การระบุยีนของสารต้านจุลชีพและลักษณะสมบัติของแอนติไลโปพอลิแซ็กคาไรด์แฟกเตอร์ของกุ้งกุลาดำ *Penaeus monodon*

นางสาวกุลยา สมบูรณ์วิวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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IDENTIFICATION OF GENES FOR ANTIMICROBIAL EFFECTORS AND CHARACTERIZATION OF ANTI-LIPOPOLYSACCHARIDE FACTOR OF THE BLACK TIGER SHRIMP *Penaeus monodon*

Miss Kunlaya Somboonwiwat

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Thesis Title	Identification of genes for antimicrobial effectors and characterization of anti-lipopolysaccharide factor of the black tiger shrimp *Penaeus monodon*
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Field of Study	Biochemistry
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กุลยา สมบูรณ์วิวัฒน์ : การระบุยีนของสารต้านจุลชีพและลักษณะสมบัติของแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ของกุ้งกุลาดำ *Penaeus monodon* (IDENTIFICATION OF GENES FOR ANTIMICROBIAL EFFECTORS AND CHARACTERIZATION OF ANTI-LIPOPOLYSACCHARIDE FACTOR OF THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษา : รศ.ดร.อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม Dr.Evelyne Bachére และ อ.ดร.รัฐ พิชญางกูร : 182 หน้า. ISBN 974-53-1746-2.

ในงานวิจัยนี้ใช้เทคนิค Differential Display PCR (DD-PCR) ในการศึกษาการเปลี่ยนแปลงการแสดงออกของยีนในเซลล์เม็ดเลือดของกุ้งกุลาดำที่ติดเชื้อวิบริโอฮาวิอาย โดยนำคู่ไพรเมอร์ oligo-dT และarbitrary จำนวน 44 คู่ มาทำการตรวจสอบหายีนที่มีการแสดงออกแตกต่างกันระหว่างกุ้งปกติ และกุ้งที่ติดเชื้อ พบแถบของ cDNA ที่มีการแสดงออกแตกต่างกันทั้งหมด 79 แถบ ซึ่งมาจากไพรเมอร์ 33 คู่ ประกอบด้วย ยีนที่มีการแสดงออกเพิ่มขึ้นจำนวน 48 แถบ และยีนที่มีการแสดงออกลดลงเมื่อติดเชื้อวิบริโอฮาวิอายจำนวน 31 แถบ นำมาเพิ่มปริมาณด้วยเทคนิคพีซีอาร์ แล้วทำการโคลนcDNA ที่ได้เข้าสู่เวคเตอร์ ซึ่งสามารถโคลนได้ 48 ชิ้น สุ่มเลือกโคลนและนำไปหาลำดับเบสบางส่วน จากการวิเคราะห์ลำดับเบสของโคลนที่ได้จากแถบเดียวกัน พบว่า 21 แถบ มีลำดับเบสชนิดเดียว และอีก 27 แถบนั้นมีลำดับเบสมากกว่าหนึ่งชนิด เมื่อนำลำดับเบสจากโคลนทั้งหมด 267 โคลน ไปเปรียบเทียบกับข้อมูลใน GenBank พบ 85 โคลน (31%) ที่มีลำดับเบสคล้ายกับยีนที่มีรายงานแล้วโดยเป็นยีนที่แตกต่างกัน 24 ยีน โดยในการศึกษานี้ได้คัดเลือกยีนที่สนใจได้แก่ caspase 3B, SERPINB3, profilin, lysozyme, interferon-related developmental regulator 1, และ glucose transporter 1 มายืนยันการแสดงออกของยีนดังกล่าวด้วยเทคนิค real-time PCR ซึ่งพบว่ายีนเหล่านี้มีการแสดงออกเพิ่มขึ้นหลังจากการติดเชื้อวิบริโอฮาวิอาย ยีนของสารต้านจุลชีพแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ ชนิดที่ 3 ซึ่งมีรายงานการว่าเป็นชนิดที่พบมากที่สุดในในเซลล์เม็ดเลือดของกุ้งกุลาดำ เป็นยีนแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ชนิดเดียวที่พบในการศึกษานี้ จึงเป็นยีนที่น่าสนใจสำหรับการศึกษาลักษณะสมบัติ แอคติวิตี และคุณสมบัติทางชีวภาพ โดยการผลิตโปรตีนแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ ชนิดที่ 3 ในระบบยีสต์ *Pichia pastroris* โปรตีนที่ผ่านการทำบริสุทธิ์แล้วมีฤทธิ์ยับยั้งการเจริญของแบคทีเรียแกรมลบ แบคทีเรียแกรมบวก และเชื้อราได้ดี ซึ่งโปรตีนนี้ออกฤทธิ์โดยการฆ่าเชื้อแบคทีเรีย (bactericidal effect) *Bacillus megaterium* และ *Escherichia coli* 363 การศึกษาระดับการแสดงออกของยีนแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ ชนิดที่ 3 ในช่วงเวลาต่างๆ ระหว่างที่กุ้งกุลาดำได้รับเชื้อวิบริโอฮาวิอาย ด้วยเทคนิค *in situ* hybridization และ real-time PCR พบว่ายีนนี้มีการแสดงออกเพิ่มขึ้นภายใน 6 ชั่วโมงแรกที่ได้รับเชื้อ และพบการกระจายตัวของเซลล์เม็ดเลือดที่แสดงออกยีนแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ ชนิดที่ 3 อยู่ทั่วไปในส่วน cepharothorax ของกุ้งกุลาดำ ในช่วงเวลาดังกล่าว หลังจากนั้นจะมีปริมาณสูงขึ้นอีกครั้งที่เวลา 72 ชั่วโมง การศึกษาโปรตีนโดยการใช้แอนติบอดีที่จำเพาะต่อโปรตีนแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ ชนิดที่ 3 พบว่าในช่วงแรกของการตอบสนองต่อเชื้อแบคทีเรียมีการเคลื่อนที่ของเซลล์เม็ดเลือดที่ผลิตโปรตีนนี้ไปยังบริเวณที่ทำการฉีดเชื้อเข้าไปยังกุ้งกุลาดำ ข้อมูลที่ได้ในการศึกษานี้ชี้ให้เห็นว่าสารต้านจุลชีพแอนติไลโปพอลิแซ็กคาไรด์แฟคเตอร์ ชนิดที่ 3 มีบทบาทสำคัญในการปกป้องกุ้งกุลาดำจากการบุกรุกของจุลชีพ

