 3 MM^{TM} filter paper was cut larger than the gel and then pre-wet in 10x SSC and placed on the porous screen. The nylon membrane was cut to the same size or slightly larger than the gel, pre-wet in 10x SSC and placed on the filter paper. The rubber window gasket was socked with distilled water and placed over the membrane. The rubber window gasket should have a template cut such that the window was 2-5 mm smaller than the gel or enough to provide for a good seal. The gel was carefully transferred into position over the opening in the rubber window gasket in contact with the membrane and the lid was placed on the base of the unit and removed air bubbles by using a 10 ml glass pipette. The sealing frame was placed on the top of the vacuum stage and locked the sealing frame onto the four latch posts. The vacuum source was started and slowed turn the bleeder valve until the gauge read at 5 inches Hg. The transfer buffer (10x SSC) was gently poured on the upper reservoir. The RNA was transferred to nylon membrane for 1.5 hour at 5 inches Hg. When completed, the pump was turn off and remaining transferred was removed. The membrane was soaked in 2x SSC for 5 minutes and air-dried between two sheets of filter paper, followed by baking at 80 °C for 2 hours. The blot may be used immediately or stored at 4 °C until required.

2.11.1.2 Preparation of labeled probe

Anti-LPS factor fragments were prepared by cutting clone pSh71 with restriction enzyme *Eco* RI at 37 °C overnight, then purified the DNA fragment by running on a 1.4 % (w / v) agarose gel at 100 volts for 1 hour. The standard ladder (100 bp marker, Biolabs) lane was cut and stained with ethidium

bromide solution (2.5 μg / ml of ethidium bromide in water) for 5-10 minutes and then destain the gel in water for 10 minutes with gentle agitation. The ethidium bromide fluorescent DNA marker bands were visualized under UV light from a transilluminator. The anti-LPS factor fragments with size about 500 bp were measured from the DNA marker and eluted from agarose gel by using QIAquick gel extraction kit (QIAGEN).

18S rRNA transcripts were designed from the sequences of *Penaeus japonicus* (kindly provided by Jiraporn Rojtinnakorn) and the 18S rRNA fragments were prepared by RT-PCR reaction, using first strand cDNA of shrimp hemocytes as a template. First-strand cDNA was synthesised from total RNA using the AMV Reverse Transcriptase First-strand cDNA synthesis kit (Life Sciences) in a total volume of 12.5 μl. The 10 μg of total RNA was annealed to 0.05 μg of pd(T)12-18 at 70°C for 10 minutes and then chilled on iced. The AMV-reverse transcriptase (12.5 units) was added to the RNA-primer mixture in a reaction containing 20 mM DTT, 12.5 units RNasin RNase inhibitor, 1x reaction buffer (Life Sciences) containing nucleotides and incubated at 41 °C for 60 minutes. After incubation, the reaction was stopped by chilling on ice and 50 ng of the first strand cDNA was used as a template for amplification by the polymerase chain reaction (PCR). The PCR was conducted in a volume of 25 μl with a final concentration of 1x PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 0.5 M of 18S-f (5'- GAGACGGCTACCACATC TAAG –3') and 18S-r (5'- ATACGCTAGTGGAGCTGGA –3') primers, 0.2 mM of each dNTP (Gibco BRL) and 1 μg of cDNA template. Reactions were amplified on a thermal cycler (Perkin Elmer) using a 95 °C, 2 minutes initial denaturation followed by a 95 °C, 30 seconds denaturation, a 50 °C, 30 seconds annealing temperature, a 72 °C, 1 minute extension for 30 cycles, and a 72 °C, 7 minutes final extension. Annealing temperatures were optimized for each primer pair. The 5 μl of each PCR sample were resolved on a 1.5 % agarose gel, and the PCR products were visualized with ethidium bromide staining and UV

illumination. The PCR product of a 182 bp fragments should be observed. The 18S rRNA fragments were purified by using QIAquick gel extraction kit (QIAGEN).

The DNA fragment was excised with razor blade from agarose gel and transferred into a 1.5 ml microcentrifuge tube. The agarose was weighed to determine approximate volume of the gel slice (100 mg equals approximately to 100 μl) and added 3 volume of buffer QG to 1 volume of gel slice. The gel was incubated at 50 °C for 10 minutes and mixed by vortexing the tube every 2 or 3 minutes during the incubation to help dissolved gel. After the gel slice has dissolved completely, added 1 gel volume of isopropanol to the sample and mixed gently. A QIAquick spin column was placed in a provided 2 ml collection tube, loaded the mixture to the QIAquick column and centrifuged at 10000 rpm for 1 minute. The supernatant was discarded and placed QIAquick column back in the same collection tube. 750 μl of buffer PE was added to washed the QIAquick column. The supernatant was removed by centrifugation as above, discarded the flow-through and additional centrifuged the QIAquick column at 12000 rpm for 2 minutes. The QIAquick column was placed into a new 1.5 ml microcentrifuge tube. The DNA fragment was eluted by adding 50 μl of buffer EB (10 mM Tris-Cl, pH 8.5) or steriled water to the center of the QIAquick column and centrifuged at 12000 rpm for 2 minutes. The supernatant was combined.

DNA probe was prepared by using ECL random prime labeling and detection system (Amersham). The labeling reaction mixture contained the denatured DNA fragment of interest (up to 100 ng), 10 μl of nucleotide mixture, 5 μl of primers, 1 μl of exonuclease-free *Klenow* (5 units/μl), and steriled distilled water was added to make the final volume to 50 μl. The denatured DNA fragment was prepared by heating for 5 minutes in a boiling water bath and immediately chilled on ice for 5 minutes. After mixed the

reaction mixture to 1.5 ml microcentrifuge tube, spun briefly and incubated at 37 °C for 1 hour. The reaction was terminated by adding EDTA to a final concentration of 20 mM and probe can then be stored in a freezer at –20 °C in the dark for at least 12 months.

2.11.1.3 Hybridization

The membrane was pre-hybridized in hybridization solution [5x SSC, 0.1 % (w/v) SDS, 5 % (w/v) dextran sulphate (Sigma), 20 fold dilution of liquid block, and 2x Denhardt ‘s solution] at the volume 10 ml/100 cm^2 of membrane. The membrane was incubated at 60 °C for at least 1 hour. The labeled probe was denatured by heating for 5 minutes in a boiling water bath and quickly cooled on ice for 5 minutes. The denature labeled probe was spun briefly, then added to the hybridization solution and further incubated at 60 °C with gently shaking overnight. After hybridization step, the hybridization solution was removed and the membrane was washed with 1x SSC containing 0.1 % (w/v) SDS and 0.5x SSC containing 0.1 % (w/v) SDS, respectively, at 60 °C for 15 minutes.

2.11.1.4 Blocking, antibody incubation and washes

After washing step, the membrane was placed in a container, rinsed the membrane with buffer A (100 mM Tris-HCl, pH 7.5 and 600 mM NaCl) for 1 minute, and discarded the washed solution. The 20 fold dilution of liquid block in buffer A was added and incubated at room temperature for 30 minutes with gently shaking. During this incubation, the anti-fluorescein-HRP conjugate was diluted 2000 fold in freshly-prepared 0.5 % (w/v) bovine serum albumin (BSA) fraction V in buffer A. After incubation step, the membrane was placed in a new container and incubated in diluted conjugate solution at room temperature for 30 minutes with gently shaking. The membrane was then placed in a clean container and removed unbound conjugate by washing for

3×10 minutes in excess 0.1 % (v/v) Tween^{TM}-20 in buffer A at room temperature with gently shaking.

2.11.1.5 Signal generation and detection

The detection solution was prepared by mixing an equal volume of detection solution 1 with detection solution 2 to give sufficient to cover the blot. The membrane was drained the excess reagent from the washed blot and placed on a sheet of plastic bag. The detection solution was added directly to the membrane on the side carried the DNA and incubated at room temperature for 1 minute. The membrane was then drained off the excess detection reagent, wrapped the membrane in plastic bag and gentle smooth out air pockets followed by autoradiography.

2.11.2 Reverse transcription PCR (RT-PCR)

First-strand cDNA was synthesised from total RNA using the AMV Reverse Transcriptase First-strand cDNA synthesis kit (Life Sciences) in a total volume of 12.5 μl. Ten micrograms of total RNA was annealed to 0.05 μg of pd(T)12-18 at 70°C for 10 minutes and chilled on iced. The AMV-reverse transcriptase (12.5 units) was added to the RNA-primer mixture in a reaction containing 20mM DTT, 12.5 units RNasin RNase inhibitor, 1x reaction buffer (Life Sciences) containing nucleotides and incubated at 41°C for 60 minutes. After incubation, the reaction was stopped by chilling on ice and 50 ng of the first strand cDNA was used as a template for amplification by the polymerase chain reaction. The PCR was conducted in a volume of 25 μl with a final concentration of 1x PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 0.5 M of forward and reverse primers, 0.2 mM of each dNTP (Gibco BRL) and 1 μg of cDNA template.

Reactions were amplified on a thermal cycler (Takara) using a 95°C, 2 minutes initial denaturation followed by a 95°C, 30 seconds denaturation, a 50 to 60°C, 30 seconds annealing temperature, a 72°C, 1 minute extension for 30 cycles, and a 72°C, 5 minutes final extension. Annealing temperatures were optimized for each primer pair. Five microliters of each PCR sample were resolved on a 1.5 % agarose gel, and the PCR products were visualized with ethidium bromide staining and UV illumination. Signal quantification was analysed by ATTO Co. densitograph software (version 2).

CHAPTER III

RESULTS

3.1 Total RNA preparation

Total RNA was extracted from *P. monodon* tissues and hemocytes using Trizol reagent (Gibco BRL) as described in section 2.5.3. The concentration of total RNA was determined by measuring the absorbance at 260 nm and assuming that 1.0 OD was equivalent to 40 μg/ml (Sambrook et al., 1989). The total RNA from obtained hemocytes of each individual shrimp was about 10-20 μg/1 ml of hemolymph and those from shrimp tissues including lymphoid organ, intestine, hepatopancreas, heart, gills, muscle, and eyes stalk were about 5-10 μg/mg tissue. In this preparation method (using Trizol reagent), if total RNA had an $OD_{260/280}$ ratio of 1.6-1.9 indicating high purity. Whereas the $OD_{260/280}$ obtained was 1.5-1.7 indicating good quality of total RNA used in this study.

3.2 Construction of transfer vector

3.2.1 Preparation of anti-LPS factor gene

DNA fragments containing a full-length and NH_2-terminal truncated anti-LPS factors were prepared. A full-length anti -LPS factor (FL-ALF) gene of *P. monodon* was prepared by *Eco* RI digestion of a cDNA clone Sh71 (Figure 3.1). The insert fragment from pSh71 was used as a probe in Northern blot analysis and subjected to construct transfer vector for baculovirus expression system. The insert fragment of the pSh71 clone was 517 bp in length, which contains an open reading frame (ORF) of 372 bp. It codes for a protein of 123 amino acids with the putative initiation methionine codon (ATG)

begining at nucleotide 17 and the stop codon (TAG) begining at nucleotide 386 (Figure 3.2). The predicted molecular weight of the protein is 13.7 kDa.

The amino acid sequences alignment of anti-LPS factor purified from the horseshoe crabs, *L. polyphemus*, *T. tridentatus*, and the black tiger shrimp *P. monodon* showed the two conserved cysteine residues (C) linked with intracellular disulfide bond and highly positive charged residues within the disulfide loop, which are necessary for disulfide bridge formation in three dimensional structure. The NH_2-terminal region is highly hydrophobic and anti-LPS factor purified from horseshoe crabs were found to lack the NH_2-terminal portion because the amino acid sequences were obtained from mature protein. The extra 26 amino acid residues at the NH_2-terminus of the *P. monodon* anti-LPS factor was proposed to be a signal peptide for protein transport (Figure 3.3).

NH_2-terminal truncated of anti-LPS factor (ΔNAL) gene was prepared by PCR amplification using pSh71 as a template and oligonucleotide primers incorporating 5' *Xho* I (5'- CCGCTCGAGCGGATGTGGGAGGCTGTG –3') and 3' *Eco* RI (5'- GGAATTCCTCATAGAGCAAAAGG –3') cleavage sites. The resulting PCR product was run on a 1.5 % agarose gel to determine whether the reaction was successfully amplified a specific 330 bp fragment of expected size was observed (Figure 3.4). The ΔNAL fragment contains an ORF of 294 bp encoding 97 amino acids (Figure 3.5). The predicted molecular weight of the protein is 11.0 kDa. The 330 bp ΔNAL fragment gene was subjected to construct a transfer vector.

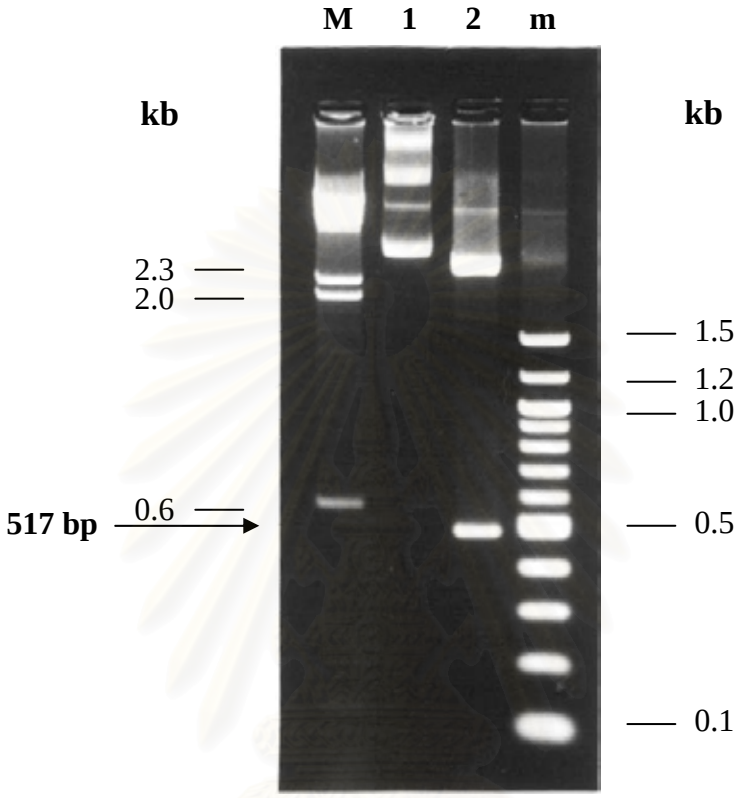


Figure 3.1 Ethidium bromide staining of clone pSh71 digested with restriction enzyme *Eco* RI. The DNA was run on 1.5 % agarose gel at 100 volts for 1 hour.