ภาควิชา……ชีวเคมี……	ลายมือชื่อ นิสิต…….
สาขาวิชา……ชีวเคมี……..…	ลายมือชื่ออาจารย์ที่ปรึกษา……...…….
ปีการศึกษา……2547…….	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม…….....…..
	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……..…

4373804723 : MAJOR BIOCHEMISTRY
KEY WORD: *Penaeus monodon* / differential display PCR / anti-lipopolysaccharide factor / haeomocytes / *Pichia pastoris* expression system

Kunlaya Somboonwiwat : IDENTIFICATION OF GENES FOR ANTIMICROBIAL EFFECTORS AND CHARACTERIZATION OF ANTI-LIPOPOLYSACCHARIDE FACTOR OF THE BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : ASSOC. PROF. ANCHALEE TASSANAKAJO N, Ph.D., THESIS COADVISOR : EVELYNE BACHÈRE, Ph.D. AND RATH PICHYANGKURA, Ph.D. 182 pp. ISBN 974-53-1746-2.

Differential Display PCR technique (DD-PCR) was used for the analysis of altered gene expression in haemocytes of *Vibrio harveyi* infected *Penaeus monodon*. Forty-four combinations of arbitrary and oligo-dT primer were used to screen for differentially expressed genes. A total of 79 differentially expessed bands could be identified from 33 primer combinations. These included 48 bands (61%) whose expression level increased and 31 bands (39%) decreased in expression after *V. harveyi* challenge Forty-eight differential display fragments were successfully reamplified and cloned. These clones were randomly selected and partially sequenced. The sequence analysis showed that 21 (44%) out of 48 bands contained unique sequences, while the remaining bands contained mixture of two to four different sequences. Eighty-five (31%) out of 267 clones matched with sequences in the GenBank database which represented 24 different genes. Caspase 3B, SERPINB3, profilin, lysozyme, glucose transporter 1, interferon-related developmental regulator 1, were selected for further confirmation for their differentially expression patterns by real-time PCR. The results indicated that all selected genes were up-regulated in *P. monodon* haemocytes upon *V. harveyi* challenge. Anti-lipopolysacharide factor type3 (ALF*Pm*3) cDNAs, an antimicrobial peptide, previously identified as the most abundant type of ALF in *P. monodon* was the only one type of ALF identified in this study. In order to characterize their biological activity and properties, we expressed the cDNA of ALF*Pm*3 in yeast *Pichia pastroris*. The purified protein exhibited a strong activity against several strains of Gram-negative and -positive bacteria and fungi. Analysis of the antibacterial action against tested strains, *Bacillus megaterium* and *Escherichia coli* 363, suggested that the inhibition was due to bacteriocidal not bacteriostatic effect. The spatio-temporal expression of ALF*Pm*3 encoding gene was studied in response to *V. harveyi* challenge by in situ hybridization and real-time PCR analysis. ALF*Pm*3 expression was significantly increased in haemocytes at the early phase (6 hr) of microbial infection. Localization of ALF*Pm*3 transcripts in shrimp tissues revealed that bacterial invasion resulted in distribution of ALF*Pm*3 expressing haemocytes throughout the shrimp cepharothorax during the first phase of infection. The ALF*Pm*3 transcripts reached high level of expression again at 72 hour-post injection. During the first phase of immune response, migraion of ALF*Pm*3 producing haemocytes to the injection site was evidenced, when detection of ALF*Pm*3 protein with a specific antibody was performed. Taken together, these results implied the crucial role of this effector in protection of shrimp against microbes.

Department…Biochemistry……	Student's signature ….....
Field of study…Biochemistry…….	Advisor's signature .....…...
Academic year…..2004…..…….	Co-advisor's signature.....
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List of Abbrevations

ALF	anti-lipopolysaccharide factor
bp	base pair
^{o}C	degree Celcius
Cfu	colony forming units
DD-PCR	differential display PCR
DEPC	diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EtBr	ethidium bromide
EST	Expressed Sequence Taq
GLUT	glucose transporter
GRP	glucose-regulated protein
hpi	Hour-post injection
HPLC	high performance liquid chromatography
IFRD	interferon-regulated developmental regulator
ISH	*in situ* hybridization
LPS	lipopolysaccharide
kb	kilobase
kDa	kilodalton
M	molar
MCS	multiple cloning sites
MDP	mandibular pulp
MIC	minimum inhibitory concentration
ml	millilitre
MT	metric ton

$MgCl_2$	magnesium chloride
mg	milligram
mM	millimolar
ng	nanogram
nm	nanometre
O.D.	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
proPO	prophenoloxidase
proppA	prophenoloxidase activating enzyme
RSL	reactive site loop
SDS	sodium dodecyl sulfate
SPI	serine proteinase inhibitor
μg	microgram
μl	microlitre
μM	micromolar
WSSV	white spot syndrome virus

CHAPTER I

INTRODUCTION

1.1 General introduction

The important cultivated shrimp species are the black tiger shrimp, *Penaeus monodon*, the pacific white shrimp, *Litopenaeus vannamei*, and the chinese white shrimp, *Fenneropenaeus chinensis*, which dominate the major global share of world shrimp products, accounting for over 75 percent of total shrimp aquaculture production in 2002 (Figure1.1). *P. monodon* only ranked 16th in terms of global aquaculture production by weight in 2002, but it ranked second in terms of value at US$ 3,371 billion. The contribution of *P. monodon* remained stable at around 600,000 metric tonnes from 1994 through 2002, and had declined from over 63 percent to 40 percent in 2002, as *F. chinensis* and now particularly *L. vannamei* productions have increased to more than 500,000 metric tons (Briggs et al., 2004).

Penaeus monodon is farmed everywhere in Asia, e.g. India, Vietnam, Indonesia, Philipines, Malaysia, and Thailand. By the mid 1970s, Thailand's *P. monodon* industry started and the intensive farming has been proceeded for more than two decades. Shrimp farms and hatcheries are scattered along the coastal areas of Thailand. Southern provinces (Nakorn Sri Thammarat, Surat Thani) account for the majority while those in the East (Chanthaburi) and Central regions (Samut Sakhon, Samut Songkhram) comprise the minority in terms of number of farm. The intensive farming system (85%) has been used for *P. monodon* farming activity resulting in the consistent increase in the outcome production (Source: Department of Fisheries).

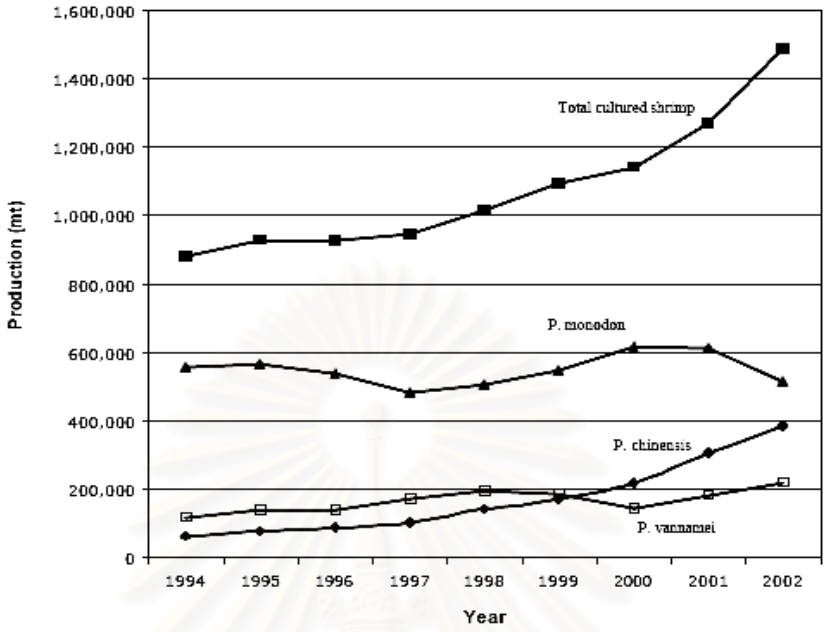


Figure 1.1 World production of cultured shrimp species (1994-2001)

Source: FAO Fishstat (2003)

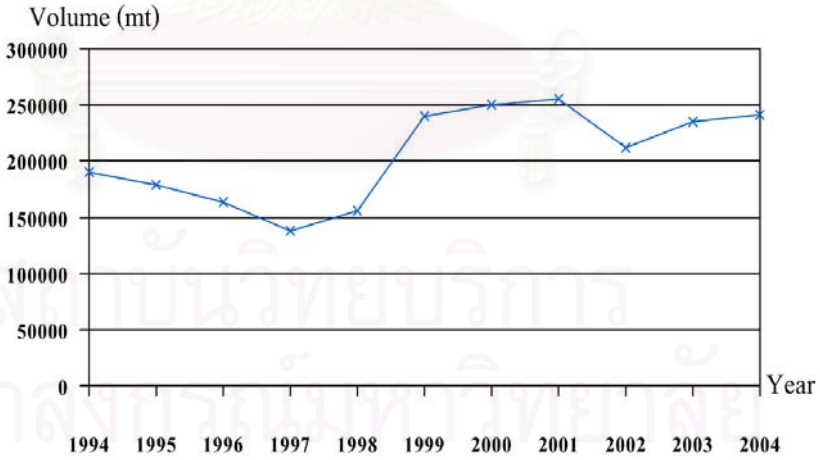


Figure 1.2 Exportation quantity of Thai shrimp during 1994-2004

Source: Thai Customs Department cited in Shrimp Culture Newsletter

Thailand has been the world's leading shrimp producer since 1993. However, a severe outbreak during 1995-1997 causing the decrease in the shrimp production at that period (Figure 1.2). In 2002, Thai shrimp production fell about 40 percent from 2001 to approximately 160,000 tons due to infectious disease at the beginning of the year, unfavorable weather, high salt concentrations in water, slow shrimp growth rates, low yield per area, switching to other shrimp species such as fresh water shrimp or white shrimp and concerning over the antibiotic residues. Other major shrimp exporting countries also increased the output in 2002, causing the Thai share in world markets, especially in the US, to shrink. The continual disease problem caused the great impact on *P. monodon* production yield. In 2004, *P. monodon* production of Thailand fell down to 75,000 tons, whereas those of other countries increased (Table 1.1). Nevertheless, the total shrimp production in Thailand was not significantly affected due to the increase production of the white shrimp *L. vannamei* According to Thai Customs Department, the export value of Thai shrimp in 2001 to 2004 was not significantly decrease.

At the end of 2004, on December, 26th, Thailand and other countries such as the Maldives, Sri Lanka, and Indonesia were affected by the recent Tsunami. Tsunami waves devastated the shores of Indonesia, Sri Lanka, South India, Thailand, and other countries. It caused serious damages and deaths as far as the east coast of Africa. Over 300,000 people have died, however, the count has not yet completed. As a result of Tsunami, the shrimp hatchery farms and the intensive shrimp farms located in the South of Thailand have been damaged. This would result in the decline of shrimp production in 2005.

The situation encouraged Thai farmers to search for alternative species such as *L. vannamei*. Production of *L. vannamei* comparing to *P. monodon* increased significantly from 49% to 80% in 2003 to 2004 and is expected to increase to 90-95% in 2005 because of the high survival rate of *L. vannamei* (Table 1.2). As the farming of *L. vannamei* is increased, there is the risk of interbreeding between the native species, *P. monodon*, and the alien species, *L. vannamei*, which may cause problems in the future, unless the good management and control are performed. The *L. vannamei* may also dominate over the native species *P. monodon*, which may have an impact on the ecological balance in this area.

Table 1.1 *Penaeus monodon* production in various countries in 2004

Country	*P. monodon* produciton (tons)
India	120,000
Vietnam	100,000
Indonesia	60,000
Philipines	35,000
Malaysia	25,000
Thailand	75,000

Source: Shrimp Culture Newsletter

Table 1.2 Comparison of percentage of *Penaeus monodon* and *P. vanamei* production during 2003 to 2005

Year	*P. monodon* (%)	*P. vannamei* (%)	Total production (Tons)
2003	51	49	350,000
2004	20	80	360,000
2005*	5-10	90-95	400,000

Source: Shrimp Culture Newsletter

* The data for 2005 is an estimate value.

Litopenaeus vannamei is a known carrier of *Baculovirus penaei* (BP), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Reo-like virus (REO), and Taura Syndrome virus (TSV). These viruses can be transmitted to native wild penaeid shrimp populations, and thus increases the concern over the spreading of the disease by the releases of infected shrimps from culture facilities (Overstreet et al., 1997). Because the first signs of Taura Syndrome Virus (TSV) in Thai *L. vannamei* and *Macrobrachium rosenbergii* has been observed (Briggs et al., 2004), there is also the possibility of viral outbreak on cultured *L. vanamei* as in Latin America.