Lane M : DNA marker (λDNA/*Hind* III)

Lane m : Standard DNA ladder (100 bp marker)

Lane 1 : undigested clone pSh71

Lane 2 : clone pSh71 digested with *Eco* RI

```
  1 TTTCCTAGTTTAGAAG ATG CGT GTG TCC GTG CTG GTA AGC CTG GTG CTG GTG GTG TCC CTG GTG 64
  1                  M   R   V   S   V   L   V   S   L   V   L   V   V   S   L   V   16

 65 GCA CTC TTC GCC CCA CAG TGC CAG GCT CAA GGG TGG GAG GCT GTG GCA GCG GCC GTC GCC  124
 17 A   A   L   F   P   Q   C   Q   A   Q   G   W   E   A   V   A   A   A   V   A    36

125 AGC AAG ATC GTA GGG TTG TGG AGG AAC GAA AAA ACT GAA CTT CTC GGC CAC GAG TGC AAG  184
 37 S   K   I   V   G   L   W   R   N   E   K   T   E   L   L   G   H   E   C   K    56

185 TTC ACC GTC AAG CCT TAT TTG AAG AGA TTC CAG GTG TAC TAC AAG GGG AGG ATG TGG TGC  244
 57 F   T   V   K   P   Y   L   K   R   F   Q   V   Y   Y   K   G   R   M   W   C    76

245 CCA GGC TGG ACG GCC ATC AGA GGA GAA GCC AGC ACA CGC AGT CAG TCC GGG GTA GCT GGA  304
 77 P   G   W   T   A   I   R   G   E   A   S   T   R   S   Q   S   G   V   A   G    96

305 AAG ACA GCC AAA GAC TTC GTT CGG AAA GCT TTC CAG AAA GGT CTC ATC TCT CAA CAG GAG  364
 97 K   T   A   K   D   F   V   R   K   A   F   Q   K   G   L   I   S   Q   Q   E   116

365 GCC AAC CAG TGG CTC AGC TCA TAG GCCTTTTGCTCTATGAAGAATTGTCAGTGTTCAGCTGCAGTTGGCAA  436
117 A   N   Q   W   L   S   S   stop

437 TGGAAGCTCTACCATTTTGATTTAAAAAAAAAAAAAAAGCGGCCGCAAATTCCTCMACCCCGGGGGATCCACTATTTCT  514
515 AAA
```

Figure 3.2 Nucleotide and deduced amino acid sequence of a cDNA clone, Sh71, encoding anti-LPS factor from the black tiger shrimp, *P. monodon*. pSh71 containing a 517 bp insert fragment with an open reading frame (ORF) of 372 bp encoding 123 amino acids with the putative initiation methionine codon (ATG) begining at nucleotide 17 and the stop codon (TAG) begining at nucleotide 386. Nucleotide (upper) and deduced amino acid sequences (lower) are shown.

```
T. Tridentatus          1: ----------------------PEGGIWTQLALALVKNLATLWQSGDFQFLGHECHYRIN  38
L. polyphemus           1: -----------------------DGIWTQLIFTLVNNLATLWQSGDFQFLDHECHYRIK  36
P. monodon              1: MRVSVLVSLVLVVSLVALFAPQCQAQGWEAVAAAVASKIVGLWRNEKTELLGHECKFTVK  60
                                                  *  :  ::..::. **:. . ::*.***:: ::

T. tridentatus         39: PTVKRLKWKYKGKFWCPSWTSITGRATKSSRSGAVEHSVRDFVSQAKSSGLITEKEAQTF  98
L. polyphemus          37: PTFRRLKWKYKGKFWCPSWTSITGRATKSSRSGAVEHSVRNFVGQAKSSGLITQRQAEQF  96
P. monodon             61: PYLKRFQVYYKGRMWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQKGLISQQEANOW  120
                           * .:*::  ***::***.**:* *.*:. *:**.. ::.::** :* ..***::::*: :

T. tridentatus         99: ISQYQ        103
L. polyphemus          97: ISQYN        101
P. monodon            121: LSS--        123
                           :*.
```

Figure 3.3 Amino acids sequences alignment of anti-LPS factor from *L. polyphemus*, *T. tridentatus*, and *P. monodon*. The asterisks indicated that the residues are identical.

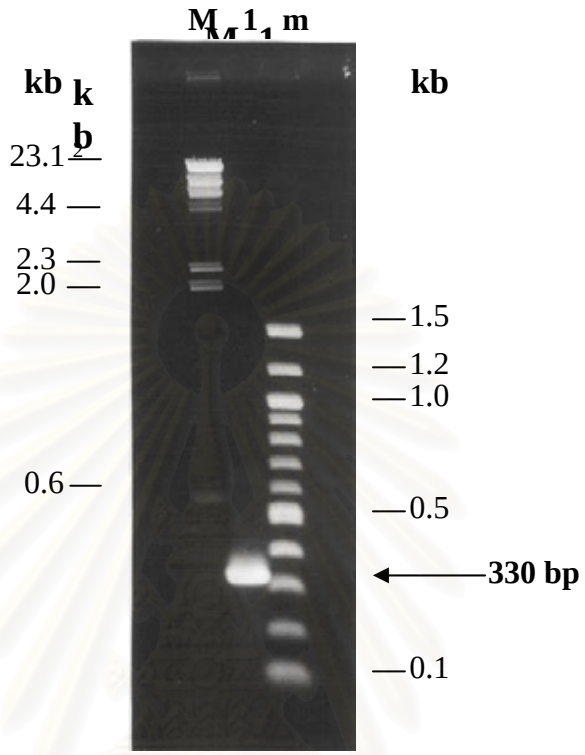


Figure 3.4 Ethidium bromide staining of NH_2-terminal truncated anti-LPS factor (ΔNAL) gene amplified by PCR, using pSh71 as a template and oligonucleotide primers incorporating 5' *Xho* I (5'- CCGCTCGAGCGGATG TGGGAGGCTGTG –3') and 3' *Eco* RI (5'- GGAATTCCTCATAGAGCAAA AGG –3') cleavage sites. The PCR product was run on a 1.5 % agarose gel at 100 volts for 1 hour.

Lane M : DNA marker (λDNA/*Hind* III)

Lane m : Standard DNA ladder (100 bp marker)

Lane 1 : amplified ΔNAL gene products

```
  1 CCGCTCGAGCGG ATG TGG GAG GCT GTG GCA GCG GCC GTC GCC AGC AAG ATC GTA GGG TTG TGG  63
  1              M   W   E   A   V   A   A   A   V   A   S   K   I   V   G   L   W   17

 64 AGG AAC GAA AAA ACT GAA CTT CTC GGC CAC GAG TGC AAG TTC ACC GTC AAG CCT TAT TTG  123
 18 R   N   E   K   T   E   L   L   G   H   E   C   K   F   T   V   K   P   Y   L    37

124 AAG AGA TTC CAG GTG TAC TAC AAG GGG AGG ATG TGG TGC CCA GGC TGG ACG GCC ATC AGA  183
 38 K   R   F   Q   V   Y   Y   K   G   R   M   W   C   P   G   W   T   A   I   R    57

184 GGA GAA GCC AGC ACA CGC AGT CAG TCC GGG GTA GCT GGA AAG ACA GCC AAA GAC TTC GTT  243
 58 G   E   A   S   T   R   S   Q   S   G   V   A   G   K   T   A   K   D   F   V    77

244 CGG AAA GCT TTC CAG AAA GGT CTC ATC TCT CAA CAG GAG GCC AAC CAG TGG CTC AGC TCA  303
 78 R   K   A   F   Q   K   G   L   I   S   Q   Q   E   A   N   Q   W   L   S   S    97

304 TAG GCCTTTTGCTCTATGAGGAATTCC 330
    stop
```

Figure 3.5 Nucleotide and deduced amino acid sequence of NH_2-terminal truncated anti-LPS factor (ΔNAL) gene amplified by PCR, using pSh71 as a template and oligonucleotide primers incorporating 5' *Xho* I (5'-CCGCTCGAGCGGATGTGGGAGGCTGTG –3') and 3' *Eco* RI (5'-GGAATTCCTCATAGAGCAAAAGG –3') cleavage sites. The specific 330 bp PCR product contains an ORF of 294 bp encoding 97 amino acids. Nucleotide (upper) and deduced amino acid sequences (lower) are shown. The nucleotides shown double underlined correspond to 5' *Xho* I site. The single underlined nucleotides correspond to 3' *Eco* RI site.

3.2.2 A full-length anti-LPS factor (FL-ALF) construct

The 517 bp full-length anti-LPS factor (FL-ALF) gene was digested with restriction enzyme *Eco* RI and the DNA fragment was purified by using QIAquick gel extraction kit (QIAGEN), then further ligated into *Eco* RI-digested and dephosphorylated pBacPAK8 vector with T4 DNA ligase. The ligation mixture was transformed into *E. coli* XL-1blue cells by electroporation as described in section 2.6.4. The recombinant clones were first selected with LB agar plates containing 50 mg/ml ampicillin, and further selected according to the presence of the insert fragment. The recombinant plasmid was digested with restriction enzyme *Eco* RI and analyzed on a 0.7 % agarose gel electrophoresis (Figure 3.6). The results show the example of 7 recombinant clones (clone no. 1, 3, 10, 12, 13, 21, and 28) which contained the inserted fragment whereas the clone no.7 does not contain the inserted fragment.

To confirm the corrected orientation of the gene was carried out a restriction mapping. The recombinant plasmid was digested with restriction enzymes *Eco* RI, *Hind* III, *Pst* I, *Not* I, *Sma* I, and *Xho* I and analyzed by a 1.4 % agarose gel electrophoresis using λDNA/*Hind* III and standard DNA ladder (100 bp markers, Biolabs) as markers to determine the fragment size (Figure 3.7). Comparison of restriction enzyme mapping from clone no.13 with restriction map applied from a DNA strider program demonstrated that the clone no.13 contains a correct oriented DNA insert fragment of a full length anti-LPS factor (FL-ALF) gene (Figure 3.8).

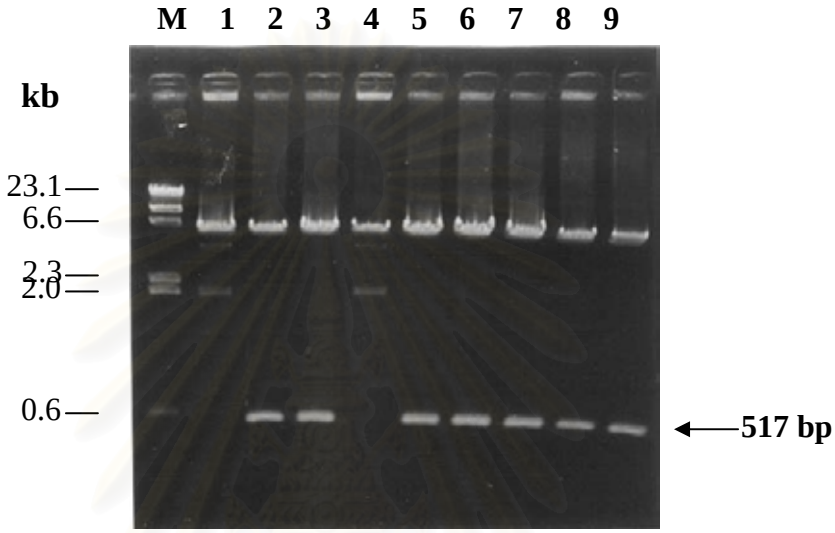


Figure 3.6 Ethidium bromide staining of recombinant FL-ALF transfer vector digested with restriction enzyme *Eco* RI to select a 517 bp insert fragment. The DNA fragment was analyzed on a 0.7 % agarose gel, electrophoresed at 100 volts for 1 hour.

Lane M : DNA marker (λDNA/*Hind* III)

Lane 1 : *Eco* RI digested pBacPAK8

Lanes 2-9 : *Eco* RI digested recombinant plasmid clones no.1, 3, 7, 10, 12, 13, 21, and 28, respectively

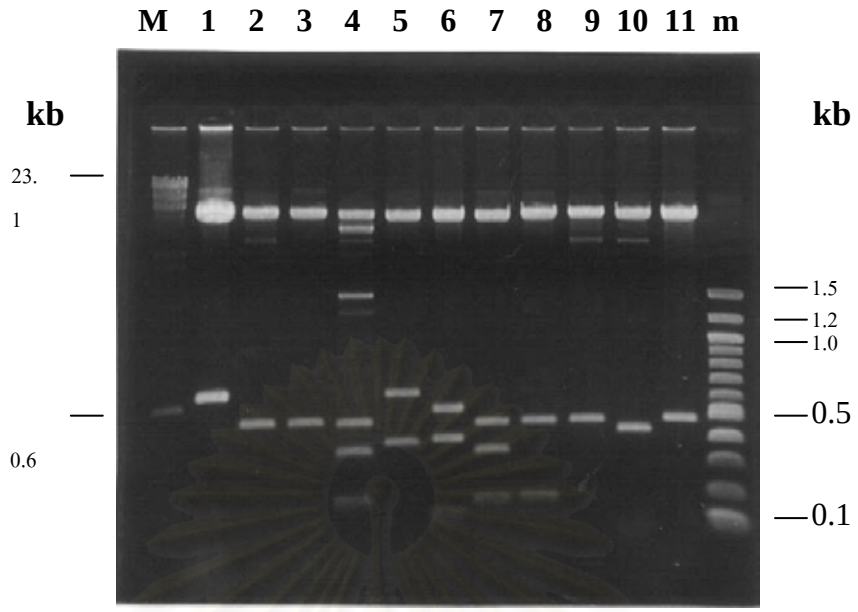


Figure 3.7 Ethidium bromide staining of a recombinant transfer vector (clone no.13 containing full-length anti-LPS factor (FL-ALF) digested with restriction enzymes *Eco* RI, *Hind* III, *Pst* I, *Not* I, *Sma* I, and *Xho*. The DNA fragment was analyzed on a 1.4 % agarose gel, electrophoresed at 100 volts for 1 hour.