The world has learned that the intensive farming system of only one species of shrimp leads to the outbreak of diseases a few years afterwards and the recovery is slow or not at all. Changing the species by importing the alien species, *L. vannamei*, believed to be pathogen-free (resistant) and give better yield, and use the intensive farming system will certainly follow the same cause as in the past. Thailand also has to pay for the postlarvae reducing the profit, if any, from the farming. Although the farms can survive the crisis by cultivation of the new shrimp species, it does not mean the sustainability of shrimp production in the long run since the actual causes of the infectious diseases are still there in the farm. On the other hand, the selection of better shrimp and the control of the diseases are utmost strategic move to sustainability of the farming. For the shrimp species, the native shrimp rather than the alien species should be considered as essential for this area. Consequently, research on *P. monodon* immunity and development of farming technology should be intensively studied.

1.2 Taxonomy of Penaeus monodon

Penaeus monodon, the giant tiger shrimp, is a shrimp specie that was classified into the largest phylum in the animal kingdom, the Arthropoda. The taxonomic definition of *Penaeus monodon* is as follow (Baily-Brook and Moss, 1992):

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Subclass Eumalacostraca
Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

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

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

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

1.3 Morphology

The shrimp body includes three regions: head, thorax, and abdomen (Figure 1.3). Each body region possesses appendages specialized for different functions. The head (five somites) and thorax (eight somites) are fused into a cephalothorax, which is completely covered by the carapace. Many internal organs, such as gills, digestive system, reproductive system and heart located in thorax are protected by carapace, while the muscles concentrate in the abdomen. The pleura of the cephalothorax form the branchiostegite or gill cover. The carapace has characteristic ridges (carinae) and grooves (sulci). The rostrum is always prominent, with a high median blade bearing dorsal teeth and, in some genera, ventral teeth as well. The compound eyes are stalked and laterally mobile and the somites of the head bear, in order, pairs of antennules, antennae, mandibles, maxillae 1 and maxillae 2 (not visible in Figure1.3). The thorax has three pairs of maxillipeds and five pairs of pereopods (legs), the first three being chelate and used for feeding, and last two simple (non-chelate) and used for walking. The abdomen consists of six somites, the first five with paired pleopods (walking legs) (Baily-Brook and Moss, 1992; Bell and Lightner, 1988). and the sixth with

uropods. The mouth is situated ventrally and the cephalic appendages surrounding it, plus the first and second maxillipeds and sometimes the third as well, may be referred to collectively as the "mouth parts". The anus is on the ventral surface of the telson towards its base (Dall et al., 1990).

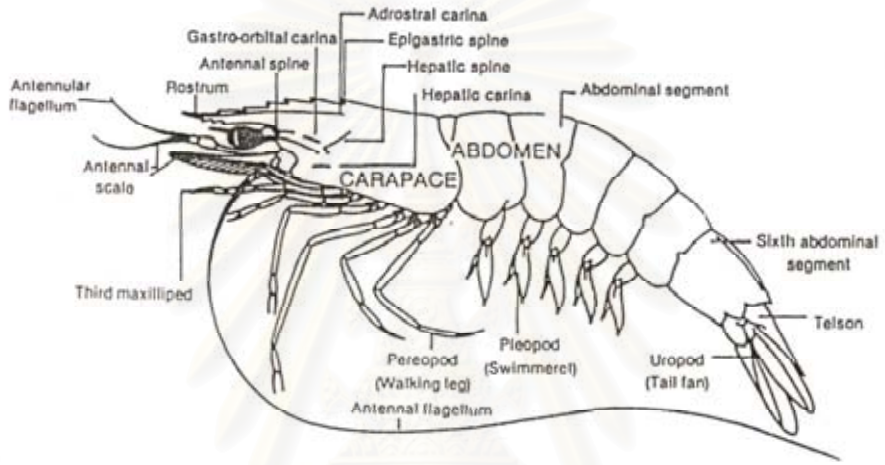


Figure 1.3 Lateral view of the external morphology of *Penaeus monodon* (Anderson, 1993).

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

The black tiger shrimp has the following characteristic coloration: carapace and abdomen are transversely banded with red and white, the antennae are grayish brown, and the pereopods and pleopods are brown with crimson fringing setae. In shallow brackishwaters or when cultured in ponds, the color changes to dark and, often, to blackish brown (Moton, 1981:cited in (Solis, 1988).

The internal morphology of penaeid shrimp is very well developed (Figure 1.4). Muscular, digestive, circulatory, respiratory, nervous, and reproductive systems are all present. Many sorts of muscles control movements of the body such as

walking, crawling, burrowing, swimming, feeding, and breathing. The digestive system is complex, in which parts of the tract are differentiated into a foregut, a midgut, and a hindgut. The circulatory system consists of a heart, dorsally located in the cephalothorax, with branching arteries conducting blood to the various organs. Gills are responsible for respiration process. The nervous system consists of two ventral nerve cords, a dorsal brain, and a pair of ganglia for each somite. Hepatopancreas connects to the gastrointestinal tract via the primary duct. It occupies a large portion of the cephalothorax in penaeid shrimp and functions on absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leaves the heart ends in the lymphoid organ where the haemolymph is filtered. This organ consists of two distinct lobes, each located ventro-lateral to the junction of the anterior and posterior stomach chambers. The lymphoid lobes are apposed slightly dorso-anterior to the ventral hepatopancreatic lobe. The haemocytes are produced in haematopoietic tissue. The hematopoietic tissue consists of densely packed lobules located at different parts of the shrimp anterior region. The first one is hematopoietic tissue surrounding the lateral arterial vessel, which joins the anterior recurrent artery at the base of the rostrum. The second one located within the 1st maxillliped. The third one is 2nd maxilliped hematopoietic tissue. The last one is epigastric hematopoietic tissue located dorsal to the anterior stomach chamber and just ventral to the dorsal cuticle. Lymphoid organ and haematopoietic tissue are shown in Figure 1.5.

1.4 Distribution and life cycle

The black tiger shrimp is mainly found throughout the Indo-Pacific region. Northern part of Indo-Pacific region ranges from Japan and Taiwan, Eastern region distributes toward Tahiti. For southern and western part, it distributes to Australia and Africa, respectively. The development of penaeid shrimps include several distinct stages in various habitats (Figure 1.6). Brackish shore is a preferable area for juveniles. The adults migrate to deeper offshore areas that have higher salinity, where mating and reproduction occur. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). Larvae hatching from the fertilized eggs pass through a series of moults and metamorphic stages before becoming adulting-like (juveniles).

After one day, the eggs hatch into the first stage, nauplius. The nauplii feed on their egg-yoke reserves for a few days and develop into the protozoeae. Around 4-5 days, the protozoeae will metamorphose into mysis by feeding on algae. The mysis feed on algae and zooplankton and then develop into early postlarvae (PLs), which the

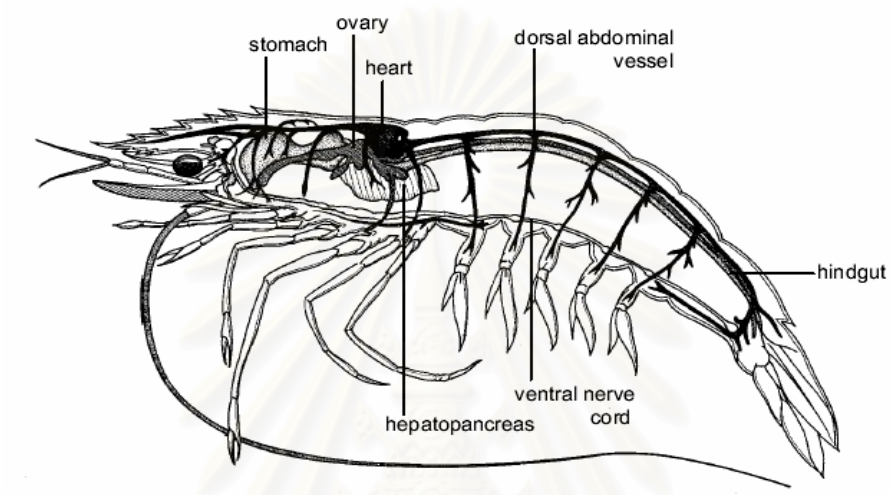


Figure 1.4 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990).

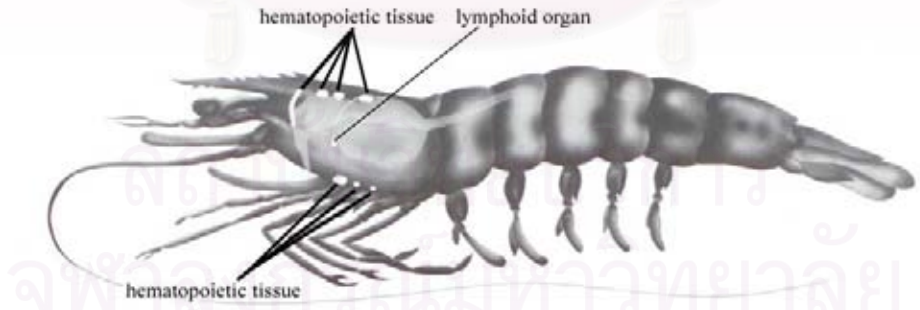


Figure 1.5 Location of hematopoetic tissue and lymphoid organ of penaeid shrimp.

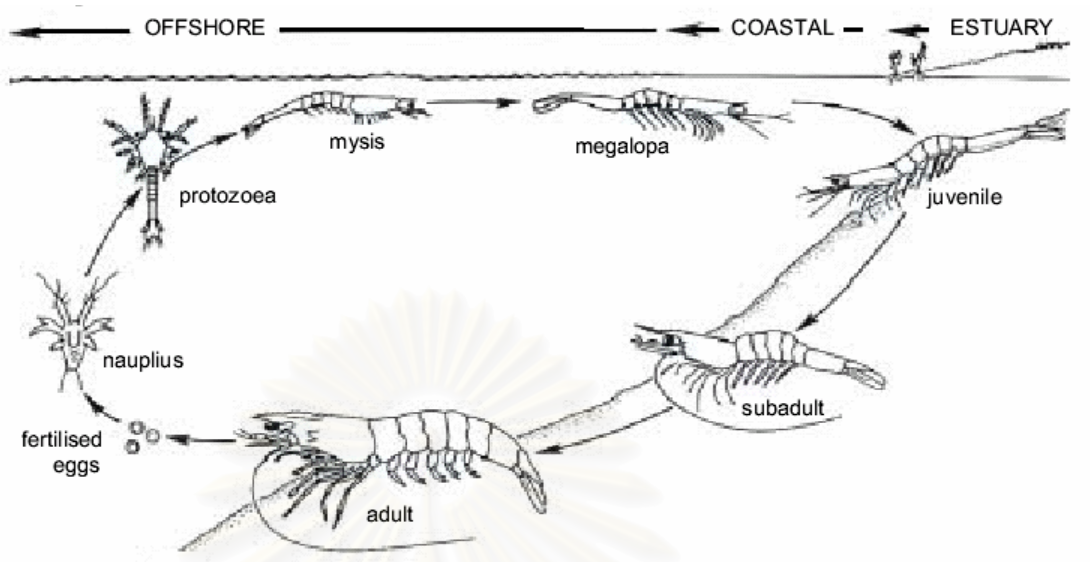


Figure 1.6 The life cycle of *Penaeus monodon* shrimp. (Baily-Brook and Moss, 1992).

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

1.5 Shrimp diseases

Diseases are the biggest obstacle to the future of shrimp farming. Viral and bacterial outbreaks have decimated the industries in many countries. At present, white spot syndrome virus and bacteria Vibrionacea family considered significance causative agents of infectious diseases in Thailand. Diseases affected the economic viability and long-term sustainability of the shrimp farming industry. It caused great loss of shrimp production. Therfore, the prevention and control of diseases turned into a priority for shrimp production. To deal with this problem, besides the development of farm management, shrimp immunity and defence effectors responsed to pathogen should be elucidated.

1.5.1 Viral disease

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

1.5.2 White spot syndrome (WSS)

White spot syndrome (WSS) is one of the most important viral disease, which affect most of the commercially cultivated marine shrimp species, not just in Asia but globally (Chou et al., 1995; Flegel, 1997; Lightner, 1996; Lotz, 1997; Span, 1997). Lightner (1996) has called this virus white spot syndrome baculovirus (WSSV). This virus belongs to a new virus family, the *Nimaviridae*, and the genus *Whispovirus* (Mayo, 2002; van Hulten et al., 2001). WSSV virions have been isolated and identifed to be a double-stranded circular DNA virus and are bacilliform to cylindrical morphology with a tail-like at one end of the particle. Its genome length is 305 kbp (van Hulten et al., 2001; Yang et al., 2001). The virons contain one nucleocapsid with a typical striated appearance and five major and at least 13 minor proteins (Huang et al., 2002a; Huang et al., 2002b; van Hulten et al., 2002; van Hulten et al., 2000a; van Hulten et al., 2000b). It has a wide host range among crustaceans (Wang et al., 1998). It is believed that the envelop proteins play important roles in

virus infection, so many researches have now focused on the analyses of envelop proteins (Wu et al., 2005; Zhang et al., 2004).