Lane M : DNA marker (λDNA/*Hind* III)

Lane m : Standard DNA ladder (100 bp marker)

Lane 1 : digested with *Hind* III

Lane 2 : digested with *Pst* I

Lane 3 : digested with *Not* I

Lane 4 : digested with *Hind* III and *Eco* RI,

Lane 5 : digested with *Hind* III and *Xho* I

Lane 6 : digested with *Hind* III and *Pst* I

Lane 7 : digested with *Hind* III and *Not* I

Lane 8 : digested with *Hind* III and *sma* I

Lane 9 : digested with *Pst* I and *Xho* I

Lane 10 : digested with *Pat* I and *Not* I

Lane 11 : digested with *Sma* I and *Not* I

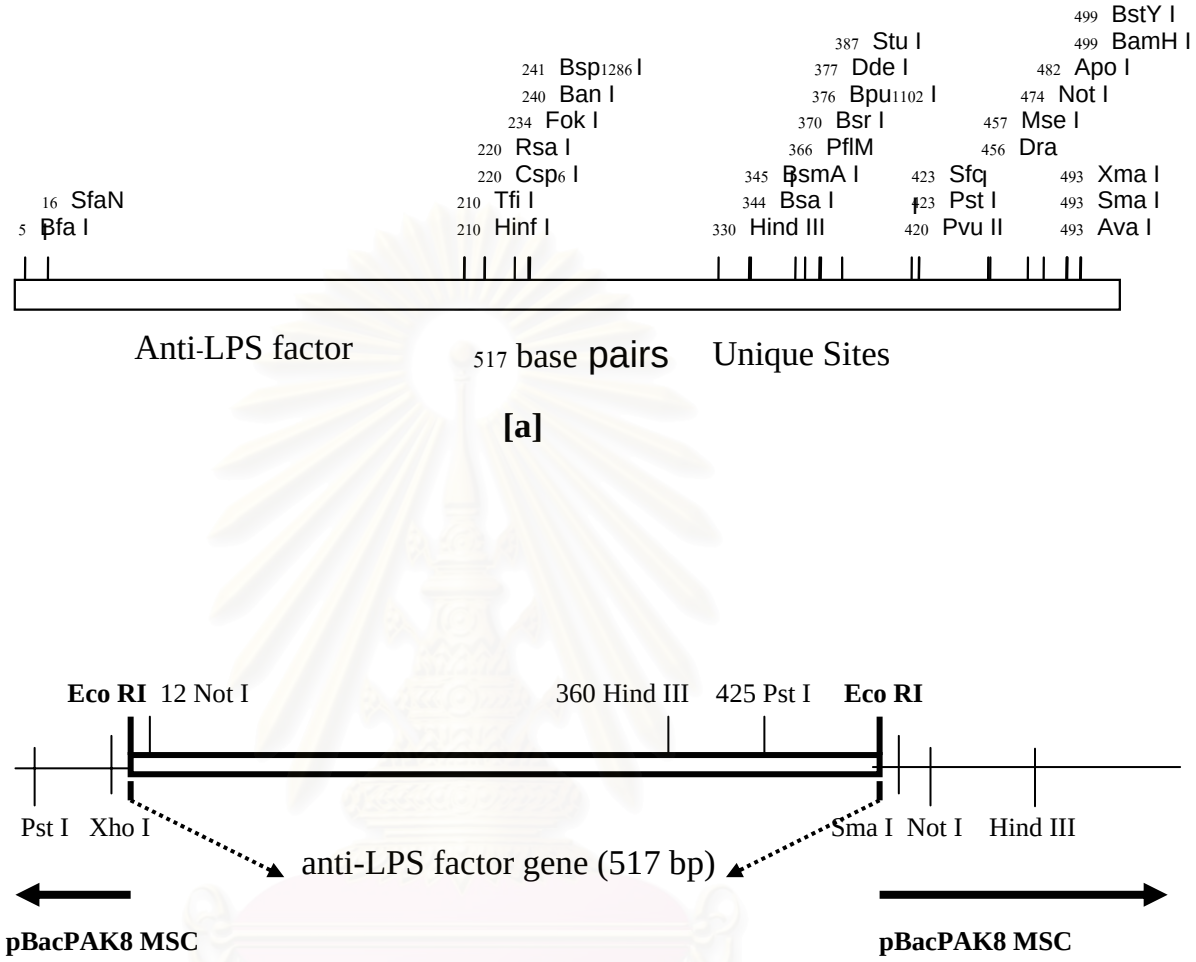


Figure 3.8 Comparison of restriction enzyme mapping of full-length anti-LPS factor (FL-ALF) gene applied from a DNA strider program that obtained from restriction digestion of clone no.13.

[a] restriction enzyme map applied from the DNA strider program

[b] restriction enzyme map from clone no.13

3.2.3 An NH_2-terminal truncated (ΔNAL) construct

The NH_2-terminal truncated (ΔNAL) fragment gene was amplified by PCR using oligonucleotide primers with contained *Xho* I and *Eco* RI cut sites. After amplification, PCR products were phenol-chloroform extracted, digested with restriction enzymes *Xho* I and *Eco* RI then purified with QIAquick gel extraction kit (QIAGEN) and further ligated into pBacPAK8/*Xho* I/*Eco* RI vector. The ligation mixture was transformed into *E. coli* XL-1blue cells by electroporation as described in section 2.6.4. The recombinant clones were first selected with LB agar plates containing 50 mg/ml ampicillin and further detected by PCR using recombinant plasmid as a template and oligonucleotide Bac1 (5'- ACCATCTCGCAAATAAATAAG –3') and Bac2 (5'- ACAACGCA CAGAATCTAGCG –3'). The resulting PCR products were analyzed by 1.4 % agarose gel electrophoresis using standard DNA ladder (100 bp markers, Biolabs) and a 330 bp ΔNAL gene as markers (Figure 3.9). The recombinant transfer vector containing a ΔNAL gene was amplified and the specific DNA fragment of 486 bp was obtained. The results in Figure 3.9 indicated that these clones contain ΔNAL gene.

The recombinant transfer vectors of FL-ALF gene (ptr13) and ΔNAL gene (pΔNAL7) were purified by using QIAprep plasmid purification kit (QIAGEN). The transfer vectors, ptr13 and pΔNAL7, were subjected to transfer anti-LPS factor gene to the viral genome of baculovirus.

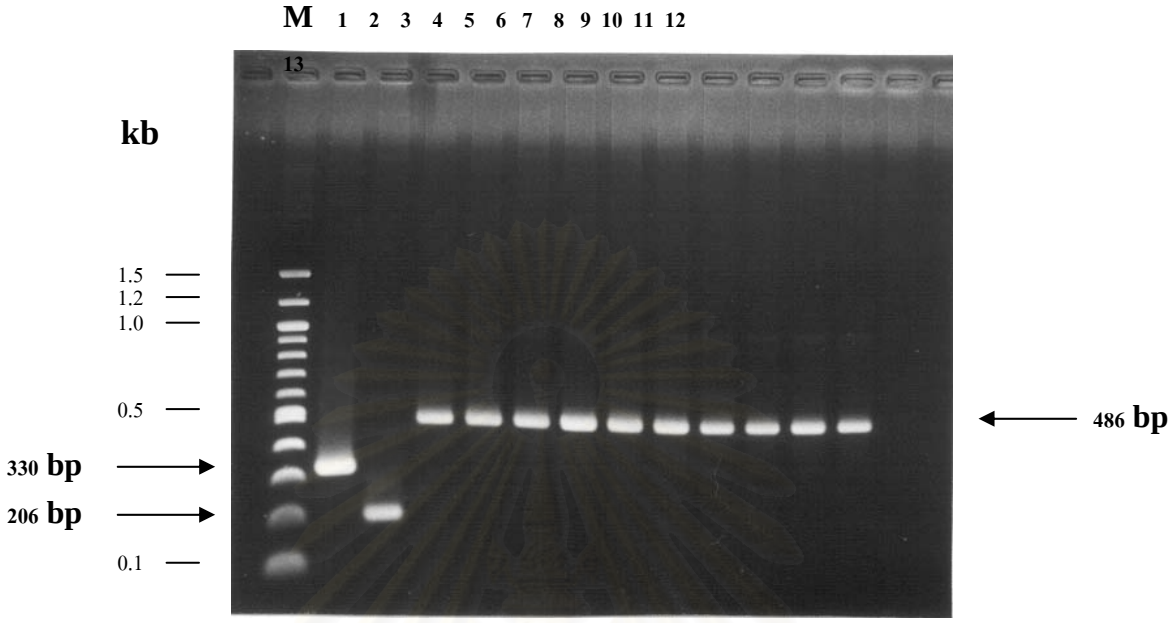


Figure 3.9 Ethidium bromide staining of NH_2-terminal truncated anti-LPS factor (ΔNAL) detected by PCR amplification using recombinant plasmid as a template and oligonucleotide Bac1 (5'- ACCATCTCGCAAATAAATAAG –3') and Bac2 (5'- ACAACGCACAGAATCTAGCG –3'). The recombinant transfer vector containing ΔNAL gene gave amplified product of 486 bp. The amplified PCR products were separated on a 1.4 % agarose gel, electrophoresed at 100 volts for 1 hour.

Lane M : Standard DNA ladder (100 bp marker)

Lane 1 : ΔNAL gene

Lane 2 : amplified pBacPAK8 vector

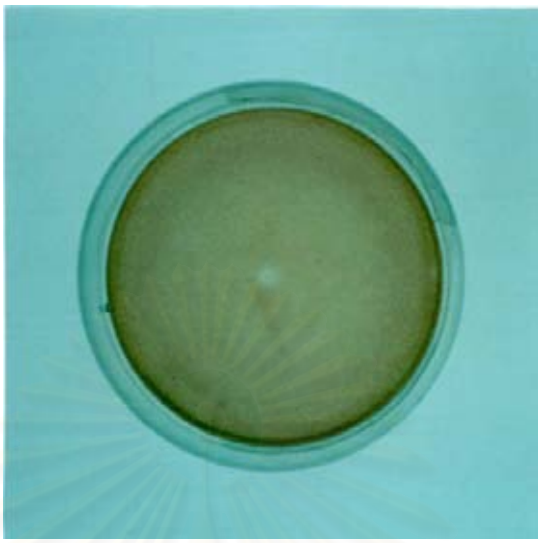
Lanes 3-12 : amplified products from each recombinant transfer vector

Lane 13 : negative control

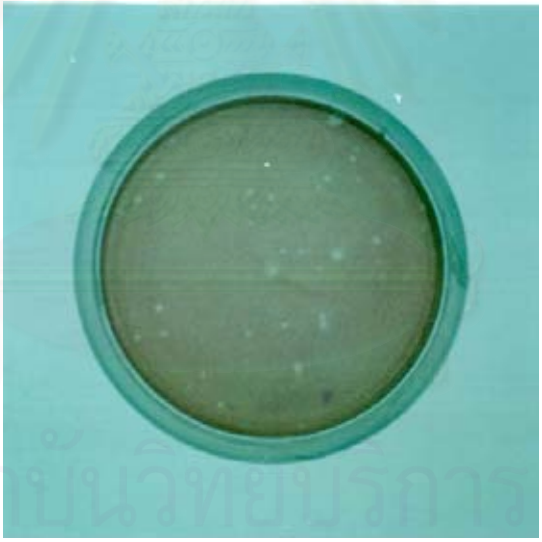
3.3 Expression of anti-LPS factor

3.3.1 Recombinant baculoviruses preparation

The recombinant baculoviruses were obtained by cotransfection of the transfer vector, ptr13 and pΔNAL7, carrying the foreign gene and *Bsu* 36I-digested AcNPV baculovirus DNA (Pharmigen, USA) into *Spodoptera frugiperda* Sf9 cells as described in section 2.7.2. The recombinant virus from cotransfection supernatant was purified by plaque purification as described in section 2.7.3. The cotransfection supernatant was infected cell monolayers with low ratio of virus and then overlaid with SeaPlaque agarose to keep the cells stable and limit the spreading of virus. After incubation, the plaques are visualized by staining with 0.03 % neutral red with PBS and kept in the dark overnight to allow the plaques to clear (Figure 3.10). The results showed plaques which appeared as clear circle about 0.3-0.5 mm in diameter against a red or pink background. Individual plaques obtained from varying dilutions of viral stock can be counted to determine the viral titer (pfu/ml). The titer of recombinant baculoviruses stock from full-length anti-LPS factor (FL-ALF) gene and NH_2-terminal truncated anti-LPS factor (ΔNAL) gene were 1.9×10^8 pfu/ml and 2.2×10^8 pfu/ml, respectively.



[a]



[b]

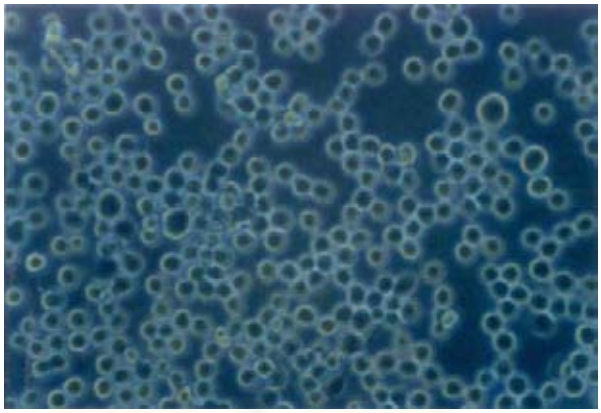
Figure 3.10 Neutral red staining of insect cells infected with recombinant baculovirus by plaque purification. Viral plaques were appeared as clear circle about 0.5-3 mm in diameter against a red or pink background.