The affected shrimp exhibit white patches or spots on the inside surface of the carapace and the shell accompanied by reddish to pinkish red discoloration of the body. The disease occurs in ongrowing juvenile shrimp of all ages and sizes. Histological changes observed from diseases shrimp showed wide sprade cellular degeneration and severe nuclear hypertrophy in most tissue derived from ectodermal and mesodermal origin, especially from subcuticular shell epithilum, gill epitelium, sublcuticular stomach epithelium, lymphoid organ, connective tissue, hematopoietic tissue and nervous tissues (The ASEAN Fisheries Sub-Working group, 1998). The other clinical sign of this disease is a rapid reduction in food consumption of shrimp. Mortalities were low during the initial two to three days. Mass mortaities occurred within seven to ten days of the first clinical signs. Few of the WSSV-infected shrimps survived, but even so most of them died. Shrimp, which survive the infection, are suspected to be life-long carriers of WSSV.

To minimize damage from WSSV infection, detection of virus at an early stage is necessary. Several diagnostic methods have been described such as PCR (Tapay et al., 1999), in situ hybridization (Wang et al., 1998), miniarray (Quere et al., 2002), observation of tissues subjected to fixation or negative staining (Inouye et al., 1994), immunological methods using monoclonal and polyclonal antibodies to WSSV or their component proteins and recently developped method (Poulos et al., 2001), a reverse passive latex agglutination (RPLA) method (Okumura et al., 2005).

To protect shrimp or other crustaceans against WSSV, the WSSV subunit vaccine, the WSSV envelop proteins VP19 and VP28 was evaluated. The VP19 and VP28 fused to MBP showed significantly better survival than the control group (Witteveldt et al., in press). The other research area concerning the control of WSSV infection is to identify and production of antiviral proteins. A *PmAV* cDNA of WSSV-resistant *P. monodon* encoding for a 170 amino acid peptide with a C-type lectin-like domain were demonstrated that it possessed a strong antiviral activity in virus infected fish cell line (Luo et al., 2003). In 2004, Zhang and co-workers showed that hemocyanin isolated from *P. monodon* had non-specific antiviral properties. In

addition, interferon-like protein from haemocytes of the virus-resistant *Marsupenaeus japonicus* was found to have also the non-specific antiviral ability by inhibiting SGIV (grouper iridovirus) in GP cells (grouper embryo cells) (He et al., 2005).

1.5.3 Bacterial disease

Species of *Vibrio* are commonly found in marine environments as the bacterial flora and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). They exist in a high numbers in both the water and sediment of shrimp ponds, especially in an intensive culture system (Direkbusarakom et al., 1998). The virulence of this species has been recognized in a small but expanding list of cultured marine animal particularly penaeids in Asia and Australia (Vandenberghe et al., 1998). Disease outbreaks attributed to *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus* and *V. penaeicida* have been observed in nursery or growout ponds of *P. monodon*, *L. vannamei*, *P. japonicus* and *P. stylirostris* (Saulnier et al., 2000). Disease signs range from localized cuticular lesions, oral and enteric infections to septicaemia (Lightner, 1996).

Gross signs of Vibriosis are light or dark brown focal lesion and necrosis of appendage tips. Melanin produces by host hemocytes involved in the inflammatory process causes the change of color. Affected shrimp exhibit decreased appetite. Some also had a darker, larger of shrunken hepatopancreas.

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

The antibiotic was used to control the pathogen caused problems of drug resistance. The use of probiotics , a marine bacterial strain *Pseudomonas* I-2, which

can produce a compound with inhibitory property against *Vibrio* is an interested strategy for controlling shrimp pathogenic *Vibrio* in cultured system (Chythanya et al., 2002). Another potential probiotic bacteria are *Bacillus subtilis* BT23, which its cell free extracts had inhibitory effect on growth of *V. harveyi* isolated by agar antagonism assay from *P. monodon* with black gill disease (Vaseeharan and Ramasamy, 2003).

1.6 The invertebrate immune system

All living organisms have their own immune system for defending themselves against microbial attack or other foreign substances. Evolutionary, immune system can be classified into two types adaptive (acquired) immunity and innate (natural) immunity. Vertebrates possess both adaptive and innate immune system, whereas invertebrates involve only in innate immunity. The adaptive immune system function by producing of highly specific regcognition molecules, namely antibodies, which can memorize foreign molecules following the first time of exposure. The latter system involves a large number of generalized effector molecules.

The innate immune system can be found both in vertebrate and invertebrate animals. Phylogenetic analysis of protein families of proteins functioning in the vertebrate immunity that have known homoloques in arthropods and of proteins of arthropod immune system having known homoloques in vertebrates, suggest a evolutionary discontinuity between vertebrate and invertebrate innate immunity. The question is how invertebrates survive in a pathogen-laden environment without an adaptive immune system. The answer is that innate immune systems including haemolymph coagulation, complement activation, cell agglutination, the use of RNA interference (RNAi), pattern-recognition receptor (PRRs), hydrolytic enzymes, antimicrobial peptides (AMPs), phagocytic cells, production of active oxygen and nitrogen metabolites, and melanization pathways, are very effective (Hughes, 1998; Iwanaga, 2002).

The powerful model for studying on invertebrate innate immunity is the dipteran insect *Drosophila melanogaster*. The efficacy of Drosophila host defense resulted from recognition of various classes of infectious microorganisms and selective activation of intracellular signaling cascade. The signalling pathways, the

Toll pathway and the Immuno deficiency (IMD) pathway, control AMPs gene expression upon microbial infection (Hetru et al., 2003). The current complete genomic data of *D. melanogaster* combining with new technologies on biochemistry, genetics, and molecular biology will facilitate study on innate immunity.