[a] mock infected as control

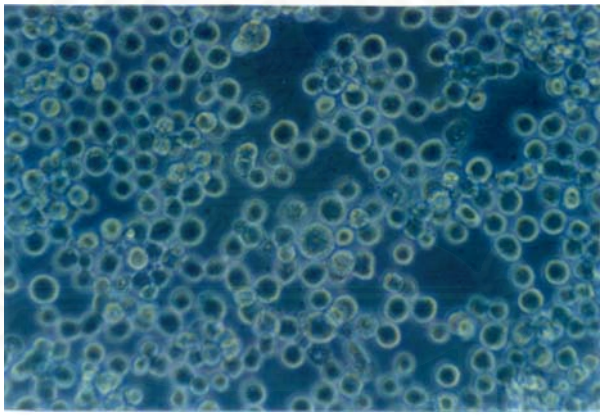
[b] recombinant virus infection

3.3.2 Preparative production of recombinant proteins

Recombinant proteins have been produced in the baculovirus expression system at level ranging between 0.1 % and 50 % of total insect cell protein. Sf9 cells were infected with 5 multiplicity of infection (MOI) of each recombinant baculovirus for 1 hour and incubated at 27 °C for 2-3 days. During incubation, the Sf9 cells were checked for sign of infection by light microscope. The infected Sf9 cells were enlarged in size (about 2 fold) and a large nucleus were visible (Figure 3.11). The infected cells and the supernatant were harvested 60-72 hours post infection (hpi) and the sample was analyzed by SDS-PAGE. The infected cells and proteins in the tissue culture fluid were resuspended and subjected to 15 % SDS-PAGE. Analysis of protein from full-length anti-LPS factor (FL-ALF) and NH_2-terminal truncated anti-LPS factor (ΔNAL) infected cells on Coomassie blue stained SDS-PAGE gels revealed over expression of a protein band of approximately 15 kDa which was not seen in uninfected cell lysates (mock infected cells) (Figure 3.12). While the proteins in the tissue culture fluid of FL-ALF and ΔNAL showed similar patterns of expression to mock infected tissue culture fluid (Figure 3.13).



[a]



[b]

Figure 3.11 Comparison of uninfected and infected Sf9 cell monolayer. The infected Sf9 cells should be enlarged in size (about 2 fold) and a large nucleus should be visible.

[a] Sf9 cells uninfected

[b] Sf9 cells infected with recombinant baculovirus

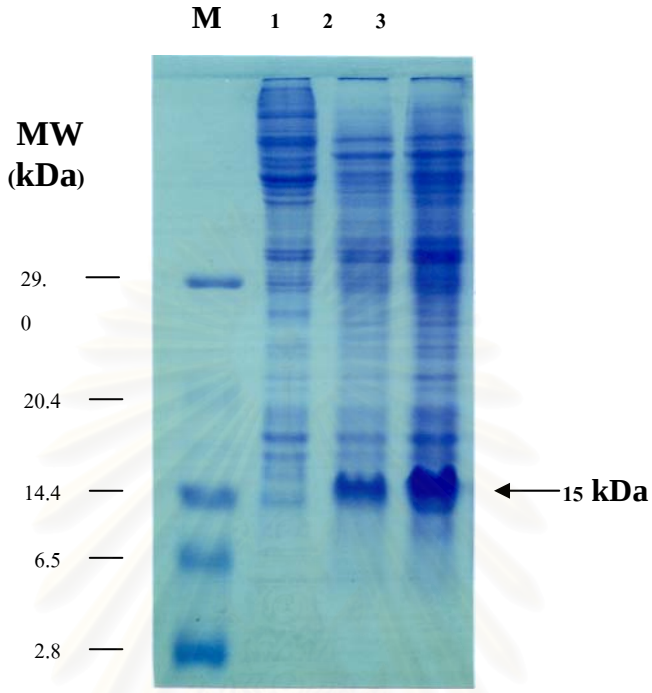


Figure 3.12 Expression of anti-LPS factor in Sf9 insect cells analyzed by SDS-PAGE. A 65 hours post infection (hpi) of infected cell lysate was subjected to 15 % SDS-PAGE.

Lane M : molecular weight standard marker (composing of carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin, and insulin with molecular weight of 29.0, 20.4, 14.4, 6.5, and 2.8 kDa, respectively)

Lane 1 : uninfected (mock) cells

Lane 2 : infected cells with virus harboring the NH_2-terminal truncated anti–LPS factor

Lane 3 : infected cells with virus harboring the full length anti-LPS gene

← Arrow indicates the expressed proteins.

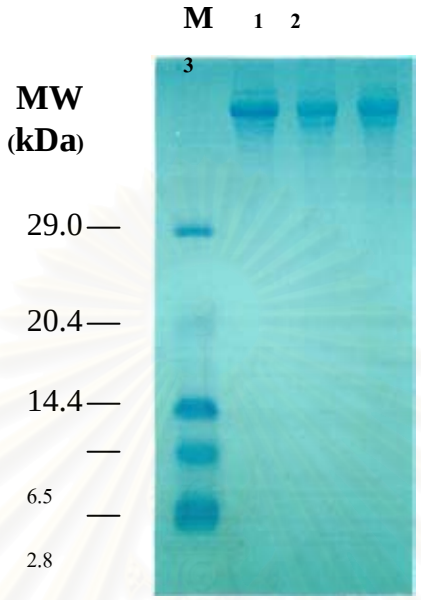


Figure 3.13 SDS-PAGE analysis of proteins expressed in the tissue culture fluid of Sf9 insect cells. A 70 hours post infection (hpi) of the proteins in the tissue culture fluid was subjected to 15 % SDS-PAGE.

Lane M : molecular weight standard marker (composing of carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin, and insulin with molecular weight of 29.0 , 20.4, 14.4, 6.5, and 2.8 kDa, respectively)

Lane 1 : uninfected (mock) tissue culture fluid

Lane 2 : tissue culture fluid from the insect cells co-transfected with the transfer vector of NH_2-terminal truncated anti-LPS factor

Lane 3 : tissue culture fluid from the insect cells co-transfected with the transfer vector of full-length anti-LPS factor

3.4 Antibacterial assay

The crude proteins of recombinant full-length (FL-ALF) and NH_2-terminal truncated anti-LPS factor (ΔNAL) were tested for their antibacterial against 3 types of bacteria by measuring bacterial growth rate at OD 600 including Gram-negative *Vibrio harveyi* 1526 and *Escherichia coli* DH5α and the Gram-positive *Staphylococcus aureus*. The bacterial growth was compared with the crude protein from total insect cells (mock infected cells) and 50 mM Tris-HCl, pH 7.4 containing 50 mM NaCl as control. The initial density of bacterial cells at 600 nm of 0.01 was measured and the cell suspensions were added to broth medium with crude protein samples and the growth rate of bacterial cells was measured every hour. *E. coli* DH5α and *S. aureus* were incubated at 37 °C in LB broth medium while *V. harveyi* 1526 was grown in LM broth medium at 30 °C. The results showed no significant change in the bacterial growth rate when the crude proteins were added against of Gram-negative *V. harveyi* 1526 (Figure 3.14) , *E. coli* DH5α (Figure 3.15) and the Gram-positive *S. aureus* (Figure 3.16) .

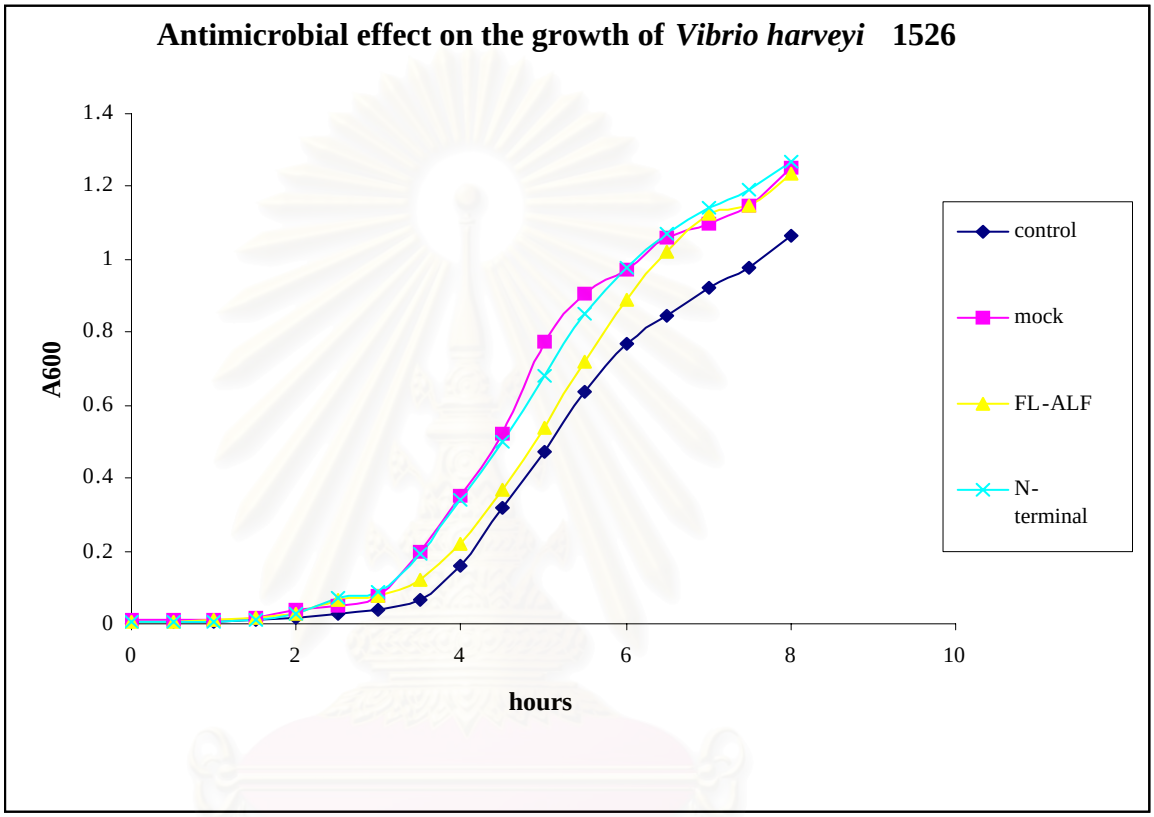


Figure 3.14 Antimicrobial effect of the crude recombinant anti-LPS factors on the growth of *V. harveyi* 1526.

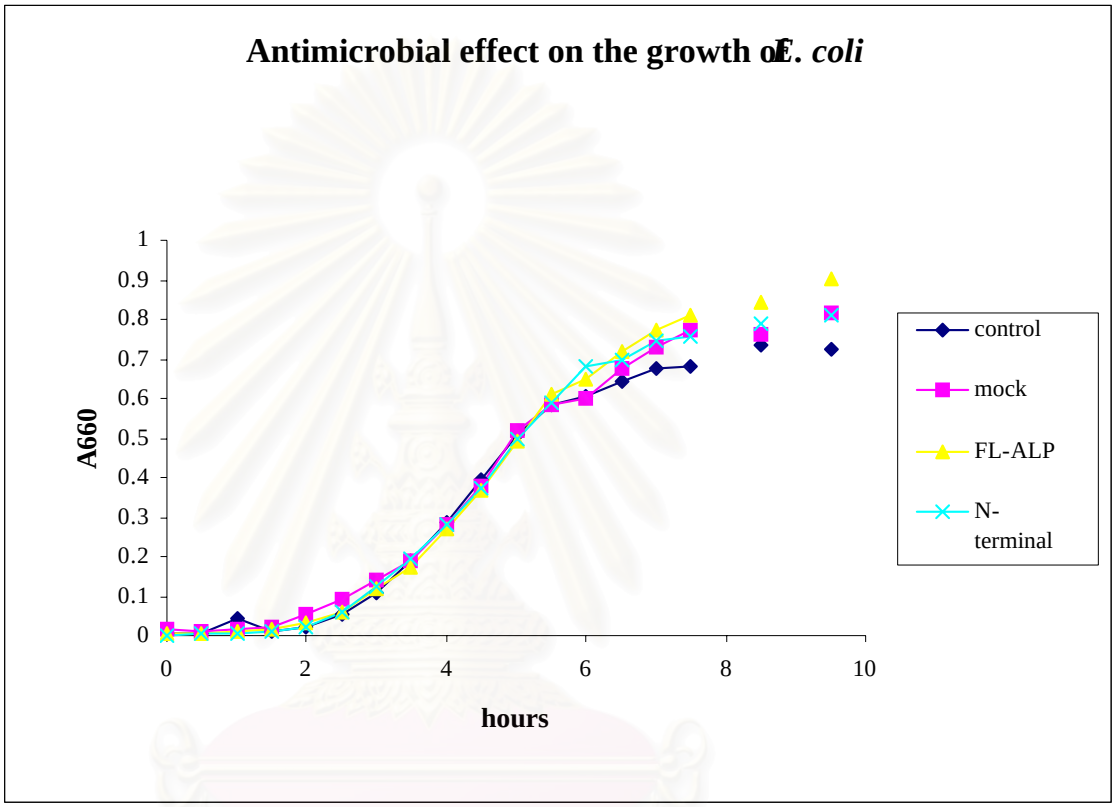


Figure 3.15 Antimicrobial effect of the crude recombinant anti-LPS factors on the growth of *E. coli* DH5α.

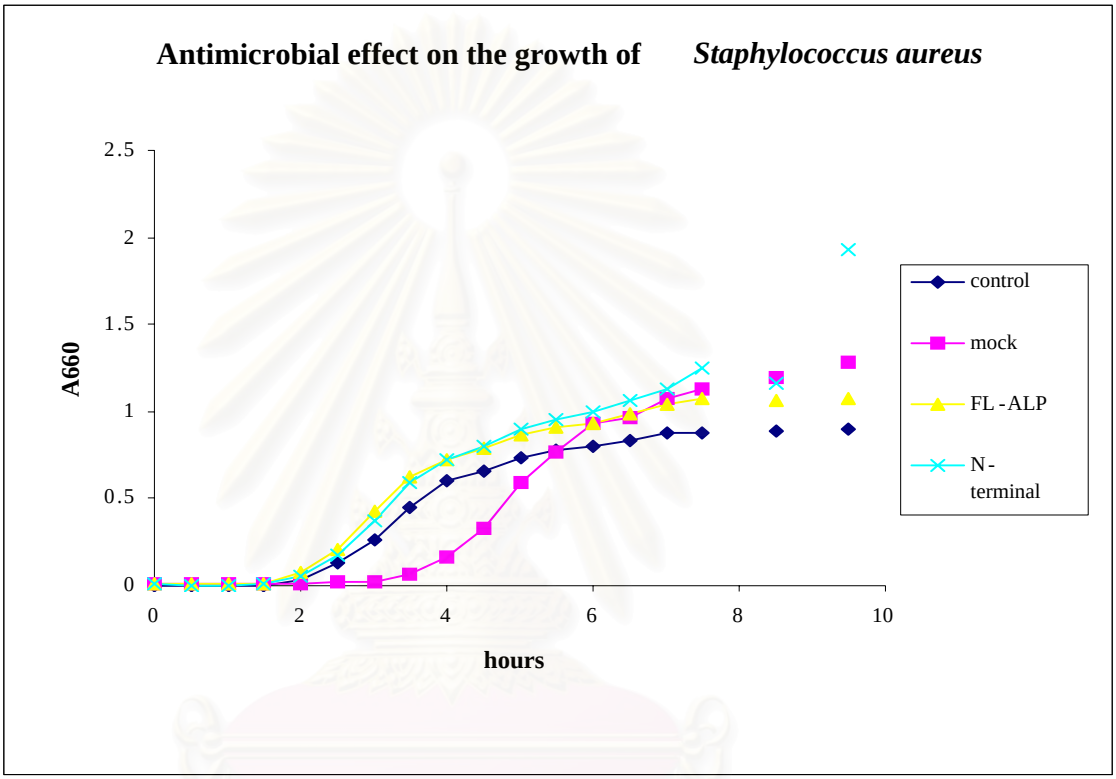


Figure 3.16 Antimicrobial effect of the crude recombinant anti-LPS factors on the growth of *S. aureus*.

3.5 Analysis of mRNA level of *P. monodon* anti-LPS factor

3.5.1 Tissue specific expression

3.5.1.1 Northern blot analysis

Anti-LPS factor expression was first studied in the blood cells of unchallenged animals (not infected experimentally with microbes). To determine whether individual shrimp expresses different mRNA level of anti-LPS factor. Total RNA was extracted from 10 individual shrimp hemocytes and the mRNA level was determined by Northern analysis, using fluorescein-11-dUTP-labeled 517 bp anti-LPS factor fragments and 182 bp 18S rRNA fragments as probes as described in section 2.5.5. Anti-LPS factor probes hybridized to an abundant mRNA of approximately 800 bp for every individual shrimp (Figure 3.17) while 18S rRNA probes hybridized to mRNA of approximately 1900 bp. The variation in the intensity of hybridization signals found in each individual shrimp indicated that the anti-LPS factor was expressed in hemocytes at different level in the shrimps.

Tissue-specific expression of anti-LPS factor was studied in various tissue types of *P. monodon*, including hemocytes, lymphoid organ, intestine, hepatopancreas, heart, gills, muscle, and eyes stalk. Ethidium bromide staining of total RNA (20 μg) isolated from different tissues indicated that intact RNA was obtained only from hemocytes but not from other tissues (Figure 3.17). Therefore, Reverse transcription-PCR (RT-PCR) which does not require high quality RNA was performed to study expression of the anti-LPS factor instead of the Northern blot analysis.

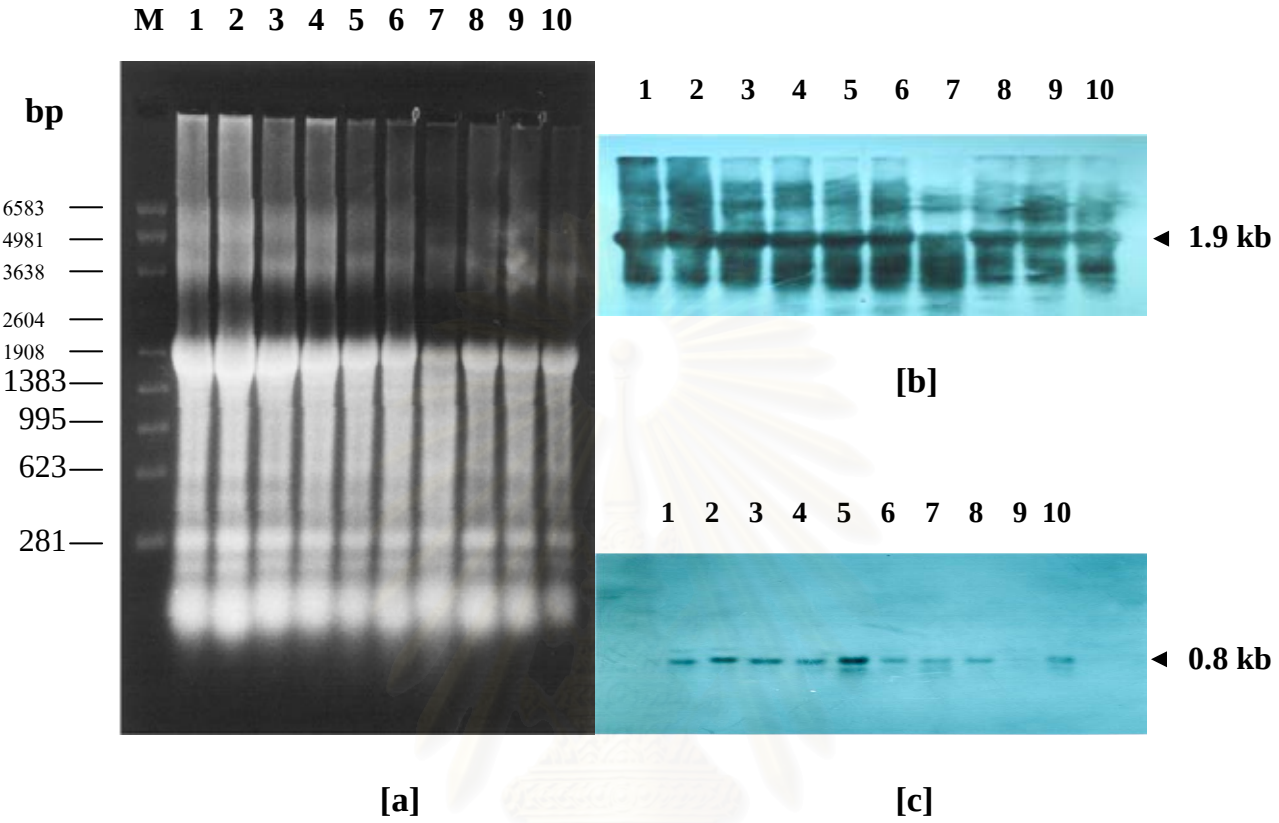


Figure 3.17 Analysis of anti-LPS factor expression from 10 individual shrimp hemocytes by Northern blot hybridization. Total RNA was run on a 1.4 % agarose-formaldehyde gel with MOPS buffer, blotted to a Nylon membrane and hybridized successively with random-primed fluorescein-11-dUTP-labeled anti-LPS factor fragment as probes and 18S rRNA fragments as control probes.

Lane M : RNA marker

Lanes 1-10 : 10 individual shrimps

[a] Ethidium bromide stained gel

[b] Northern blot hybridization with 18S rRNA probes

[c] Northern blot hybridization with anti-LPS factor probes

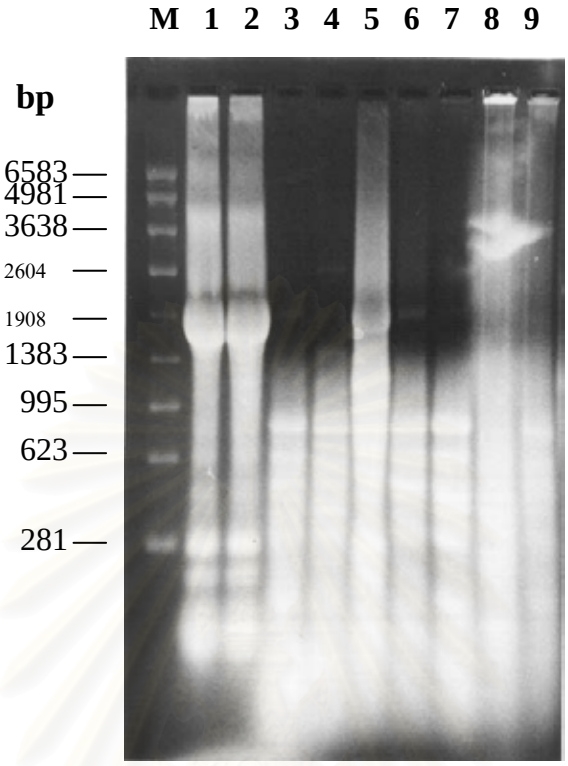


Figure 3.18 Ethidium bromide staining of total RNA from various shrimp tissues on a 1.4 % agarose-formaldehyde gel with MOPS buffer, electrophoresed at 56 volts for 3.5 hours.

Lane M : RNA marker

Lane 1 : normal shrimp hemocytes

Lane 2 : infected shrimp hemocytes

Lane 3 : lymphoid organ

Lane 4 : intestine

Lane 5 : hepatopancreas

Lane 6 : heart

Lane 7 : gills

Lane 8 : muscle

Lane 9 : eyes stalk

3.5.1.2 Reverse transcription PCR (RT-PCR) analysis

Reverse transcription PCR (RT-PCR) was used to examine mRNA expression of anti-LPS factor in several tissues including hemocytes, heart, intestine, gills, lymphoid organ, and hepatopancreas. RT-PCR was performed using specific primers designed from the nucleotide sequences of the cDNA encoding defense molecules obtained from the *P. monodon* ESTs using PrimerExpress 1.0 (ABI). The specific primer sequences for amplification of the 18S rRNA transcript was showed forward primer (5'- GAGACGGCTACC ACATCTAAG -3') and reverse primer (5'- ATACGCTAGTGGAGCTGGA -3'), and anti-LPS factor was showed forward primer (5'- CGCCAGCAAGAT CGTAGGTTG -3') and reverse primer (5'- AGGCCTATGAGCTGAGCCA CTG -3').

After optimizing the annealing temperature for each primer pairs, expected PCR products were obtained. RT-PCR using primers specific to 18S rRNA (annealing temperature was 50 °C) and anti-LPS factor (annealing temperature was 60 °C) yield the products of 182 bp and 250 bp, respectively. The 18S rRNA gene was equally expressed in all tissues examined and was used as the control. Anti-LPS factor were abundantly expressed in hemocytes but low mRNA concentrations were found in heart, gills, intestine, and lymphoid organ. No mRNA of anti-LPS factor was detected in hepatopancreas. No products were amplified in negative controls which contained water in place of cDNA (Figure 3.19).

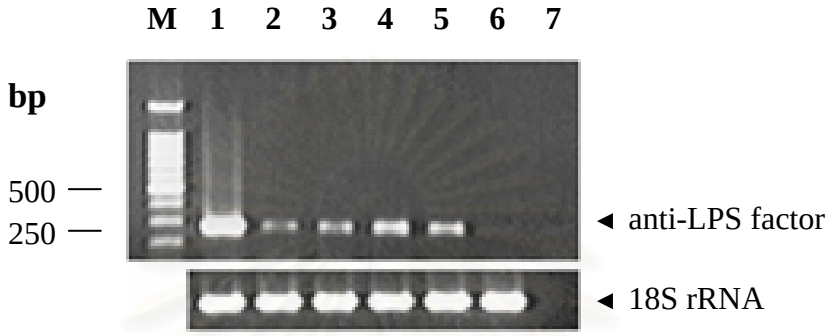


Figure 3.19 Analysis of the sites of expression of anti-LPS factor in *P. monodon*. RT-PCR was performed with cDNA from various tissues. Amplification of 18S rRNA transcript was performed as control.

Lane M : Standard DNA ladder (100 bp marker)

Lane 1 : hemocytes

Lane 2 : intestine

Lane 3 : heart

Lane 4 : gills

Lane 5 : lymphoid organ

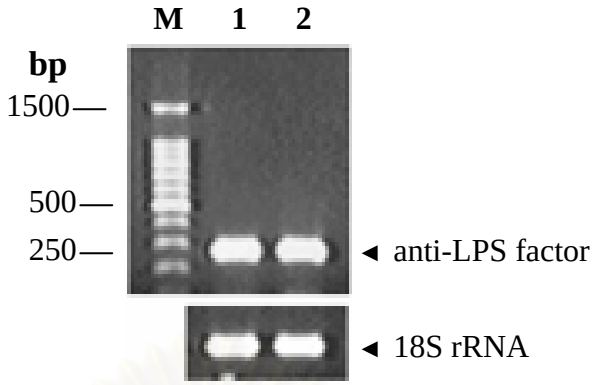
Lane 6 : hepatopancreas

Lane 7 : negative control

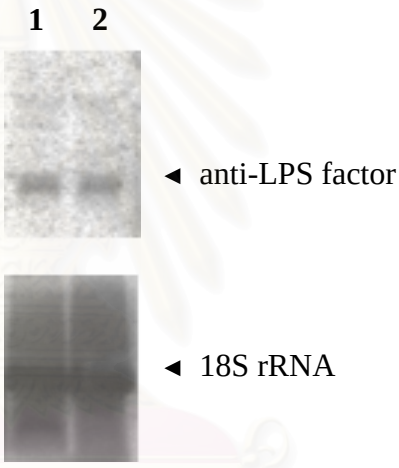
3.5.2 mRNA level in *V. harveyi* infected *P. monodon*

The influence of bacterial stimulation on transcription of the anti-LPS factor in hemocyte of the black tiger shrimp was determined. Shrimps were experimentally challenged by injection with *Vibrio harveyi* 1526, as described in section 2.8, and the hemolymph was collected at 3, 6, 12, 24 and 48 hours post infection, and total RNA was extracted. Detection of *vibrio harveyi* in shrimps was performed by spreading suspensions of hepatopancreas onto thiosulfate citrate bile-salt sucrose agar (TCBS-agar). The presence of the luminescent bacteria was observed in hepatopancreas at 42-48 hours post infection. Therefore, the induction of anti-LPS factor was first compared between unchallenged and 48 hours bacterial challenged shrimps. Both the RT-PCR and Northern analysis showed no difference in the mRNA level after 48 hours of infection (Figure 3.20). For a better quantification of mRNA level, RT-PCR was also performed by addition of 2 pairs of primers into the reaction tube, one pair amplified a constitutive 18S rRNA gene (as internal control) and another amplified the cDNA of anti-LPS factor. No significant increase in mRNA level was observed (Figure 3.21).