1.7 The crustacean immune system

In crustaceans, the innate immune system is based on cellular and humoral components of the circulatory system. The hard cuticle which covers all external surfaces of crustaceans is the first line of defense between them and the environment. However, the innate immunity responses rapidly if microorganisms can invade the animals. The major defense systems of crustaceans are carried in the hemolymph, which contains a cells called haemocyte. Haemocyte and plasma protein recognizes large groups of pathogens by mean of common molecular patterns of particular microbes. The recognition molecules, including the lipopolysaccharides (LPS) of Gram negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gramp positive, bacteria, the mannans of yeasts, the β–1,3-glucan of fungi and double-stranded RNA of viruses (Hoffmann et al., 1999), may interact with and activate the hemocytes. Several types of pattern recognition proteins (PRPs) have been isolated and characterized in several invertebrates (Lee et al., 2004; Lee and Soderhall, 2001). Some PRPs are lectins which can promote the agglutination. Therefore, they are likely to have a potential role in invertebrate non-self-recognition reactions. In crustaceans, examples of PRPs are lectins (Kopacek et al., 1993; Vargas-Albores et al., 1996), β–1,3-glucan binding protein (BGBP) in many crustaceans. The crustacean BGBPs were reported in freshwater crayfish, *Pacifastacus leniusculus* (Duvic and Soderhall, 1992), and two marine shrimp species, *P. californiensis* (Vargas-Albores et al., 1996), *L. vannamei* (Jimenez-Vega et al., 2002; Vargas-Albores et al., 1997) and *P. monodon* (Sritunyalucksana et al., 2002). Haemocyte activation results in release of various types of immune effectors, such as antimicrobial peptides, coagulation factors, proteinase, and proteinase inhibitors, which function on elimination of invading pathogens. The current knowledge of crustacean immunity was summarized as shown in Figure 1.7.

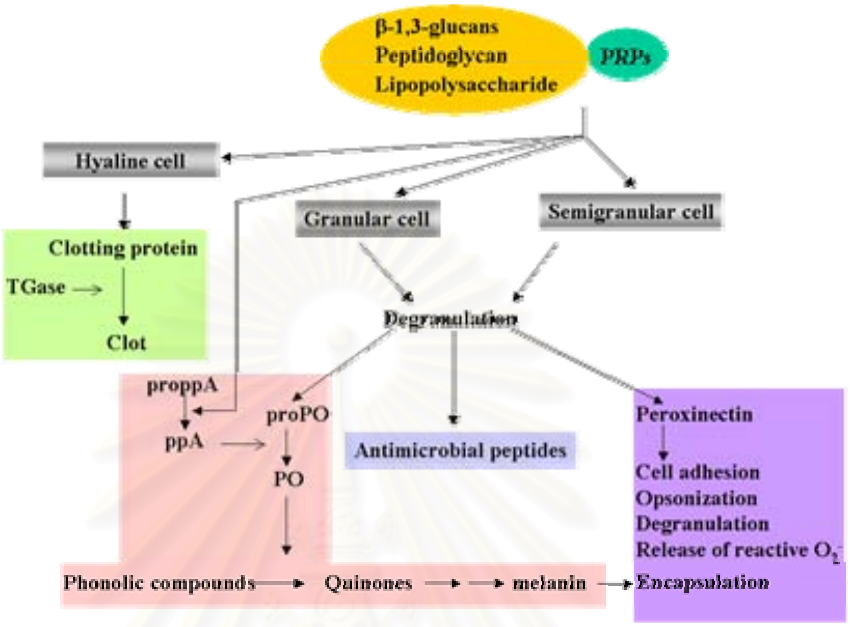


Figure 1.7 Simplified flow overview of present-day knowledge of crustaceans immunity that are mediated by the haemocytes (Smith et al., 2003).

1.7.1 Haemocytes

In general, crustacean circulating haemocytes are morphologically divided into three types: hyaline cells, semigranular cells (contain a variable number of small granules), and granular cells (contain a large number of large granules) (van de Braak et al., 2000). Indeed, they are also described in penaeid shrimp and freshwater crayfish (Rodriguez et al., 1995). The hyaline cell lacks of cytoplasmic granules with a high nucleus/cytoplasm ratio, which involved in coagulation processes. The granular cell involved in phagocytosis, encapsulation and storage of the prophenoloxidase (proPO) system (Gargioni and Barracco, 1998; Hose et al., 1990).

1.7.2 Proteolytic cascades

Proteolytic cascades triggered by nonself recognition molecules have major roles in innate immunity. In crustacean, haemolymph coagulation best described in horseshoe crab and the prophenoloxidase (proPO) cascade extensively studied in freshwater crayfish (Cerenius and Soderhall, 2004). The proteinase cascades were regulated by protease inhibitors such as serine proteinase inhibitors from the Kazal and Serpin families (De Gregorio et al., 2002; Kanost et al., 2001), α-macroglobulin (Armstrong and Quigley, 1999; Vargas-Albores et al., 1996).

1.7.2.1 The prophenoloxidase (proPO) system

The proPO activating system comprises several proteins involved in the immune defense in invertebrates leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Sritunyalucksana and Söderhäll, 2000). It is activated by extremely low quantities of lipopolysaccharides or peptideoglycans from bacteria and β–1, 3-glucans from fungi, through pattern-recognition proteins (PRPs)(Ariki et al., 2004). The proPO system is a proteinase cascade involved several zymogenic proteinases, and proPO (Cerenius and Soderhall, 2004). Upon stimulation, proprophenoloxidase activating enzyme (proppA) was changed to an active ppA, a serine proteinase, which will then convert proPO to the active phenoloxidase(PO) by proteolysis. PO catalyses two successive reactions: hydroxylation of a monophenol to o-diphenol (monophenoloxidase activity) and the oxidation of the o-diphenol to o-quinone (diphenoloxidase activity) (Cerenius and Soderhall, 2004; Decker et al., 2000; Decker and Tuczek, 2000). Production of o-quinones by PO leads to melaninsation. The production of melanin pigment can often be seen as dark spots in the cuticle of arthropods involving in the process of sclerotisation, wound healing and encapsulation of foreign materials (Theopold et al., 2004). Several components and associated factors of the proPO system have been also found to initiate several molecules in the defense reaction of the freshwater crayfish (Söderhäll and Cerenius, 1998).

Shrimp proPO system have been elucidated in *Penaeus californiensis* (Gollas-Galvan et al., 1999; Vargas-Albores et al., 1996), *P. panlensis* (Perazzolo

and Barracco, 1997), *P. stylirostris* and *P. monodon* (Sritunyalucksana et al., 1999). Shrimp proPO is synthesized in the haemocytes. Shrimp proPO is more closely related to crayfish proPO than to the insect proPO according to amino acid comparison.