To further investigate the influence of bacterial stimulation, time course of mRNA expression was performed by RT-PCR on total RNA from hemocytes of unchallenged and bacterial challenged shrimps at 3, 6, 12, 24 hours post infection (Figure 3.22). Significant increase transcript level was observed only at 3 hours post infection.



[a]



[b]

Figure 3.20 Analysis of anti-LPS factor mRNA level in hemocytes of unchallenged and *V. harveyi* 1526 challenged *P. monodon* by RT-PCR analysis compared with Northern analysis.

Lane M : Standard DNA ladder (100 bp marker)

Lane 1 : hemocytes of unchallenged shrimp

Lane 2 : hemocytes of *V. harveyi* challenged shrimp

[a] Ethidium bromide stained gel of RT-PCR analysis

[b] Northern blot analysis with anti-LPS factor and 18S rRNA probes

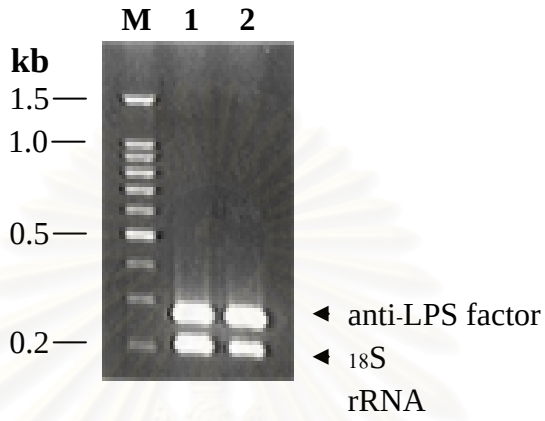


Figure 3.21 RT-PCR analysis of anti-LPS factor mRNA level in hemocytes of unchallenged and *V. harveyi* 1526 challenged *P. monodon* quantified by addition of 2 pairs of primers in one reaction tube (one pair amplified 18S rRNA as internal control).

Lane M : Standard ladder (100 bp marker)

Lane 1 : hemocytes of unchallenged shrimp

Lane 2 : hemocytes of *V. harveyi* challenged shrimp

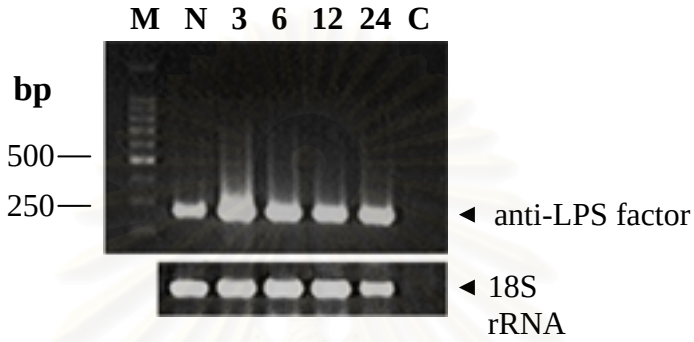


Figure 3.22 Time course of mRNA expression of anti-LPS factor in hemocytes of bacterial challenged *P. monodon* detected by RT-PCR at 3, 6, 12, and 24 hours post infection.

Lane M : Standard DNA ladder (100 bp marker)

Lane N : unchallenged shrimp

Lane C : negative control without cDNA

CHAPTER IV

DISCUSSIONS

Anti-LPS factor is a family of antimicrobial effectors found in the hemocytes of the black tiger shrimp *Penaeus monodon*. From Expressed Sequence Tags (ESTs) analysis of *P. monodon* hemocytes, several cDNA clones which showed homology to anti-LPS factor from horseshoe crab were isolated. These clones were redundant indicating the abundance of mRNA of anti-LPS factor in *P. monodon* hemocytes. The deduced amino acid of the cDNA clone, Sh71, shows an open reading frame of 372 nucleotides encoding a protein of 123 amino acids, as shown in Figure 3.2. The entire amino acid sequence of the protein shows 57 % homology to anti-LPS factor from the Atlantic horseshoe crab, *Limulus polyphemus*. The putative gene of the anti-LPS factor has also been reported in the Atlantic white shrimp, *Litopenaeus setiferus* (Gross et al., 2001).

Anti-LPS factor was initially characterized in horseshoe crabs *L. polyphemus* and *Tachypleus tridentatus* (Aketagawa et al., 1986 and Muta et al., 1987). It is a small basic protein found in hemolymph, which binds and neutralizes bacterial endotoxin (LPS) and has a strong antibacterial effect especially on the growth of Gram-negative R-type bacteria (Morita et al., 1985).

The amino acid sequences alignment of anti-LPS factor purified from the horseshoe crabs, *L. polyphemus* and *T. tridentatus*, and the black tiger shrimp *P. monodon* deduced amino acid sequence using the clustalX program showed the two conserved cysteine residues and conserved clusters of positively charged residues between the 2 cysteine residues. The cysteine residues are necessary for disulfide bridge formation in the three dimensional structure and the positively charge residues are involved in the binding with

phosphate groups in lipid A portion of LPS, respectively (Hoess et al., 1993). The NH_2-terminal region of the putative anti-LPS factor contains an extra 26 amino acid residues which was proposed to be a signal peptide for protein transport. Regardless of the signal peptide, the NH_2-terminal region of the putative anti-LPS factor is also highly hydrophobic, which is similar to those found in the horseshoe crab proteins. These results suggested that the structure, function and mechanism of action of anti-LPS factor protein from *P. monodon* should be similar to that of the homologous found in horseshoe crabs. To further characterize *P. monodon* anti-LPS factor, two constructs of the gene expressing full length (FL-ALF) and NH_2-terminal truncated (ΔNAL) derivative of the proteins were expressed in the baculovirus expression system.

Because of the antimicrobial activity of this protein, the eukaryotic expression system is preferred to a bacterial expression system. Pichyangkura has shown that the bacterial expression system did not expressed anti-LPS factor both of FL-ALF and ΔNAL, which suggested that anti-LPS factor may have a toxic effect to bacterial host cells, *E. coli*, (unpublished data). Bachere et al. (1999) has successfully expressed penaeidins, antimicrobial peptides of penaeid shrimp, in the yeast, *Saccharomyces cerevisiae*, but several differences were observed between native and recombinant penaeidins. The baculovirus expression system can be used to produce large amounts of target protein in insect host cells and the system was chosen in this study because shrimp and insect are both arthropods, consequently the expression and modification of the gene products are probably more similar than other eukaryotic expression system.

Baculovirus expression system is one of the major recombinant DNA expression systems used today for the production of a wide variety of heterologous proteins. It offers a number of advantages over prokaryotic, yeast, and mammalian expression systems, including high expression levels, limitless size of the expressed protein, efficient cleavage of signal peptides and

processing of the protein, post-translational modifications and simultaneous expression of multiple genes. In addition to these advantages, proteins are correctly folded and expressed functional.

For secreted and many membrane-associated proteins, a signal peptide at the amino-terminus is responsible for targeting the polypeptide to the endoplasmic reticulum (ER). The full-length version of anti-LPS factor with a signal peptide was proposed to secrete out of the cells. The recombinant baculovirus containing FL-ALF and ΔNAL gene was expressed in Sf9 cells using 5 MOI of each recombinant baculovirus under regulation of the polyhedrin (PH) promoter. The protein band of expected size (approximately 15 kDa) was observed in the lysate of insect cells infected with the recombinant viruses, but was not seen in the mock infected (uninfected) cells, or cells infected with other viral constructs. Proteins from the supernatant were analyzed, no recombinant protein was found indicating that the recombinant protein of FL-ALF and ΔNAL were not secreted into the medium. This result indicates that the putative signal peptide of the *P. monodon* anti-LPS factor was not recognized by the insect cells or the extra 26 amino acids residues of *P. monodon* anti-LPS factor may not function as a signal peptide.

Modification of signal peptide of anti-LPS factor may allow translated recombinant proteins to be translocated across the endoplasmic reticulum (ER) membrane. For example, the use of alternative signal peptides including bee mellitin (Tessier et al., 1991) cecropin B, alkaline phosphatase (Bielefeldt-Ohmann et al., 1997), and baculovirus structural 64K glycoprotein in insect cells have been reported.

From antimicrobial effect on the growth of bacteria, we did not observe any significant differences in the growth rate of *V. harveyi* 1526, *E. coli* DH5α, and *S. aureus*, which could explain the difference in the outcome of antimicrobial activity by the recombinant infected cell lysates as a crude

protein. This result indicated that the recombinant proteins of both of FL-ALF and ΔNAL may not function properly. It was due to the formation of insoluble inclusion bodies of recombinant proteins. Therefore, purification of the proteins are required for antimicrobial activity tested.

Inclusion bodies are formed when expression levels of heterologous proteins are very high, or in response to toxic effects exerted by the expressed product on host cell metabolism. These considerations are particularly valid for baculovirus infected cells, because the polyhedrin (PH) promoter can drive the expression of heterologous genes to considerably high levels (up to one third of total cell proteins). Moreover, the presence of hydrophobic stretches of amino acids in heterologous proteins, or their improper folding might cooperate in promoting the intracellular precipitation of recombinant antigens: particularly in late phases of baculovirus infection, when host cell function decline (Russo et al., 1998).

Royer et al. (1997) reported that the insoluble inclusion bodies and aggregated forms of recombinant human immunodeficiency virus type1 proteinase (HIV-1PR) have been observed and have been found to involve, at least in part, intermolecular disulfide bonds (Hostomsky et al., 1989). The primary structure of anti-LPS factor showed the 2 cysteine residues linked with intramolecular disulfide bond. When over expressed these cysteine residues may be linked intermolecularly. Thus, the high expression levels with the strong baculovirus polyhedrin (PH) promoter and the possible presence of intermolecular interaction increases the likelihood of the formation of intracellular inclusion bodies entrapping the recombinant protein in an insoluble form.

To improve the yield of purified recombinant protein from crude cell lysates, we may infected Sf9 cells with lower amount of recombinant baculoviruses and vary infection time to obtained soluble recombinant proteins.

Iwanaga et al. (1985) has successfully purified soluble anti-LPS factor from hemolymph of horseshoe crab by column chromatographies on dextran sulfate-Sepharose CL-6B and Sephadex G-50, which we can apply this method to purified recombinant proteins, both of FL-ALF and ΔNAL from recombinant infected Sf9 cells.

To understand the function of these antimicrobial peptides in shrimp defense, we examined the tissue localization of anti-LPS factor mRNA in normal shrimp, unchallenged animals, as well as the anti-LPS factor expression pattern in response to an experimental microbial challenge by Northern blot hybridization. In these experiments, we used anti-LPS factor specific probes (cDNA probes) and 18S rRNA probes as control. Anti-LPS factor expression in unchallenged animals was first studied in hemocytes from which the peptide cDNA had been cloned. A high level of anti-LPS factor mRNA was measured in all of shrimp analyzed, suggesting that these peptides are constitutively expressed in hemocytes. However, individual variability can be seen, which could reflect either different immune stimulation states of animals or a genetic variability in anti-LPS factor gene expression. Furthermore, Northern blot analyses performed with the total RNA extracted from hemocytes and other tissues indicated that hemocytes were the main site of anti-LPS factor synthesis.

Tissue-specific expression of anti-LPS factor in unchallenged animals was studied in various tissues, but total RNA isolated from all tissues samples were degraded except that obtained from hemocytes, as shown in Figure 3.17. Therefore, tissue-specific expression of the defense molecules by Northern blot analysis was not carried out further due to poor quality of the RNA samples. RT-PCR provided a more reliable results than the Northern blot analysis because short regions can be amplified and partially degraded samples may be used for the expression analysis (Kawasaki and Wang, 1989).

From RT-PCR analysis, anti-LPS factor showed strong expression in hemocytes and significant mRNA concentration were observed in heart, gills, intestine, and lymphoid organ, probably due to infiltrating hemocytes. No mRNA of these genes was found in hepatopancrease. These results were similar to those found in other arthropods in which hemocytes are the main sites of antimicrobial peptides synthesis (Iwanaga and Kawabata, 1998). Secondary expression sites for antibacterial peptides such as intestine or gonads were found in insects (Hoffmann et al., 1997; Manetti et al., 1998).

While there are no reports on possible translational regulation of antimicrobial peptide expression in horseshoe crab hemocytes, *in vivo* microbial challenge in shrimp showed that anti-LPS factor mRNA level was not stimulated in hemocytes when the level of mRNA was measured 24-48 hours after the microbial challenge. This is similar to the data on penaeidin gene regulation in white shrimp, *Penaeus vannamei*. Microbial stimulation is known to trigger hemocyte degranulation as one of the most immediate hemocytic reactions in crustaceans (Johansson and Soderhall, 1985). The level of antibacterial activity was found in the hemocytes did not changed in bacterial-challenged of insect, shrimp, and bivalve mollusks (Ehret-Sabatier et al., 1996; Destoumieux et al., 1997; Mitta et al., 1999), suggesting that the antimicrobial peptides are in fact stored in the hemocytes and may be released into the plasma upon infection.

However by RT-PCR analysis, significantly increase in mRNA concentration level of anti-LPS factor was found at 3 hours post infection, then drops to initial levels after 6 hours post infection, indicated that anti-LPS factor is stimulated in hemocytes at early stage of infection in response to bacterial challenged. On the other hand, the microbial challenge leads to a decrease in penaeidin gene transcription subsequent to degranulation and release of the peptides into the blood (Destomieux et al., 2000). Data analysis revealed a four to fivefold decrease in penaeidin mRNA levels at 3 hours post infection, then

returned to the basal levels at 12 hours and a slight increase was noticed at 24 hours post infection, indicated that penaeidin synthesis in hemocytes is not enhanced by microbial infection. A decrease in penaeidin messenger concentration could be associated with partial decrease of penaeidin-producing hemocytes from the blood stream.

A more reliable technique for quantification of mRNA level by Real-time PCR will be needed to study the response of these defense mRNA to bacterial infection. In addition, immunodetection of the proteins released into the plasma upon stimulation could provide a better understanding on the response of these defense molecules to the infection.

CHAPTER V

CONCLUSIONS

1. Full-length anti-LPS factor (FL-ALF) gene was prepared from a cDNA clone Sh71 and showed an open reading frame (ORF) of 372 nucleotides encoding a protein of 123 amino acids. NH_2-terminal truncated anti-LPS factor (ΔNAL) gene was prepared by PCR amplification and the specific PCR product was about 330 bp containing an ORF of 294 nucleotides encoding 97 amino acids.

2 Baculovirus expression system was used to express the recombinant proteins of anti-LPS factor, both of full-length (FL-ALF) and NH_2-terminal truncated anti-LPS factor (ΔNAL), from *P. monodon*.

3 The recombinant baculoviruses containing anti-LPS factor gene were obtained by *in vivo* homologous recombinant in *Spodoptera frugiperda* Sf9 cells between the transfer vector carrying anti-LPS factor and baculovirus DNA, which expressed recombinant protein under the polyhedrin (PH) promoter.

4 The titer of recombinant baculoviruses from full-length (FL-ALF) and NH_2-terminal truncated anti-LPS factor (ΔNAL) were 1.9×10^8 and 2.2×10^8 pfu/ml, respectively.

5 The recombinant proteins of full-length (FL-ALF) and NH_2-terminal truncated anti-LPS factor (ΔNAL) that were analyzed by SDS-PAGE showed an over expression of a protein band of approximately 15 kDa from infected cell lysates but the protein was not found in the tissue culture fluid.

These results indicated that the recombinant proteins of FL-ALF and ΔNAL were stored within infected Sf9 cells and did not secrete from cells.

6 Addition of crude proteins from infected cell lysates containing full-length (FL-ALF) and NH_2-terminal truncated anti-LPS factor (ΔNAL) showed no inhibition effect to the growth rate of Gram-negative, *V. harveyi* 1526 and *E. coli* DH5α, and Gram-positive bacteria, *S. aureus*. Because, the recombinant proteins may be formed insoluble inclusion bodies.

7 Northern blot analysis and RT-PCR indicated that hemocytes of *P. monodon* were the main sites of anti-LPS factor synthesis.

8 Variation in mRNA level was observed in hemocytes of individual challenged shrimp.

9 Analysis of mRNA level showed that the mRNA of anti-LPS factor was present in the hemocytes of both unchallenged and *V. harveyi* challenged shrimps and that slightly increase of mRNA was observed only at early stage of infection (3 hours post injection).

REFERENCES

Adams, A. 1991. Response of penaeid shrimp to exposure to *Vibrio* species. Fish Shellfish Immunol. 1: 59-70.

Aketagawa, J., Miyata, T., Ohtsubo, S., Nakamura, T., Morita, T., Hayashida, H., Iwanaga, S., Takao, T., and Shimonishi, Y. 1986. Primary structure of *Limulus* anticoagulant anti-lipopolysaccharide factor. J. Biol. Chem. 261: 7357-7365.

Anderson, I. 1993. The veterinary approach to marine prawns. In: Brown (ed.). Aquaculture for veterinations: Fish husbandry and medicine. 270-290.

Aono, H., Mori, K. 1996. Interaction between hemocytes and plasma is necessary for hemolymph coagulation in the spiny lobster, *Panulirus japonicus.* Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 113: 301-305.

Ashida, M., Yamazaki, H. 1990. Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In: Ohnishi, E., Ishizaki, H. (eds.). Molting and Metamorphosis. Springer, Berlin. 239-265.

Aspan, A., Soderhall, K. 1991. Purification of prophenaloxidase from crayfish blood cells, and its activation by an endogeneous serine proteinase. Insect Biochem. 21: 363-373.

Aspan, A., Hall, M., Soderhall, K. 1990a. The effect of endogeneous proteinase inhibitors on the prophenoloxidase activiting enzyme, a serine proteinase from crayfish haemocytes. Insect Biochem. 20: 485-492.

Aspan, A., Structevant, J., Smith, V.J., Soderhall, K. 1990b. Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. Insect Biochem. 20: 709-718.

Austin, B. 1988. Aquatic microbiology. Cambridge press. 222 pp.

Bachere, E. 1999. Shrimp immunity and disease control. Aquaculture, in press.

Bachere, E., Mialhe, E., Rodriguez, J. 1995. Identification of defence effectors in the haemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (bate)–prospects and applications. Fish Shellfish Immunol. 5: 59-162.

Baily-Brook, J.H. and Moss, S.M. 1992. Penaeid taxonomy, biology and zoogeography. In: Fast, A.W. and Lester, L.J. (eds.). Marine shrimp culture: Principle and practices. 9-27.

Barracco, M.A., Duvic, B., Soderhall, K. 1991. The β-1,3-glucan-binding protein from the crayfish *Pacifastacus leniusculus*, when reacted with a β-1,3-glucan, induces spreading and degranulation of crayfish granular cells. Cell Tissue Res. 266: 491-497.

Bauchau, A. 1981. Crustaceans. In: Rowley, R. (ed.). Invertebrate Blood Cells. Acedemic Press, New York. 386-420.

Bielefeldt-Ohmann, H., Beasley, D.W.C., Fitzpatrick, D.R., Aaskov, J.G. 1997. Analysis of a recombinant dengue-2 virus-dengue-3 virus hybrid envelope protein expressed in a secretory baculovirus system. J. Gen. Virol. 78: 2723-2733.

Boonyaratpalin, S., Supamattaya, K., Kasornchandra, J., Direkbusaracom, S.,

Ekpanithanpong, U., Chantanachooklin, C. 1993. Non–occluded baculo–like virus, the causative agent of yellow head disease in the black tiger shrimp (*Penaeus monodon*). Fish Pathol. 28: 103-109.

Chen, C., Ratcliffe, N.A., Rowley, A.F. 1993. Detection, isolation and characteization of multiple lectins from the haemolymph of the cockroach, *Blaberus discoidalis*. Biochem. J. 294: 181-190.

Chisholm, J.R.S., Smith, V.J. 1992. Antibacterial activity in the haemocytes of the shore crab, *Carcinus maenas*. J. Mar. Biol. Assoc. U.K. 72: 529-542.

Chou, H.Y., Huang, C.Y., Wang, C.H., Chiang, H.C., Lo, C.F. 1995. Pathologenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis. Aquat. Org. 23: 165-173.

Destoumieux, D., Munoz, M., Cosseau, C., Rodriguez, J., Bulet, P., Comps, M., and Bachere, E. 2000. Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. J. Cell Sci. 113, 461-469.

Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J. and Bachere, E. 1997. Penaeidins: A new family of antimicrobial peptides in the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. 272: 28398–28406.

Durliat, M. 1985. Clotting processes in crustaece decapod. Biol. Rev. 60: 473-498.

Duvic, B., Soderhall, K. 1990. Purification and characterization of β-1,3-glucan binding protein from the plasma of the crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 265: 9332-9337.

Ehret-Sabatier, L., Loew, D., Goyffon, M., Fehlbaum, P., Hoffman, J.A., Van Dorsselaser, A., and Bulet, P. 1996. J. Biol. Chem. 271: 29537-29544.

Engstrom, Y. 1998. Insect immune gene regulation. In: Brey, P.T., Hultmark, D. (eds.). Molecular Mechanisms of Immune Responses In Insects. Chapman and Hall, London. 211-244.

Flegel, T.W. 1997. Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World journal of microbiology & Biotechnology. 13: 433-442.

Flegel, T.W., Fegan, D.F., and Sriuratana, S. 1995. Environmental control of infectious shrimp diseases in Thailand. In: Shariff, M., Arthus, J.R., and Subasinghe, R.P..(eds.). Disease in Asian Aquaculture II, Fish Health Section, Asian Fisheries Society, Manila, 65–79.

Fuller, G.M., Doolittle, R.F. 1971. Studies of invertebrate fibrinogen I, purification and characterization of fibrinogen from spiny lobster. Biochemistry. 10: 1305-1310.

Gargioni, R. and Barracco, M.A. 1998. Hemocytes of the palaemonids *Macrobrachium rosenbergii* and *M. acenthurus*, and of the Penaeid *Penaeus paulensis*. J. Morphol. 236: 209-221.

Gallas-Galvan, T., Hernandez-Lopez, J., Vargas-Albores, F. 1997. Effect of calcium on the brown shrimp (*Penaeus californiensis*, Holmes) prophenol–oxidase system activation. Comp. Biochem. Physiol. 117A: 419-425.

Gross, P.S., Bartlett, T.C., Browdy, C.L., Chapman, R.W., Warr, G.W. 2001.

Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. Developmental and Comparative Immunology. 25: 565-577.

Hall, M., Wang, R., Van Antwerpen, R., Soderhall, K. 1999. The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proc. Natl. Acad. Sci. U.S.A. 96: 1965-1970.

Hall, M., Van Heusden, M.C., Soderhall, K. 1995. Identification of the major lipoproteins in crayfish hemolymph as proteins involved in immune recognition and clotting. Biochem. Biophys. Res. Commun. 216: 939-946.

Hasson, K.W., Lightner, D.V., Poulos, B.T., Redman, R.M., White, B.L., Brock, J.A., Bonami, J.R. 1995. Taura syndrome in *Penaeus vannamei*, demonstration of a viral etiology. Dis. Aquat. Org. 23: 115-126.

Hernandez-Lopez, J., Gallas–Galvan, T., Vargas-Albores, F. 1996. Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis*, Holmes). Comp. Biochem. Physiol. 113C: 61-66.

Hetru, C., Hoffmann, D., Bulet, P. 1998. Antimicrobial peptides from insects. In: Brey, P.T., Hultmark, D. (eds.). Molecular Mechanisms of Immune Responses In Insects. Chapman and Hall, London. 40-66.

Hoess, A., Watson, S., Siber, G.R., and Liddington, R. 1993. Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, *Limulus* anti-LPS factor, at 1.5 A resolution. EMBO Journal. 12(9): 3351–3356.

Hoffmann, J.A. and Reichart, J.M. 1997. *Drosophila* Immunity. Trends Cell Biol. 7: 309-316.

Hostomsky, Z., Appelt, K., and Ogeden, R. 1989. High-level expression of self-processed protease in *Escherichia coli* using a synthetic gene. Biochem. Biophys. Res. Commun. 161: 1056-1063.

Iwanaga, S. and Kawabata, S. I. 1998. Evolution and phylogeny of defense molecules associated with the innate immunity in horseshoe crab. Front. Biosci. 3: D973-D984.

Iwanaga, S., Kawabata, S. and Muta, T. 1998. New types of clotting factors and defense molecules found in horseshoe crab hemolymph: Their structures and functions. J. Biochem. 123: 1-15.

Jiravanichpaisal, P., Miyazaki, T., Limsuwan, C. 1994. Histopathology, biochemistry and pathogenicity of *Vibrio harveyi* infecting black tiger prawn *Penaeus monodon*. J. Aquat. Anim. Health. 6: 27-35.

Johansson, M.W., Soderhall, K. 1992. Cellular defence and cell adhesion in crustacean. Anim. Biol. 1: 97-107.

Johansson, M.W., Soderhall, K. 1989. Cellular immunity in crustaceans and the proPO system. Parasitol Today. 5: 171-176.

Johansson, M.W., Soderhall, K. 1985. Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J. Comp. Physiol. B 156: 175-181.

Kang, D., Liu, G., Lundstrom, A., Gelius, E., Steiner, H. 1998. A peptidoglycan

recognition protein in innate immunity conserved from insects to humans. Proc. Natl. Acad. Sci. U.S.A. 95: 10078-10082.

Kawasaki, E.S. and Wang, A.M. 1989. Detection of gene expression. In: Erlich, H.A. (ed.). PCR technology: Principles and applications for DNA amplification. Stockton Press, New York, NY. 89-97.

Komatsu, M., Ando, S. 1998. A-very-high-density lipoprotein with clotting ability from hemolymph of sand crayfish, *Ibacus ciliatus*. Biosci. Biotechnol. Biochem. 62: 459-463.

Kopacek, P., Hall, M., Soderhall, K. 1993. Characterization of a clotting protein, isolated from plasma of the freshwater crayfish *Pacifastacus leniusculus*. Eur. J. Biochem. 213: 591-597.

Kroll, R.M., Hawkins, W.E., Overstreet, R.M. 1991. Rickettsial and mollicute infection in hepatopancreatic cells of cultured Pacific white shrimp (*Penaeus vannamei*). J. Invertebr. Pathol. 57: 3622-3660.

Leangphibul, P., Nilakul, C., Somchai, C., and et al. 1985. Investigation of pathogenic bacteria from shrim farms. J. Kasetsart Sci. 7: 171-199.

Lester, H.K., Noga, E.J., Robinette, D.W. 1997. Callinectin, an antibacterial peptide from blue crab haemocytes.In: Clem, L.L., Warr, W. (eds.). Special Issue Abstracts of the 7th Congress of the ISDCI, 21-25 July 1997, Williamburg, USA. Dev. Comp. Immunol. 21: 207.

Lightner, D.V. 1996. A Handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Soc. Baton Rouge, LA. Section 3.11.

Lightner, D.V., Bell, T.A., Redman, R.M., Mohney, L.L. 1992. A review of some major diseases of economic significance in penaeid prawns/shrimps of the Americas and Indopacific. In: Shariff, M., Subasinghe, R.P., Arthur, J.R. (eds.). Disease in Asian Aquaculture I. Asian Fish Soc, Manilla. 57-80.

Lightner, D.V. 1988. *Vibrio* disease of penaeid shrimp. In: Sindermann, C.J., Lightner, D.V. (eds.). Disease Dianosis and Control in North American Marine Aquaculture. 2nd.ed. Elsevier, Amsterdam. 42-47.

Lightner, D.V. 1983. Disease of culture penaeid shrimp. In: Mcvey, J. (ed.). CRC Press, Boca Raton, FL. 289-320.

Limsuwan, C. 1991. Handbook for cultivation of black tiger prawns. Tansetaki Co Ltd, Bangkok (in Thai).

Lo, C.F., Ho, C.H., Chen, C.H., Liu, K.F., Chiu, Y.L., Yeh, P.Y., Peng, S.E., Hsu, H.C., Liu, H.C., Chang, C.F., Su, M.S., Wang, C.H., Kou, C.H. 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthopods. Dis. Aquat. Org. 27: 215-225.

Lotz, J.M. 1997. Special review: Viruses, biosecurity and specific pathogen–free stocks in shrimp aquaculture. World journal of microbiology and Biotechnology. 13: 405-413.

Madaras, F., Chew, M.Y., Parkin, J.D. 1981. Purification and characterization of the sand crab (*Ovalipes bipustulauss*) coagulogen (fibrinogen). Thrombos Haemostas. 45: 77-81.

Maheswari, R., Mullainadhan, P., Arumugam, M. 1997. Charcterizaton of a

natural haemagglutinin with affinity for acetylated aminosugars in the serum of the marine prawn, *Penaeus indicus* (Edwards, H.Milne). Fish Shellfish Immunol. 7: 17-28.

Manetti, A.G.O., Rosetto, M., and Marchini, M. 1998. Antibacterial peptides of insect reproductive tract. In: Molecular Machanisms of Immune Responses in Insects. Ed: Brey, P.T. and Hultmark, D. 67-91. London: Chapman & Hall.

Marra, M.N., Wide, C.G., Collins, M.S., Snable, J.L., Thornton, M.B., Scott, R.W. 1992. The role of bactericidal/permeability-increasing protein as natural inhibitor of bacterial endotoxin. J. Immunol. 148: 532-537.

Martin, G.G. Hose, J.E., Omori, S., Chong, C., Hoodbhoy, T., Mckrell, N. 1991. Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans. Comp Biochem Physiol. 100B: 517-522.

Martin, G.G. and Graves, B.L. 1985. Fine structure and classification of shrimp hemocytes. J. Morphol. 185: 339-348.

Mitta, G., Hubert, F., Noel, T., Roch, P. 1999. Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel, *Mytilus galloprovincialis*. Eur. J. Biochem. 264: 1-9.

Mohney, L.L., Lighter, D.V., Bell, T.A. 1994. An epizootic of vibriosis in Ecuadorian pond-reared *Penaeus vannamei* Boone(Crustacea Decapoda). J. World Aquacult. Soc. 25.

Montano-Perez, K., Yepiz-Plascencia, G., Higuera-Ciapara, I., Vargas-Albores,

F. 1998. Purification and chracterization of the clotting protein from the white shrimp (*Penaeus vannamei*). Comparative Biochemistry and Physiology In press.

Morita, T., Ohtsubo, S., Nakamura, T., Iwanaga, S.,Ohashi, K., and Niwa, M. 1985. Isolation and biological activities of *Limulus* anticoagulant (anti-LPS factor) which interacts with lipopolysaccharide (LPS). J. Biochem. (Tokyo) 97: 1611-1620.

Muta, T., Miyata, T., Tokunaga, F., Nakamura, T.and Iwanaga, S. 1987. Primary structure of anti–lipopolysaccharide factor from american horseshoe crab, *Limulus polyphemus.* J. Biochem. 101: 1321-1330.

Nappi, A.J., Vass, E., Frey, F., Carton, Y. 1995. Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. Eur. J. Cell Biol. 68: 450-456.

Nappi, A.J., Vass, E. 1993. Melanogenesis and generation of cytotoxic molecules during insect cellular immune reactions. Pigm. Cell Res. 6: 117-126.

Nash, G., Nithimathachobe, C., Tungmadi, C., and et al. 1992. Vibriosis and its control in pond reared *Penaeus monodon* in Thailand. In: Shariff, M., Subasinghe, R.P., Arthur, J.R. (eds.). Disease in Asian Aquaculture I. Asian Fish Soc, Manila. 143-155.

Noga, E.J., Arroll, T.A., Fan, Z. 1996. Specificity and some physiochemical characteristics of the antibacterial activity from blue crab *Callinectes sapidus*. Fish Shellfish Immunol. 6: 403-412.

Noga, E.J., Engel, D.P., Arrol, T.W., McKenna, S., Davidian, M. 1994. Low

serum antibacterial activity coincides with incressed prevalence of shell disease in blue crabs *Callinectes sapidus*. Dis. Aquat. Org. 19: 121-128.

Ochiai, M., Ashida, M., 1999. A pattern recognition protein for peptidoglycan. J. Biol. Chem. 274: 11854-11858.

Omori, S.A., Martin, G.G., Hose, J.E. 1989. Morphology of haemocyte lysis and clotting in the ridgeback prawn *Sicyonia ingentis*. Cell Tissue Res.

Perazzolo, M.L. and Barracco, M.A. 1997. The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors. Dev. Comp. Immunol. 21(5): 385-395.

Persson, M., Vey, A., and Soderhall, K. 1987. Encapsulation of foreign particles *in vitro* by separated blood cells from cryfish, *Astacus leptodactylus*. Cell Tissue Res. 247: 409-415.

Ratanapo, S., Chulavatnatol, M. 1992. Monodin, a new sialic acid specific lectin from black tiger prawn (*Penaeus monodon*). Comp. Biochem. Physiol. 97B: 515-520.

Roch, P. 1999. Defense mechanism and disease prevention in farmed marine Invertebrates. Aquculture. 172: 125-145.

Rodriguez, J., Boulo, V., Mialhe, E., Bachere, E. 1995. Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. J. Cell Sci. 108: 1043-1050.

Roque, A., Gomez-Gil, B., Guerra-Flores, A.L. 1998. Standardisation of three techniques for experimental *Vibrio* infections in the marine shrimp *Penaeus vannamei*. In Flegel, T.W. (ed). Advances in shrimp

biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok. 199-203.

Royer, M., Bardy, M., Gay, B., Tournier, J., and Boulanger, P. 1997. Proteolytic activity *in vivo* and encapsidation of recombinant human immunodeficiency virus type1 proteinase expressed in baculovirus-infected cells. J. Gen. Virol. 78: 131-142.

Ruangpan, L., Kitao, T. 1991. *Vibrio* bacteria isolated from black tiger shrimp, *Penaeus monodon* Fabricius. J. Fish Dis. 14: 383-388.

Ruangpan, L. 1987. Diseases of cultured marine shrimp in Thailand: *Penaeus monodon* and *P. merguiensis*. In: Limsuwan, C. and et al. (eds.). Tech. Paper for the 1th seminar on Marine Shrimp Culture, Fisheries Science Club. Kasetsart Univ. Bangkok. 46-62 (In Thai).

Russo, S., Montermini, L., Berkovitz-Siman-Tov, R., Ponti, W. 1998. Expression of bovine leukemia virus *ENV* glycoprotein in insect cells by recombinant baculovirus. FEBS Letters. 436: 11-46.

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular cloning: A laboratory manual (Second edition). New York: Cold Spring Harbor Laboratory Press.

Schnapp, D., Kemp, G.D., Smith, V.J. 1996. Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. Eur. J. Biochem. 240: 532-539.

Schumann, R.R., Leong, S.R., Flaggs, G.W., and et al. 1990. Structure and

function of lipopolysaccharide binding protein. Science. 249: 1424-1430.

Smith, V.J. 1997. Antibacterial proteins in crustacean. In: Clem, L.W., Warr, G.W. (eds.). Special Issue Abstracts of the 7th Congress of the ISDCI, 21-25 July 1997. Williamsburg, USA. Dev. Comp. Immunol. 21: 207.

Soderhall, K., Cerenius, L., Johansson, M.W. 1994. The prophenoloxidase activating system and its role in invertebrate defense. Ann. NY Acad. Sci. 712: 155-161.

Soderhall, K. 1992. Biochemical and molecular aspects of cellular communication in arthropods. Boll. Zool. 59: 141-151.

Soderhall, K., Cerenius, L. 1992. Crustacean immunity. Annu. Rev. Fish Dis. 2: 3-23.

Soderhall, K., Smith, V.J., Johansson, M.W. 1986. Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. Cell Tissue Res. 245: 43-49.

Solis, N.B. 1988. Biology and ecology. In Taki, Y., Premavara, J.H., and Lobrera, J. (eds.). Biology and culture of *Penaeus monodon*, Aquaculture Department, Southeast Asia Fisheries Development Center.

Song, Y.L., Hsieh, Y.T. 1994. Immunostimulation of tiger shrimp (*Penaeus monodon*) haemocytes for generation of microbial substances: analysis of reactive oxygen species. Dev. Comp. Immunol. 18: 201-209.

Songserm, M., Suksringam, B., Sriwopaporn, P. 1990. Study on the bacteria

luminescent disease in jumbo tiger shrimp (*Penaeus monodon*). Srinakarin–tarawiroj Research and Development Journal. 4(1): 15-24 (In Thai with English abstract).

Span, K.M., Lester, R.J.G. 1997. Special topic review: Viral diseases of penaeid shrimp with particular reference to four viruses recently found in shrimp from Queensland. World journal of microbiology and Biotechnology. 13: 419-426.

Sritunyalucksana, K., Cerenius, L., and Soderhall, K. 2000. The proPO and clotting system in crustaceans. Aquaculture. 191: 53-69.

Sritunyalucksana, K., Cerenius, L. and Soderhall, K. 1999. Molecular clonning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon.* Dev. Comp. Immunol. 23: 179-186.

Tessier D.C., Thomas, D.Y., Khouri, H.E., Laliberte, F., Vernet, T. 1991. Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. Gene. 98: 177-183.

Thornqvist, P.O., Johansson, M.W., Soderhall, K. 1994. Opsonic activity of cell adhesion protein and β-1,3-glucan binding protein from two crustaceans. Dev. Comp. Immunol. 18: 3-12.

Toh, Y., Mizutani, A., Tokunaga, F., Muta, T., Iwanaga, S. 1999. Structrure of hemocytes of the Japanese horseshoe crab *Tachypleus tridentatus*. Fine structure, morphological changes during coagulation and localization of clotting factors and antimicrobial substances. Cell Tissue Res. 266: 137-147.

Tsing, A., Arcier, J.M., Brehelin, M. 1989. Haemocytes of penaeid and

palaemonid shrimps, morphology, cytochemistry, and haemograms. J. Invertebr. Pathol. 53: 64-77.

Van de Braak, C.B.T., Faber, R., Boon, J.H. 1996. Cellular and humoral characteristics of *Penaeus monodon* (Fabricius, 1798) haemolymph. 6 Springer–Verlag, London. 194-203.

Vargas-Albores, F., Hernandez-Lopez, J., Gallas-Galvan, T., Montano-Perez, K., Jimenez-Vega, F., Yepiz–Plascencia, G. 1998. Activation of shrimp cellular defence functions by microbial products. In: Advances in shrimp biotechnology. Flegel, T.W. (ed.). Bangkok: National Center for Genetic Engineering and Biotechnology.

Vargas-Albores, F., Jimenez-Vega, F., Yepiz–Plascencia, G. 1997. Purification and comparison of β-1,3 glucan binding protein from white shrimp (*Penaeus vannamei*). Comp. Biochem. Physiol. 166B: 453-458.

Vargas-Albores, F., Jimenez-Vega, F., Soderhall, K. 1996. A plasma protein isolated from brown shrimp (*Penaeus californiensis*) which enhances the activation of prophenoloxidase system by β-1,3-glucan. Develop. Comp. Immunol. 20: 299-306.

Vargas-Albores, F. 1995. The defense system of brown shrimp (*Penaeus californiensis*): Humoral recognition and cellular responses. J. Mar. Biotechnol. 3: 153-156.

Vargas-Albores, F., Guzman–Murillo, A., Ochoa, J. L. 1993a. A lipopolysaccharide–binding agglutinin isolated from brown shrimp (*Penaeus californiensis* HOLMES) haemolymph. Comp. Biochem. Physiol. 104A: 407-4013.

Vargas-Albores, F., Guzman, M.A., Ochoa, J.L.A. 1993b. An anticoagalant solution for hemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*). Comp. Biochem. Physiol. 106A: 299-303.

Vargas-Albores, F., Guzman-Murillo, M.A., Ochoa, J.L. 1992. Size dependent haemagglutinating activity in the haemolymph from sub–adult blue shrimp (*Penaeus stylirostris* Srimpson). Comp. Biochem. Physiol. 103A: 487-491.

Wilson, R., Chen, C., Ratcliffe, N.A. 1999. Innate immunity in insects: the role of multiple endogenous serum lectins in the recognition of foreign invaders in the cockroach, *Blaberus discoidalis*. J. Immunal. 162: 1590-1596.

Wongteerasupaya, C., Vickers, J.E., Sriurairatana, S., Nash, G.L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegal, T.W. 1995. A non–occluded, systmic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis. Aquat. Org. 21: 69-77.

Yeh, M.S., Chen, Y.L., Tsai. I.H. 1998. The hemolymph clottable protein of tiger shrimp, *Penaeus monodon*, and related species. Comp. Biochem. Physiol. 121B: 169-176.

BIOGRAPHY

Miss Wilairat Anurakolan was born on February 18th 1976 in Nakhonpathom. She graduated with the Bachelor degree of Science from the Department of Biology, Faculty of Science, Silpakorn University in 1997. She has further study in Master' s degree of Science, department of Biochemistry, Chulalongkorn University in 1998.