1.7.2.2 The coagulation system/the clotting system

A coagulation system entraps foreign molecules and prevents the excess haemolymh loss from a wound. Coagulation of invertebrates, chelicerates and crustaceans, has been characterized in molecular detail. In horseshoe crab, *Tachypleus tridentatus*, clotting formation is activated by either lipopolysaccharide or β–1, 3-glucans through proteinase cascade in the large granules of haemocytes (Iwanaga et al., 1998; Kawabata and Tsuda, 2002). In this process, coagulagen was converted by proteinase into coagulin which further spontaneously aggregates to form insoluble clots. In crustacean, e.g. lobster (Fuller and Doolittle, 1971), crayfish (Hall et al., 1999; Komatsu and Ando, 1998; Kopacek et al., 1993) and shrimp (Huang et al., 2004; Yeh et al., 1998) transglutaminase (TGase) were identified. This enzyme is shown to be involved in the clotting process. TGases are Ca^{2+}-dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on certain proteins resulting in polymerization. Type C coagulation involving in lysis of explosive corpuscles or hyaline cell is present in the spiny lobster and shrimp (Yeh et al., 1999).

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 reaction in crustaceans.

1.7.3 Antimicrobial peptides or proteins

Antimicrobial peptides (AMPs) are effector molecules that play important role in innate immune system. In all kingdoms, from bacteria to human, a variety of AMPs have been identified and characterized (Bulet et al., 1999; Hancock and Diamond, 2000). AMPs share common biochemical features such as small size, generally less than 150-200 amino acid residues, amphipathic structure and cationic

property. However, the anionic peptides also exist. AMPs have wide variety and diversity in amino acid sequences, structure, and range of activity. AMPs are active against a large spectrum of microorganisms; bacterial and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Hancock and Diamond, 2000; Murakami et al., 1991; Pan et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991). In Artropoda, a broad activity of AMPs against bacteria and filamentous fungi, have been reported in insects, horseshoe crab, and shrimp (Bachere et al., 2004; Bulet et al., 1999; Vizioli and Salzet, 2002). Moreover, depending on their distribution, antimicrobial peptide expression appears to be regulated by different tissue-specific pathways and these effectors may consequently participate in either a local or a systemic reaction. Some constitutively expressed within secretory cells, others are induced upon microbial stimulation (Hancock and Diamond, 2000). In 2004, Bulet et al. evaluated that over 1000 AMPs have been isolated and characterized from multicellular organisms at the level of their primary structure which most of them have been identified in insects.

AMPs are tentatively classified into four distinct groups base 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 haemolymph; (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) one or two amino acids-rich peptides, such as arginine-, tryptophan-, histidine-, glycine- and proline-rich peptides, for example glycine-rich peptides;attacins, diptericin, and sarcotoxins, proline-rich peptides;apidaecins anddrosocin; (iv) peptides derived from partial hydrolysis of large precursor proteins, e.g. histone, hemocyanin (Bulet et al., 2004).

In general, the net positive charge together with amphipathic structure accounts for preferential binding to the negatively charged membrane interface of microorganisms, which is different from the predominantly zwitterionic surface of normal mammalian cells so that they are toxic to microbes and not to mammals. The features required for AMPs are (i) selective toxicity on microbial cells, short bacterial

killing time, broad antimicrobial spectra and no bacterial resistance development (Matsuzaki, 2001). Many antimicrobial peptides act directly on the membrane of the target microorganisms, however, there is an exception evidence that the antimicrobial peptide nisin Z uses the membrane-anchored cell wall precursor Lipid II as a receptor (Shai and Oren, 2001). AMPs can interact and insert into the lipid bilayer, causing collapse of electrochemical gradients of membrane. Therefore, microorganisms lose their cellular component and source of energy for surviring.

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

There are few reports on antimicrobial peptides in crustaceans. Tachyplesin family and anti-LPS factors which acting against Gram negative bacteria were observed in horseshoe crab (Aketagawa et al., 1986; Muta et al., 1990; Nakamura et al., 1988; Ohashi et al., 1984). In 1997, a small peptide named callinectin was reported to be responsible for the majority of antimicrobial activity observed in the haemolymph of blue crab, *Callinectes sapidus* (Khoo et al., 1999) and recently, penaeidins, a new family of antimicrobial peptide which acting against Gram-positive bacteria and fungi were reported in penaeid shrimp *L. vannamei* (Destoumieux et al., 1997). These peptides contain both a proline rich domain at the N-terminal and a carboxy-terminal domain containing 6 cysteines which from 3 disulfide linkages. cDNA clones of penaeidin isoform were also isolated from the haemocytes of *L. vannamei* and *L. setiferus* (Gross et al., 2001) and *P. monodon* (Supungul et al., 2004). A cystein-rich 11.5 kDa antibacterial protein was purified and characterized from haemolymph of shore crab, *Carcinus maenas* (Relf et al., 1999).Crustins, an antimicrobial peptide homologues of an 11.5 kDa antibacterial

peptide were identified from 2 species of *Penaeid* shrimp, *L. vannamei* and *L. setiferus* (Bartlett et al., 2002 (Vargas-Albores et al., 2004). Peptides derived from the hemocyanin of *L. vannamei and P. stylirostris* and hemocyanin of *P. monodon* posses antiviral activity has been also identified (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004). Recently, the histones and histone derived peptides of *L. vannamei* has been reported as an innate immune effectors because they can inhibit growth of Gram-positive bacteria (Patat et al., 2004).

1.8 Antilipopolysaccharide factors

Anti-lipopolysaccharide factor (ALF) is an AMP previously found in haemocytes of horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus,* defined as LALF and TALF, respectively (Morita et al., 1985; Tanaka et al., 1982). This molecule is a small basic protein located in large granule haemocytes of horseshoe crab. It binds and neutralized the toxic lipid A moiety of LPS leading to inhibition of the endotoxin-mediated activation of the coagulation cascade. It exhibits strong antibacterial activity on the growth of Gram-negative R-type bacteria (Morita et al., 1985). Due to LPS-binding activity, these molecules and its derivatives are predominant candidates for potential therapeutic agents for prophylaxis and therapy of viral and bacterial infectious diseases, as well as for septic shocks (Vallespi et al., 2000).

Recently, ALF cDNA clones were identified from the haemocytes of the black tiger shrimp, *Penaeus monodon* by Expressed sequence tag (EST) analysis. (Supungul et al., 2004; Supungul et al., 2002). Further sequence alignment indicated that at least 4 different types (ALF*Pm*1- ALF*Pm*5) exist in the *P. monodon* haemocytes. They have open reading frames of 252, 360, 369, and 396 bp, coding for proteins of 84, 120, 123, and 132 deduced amino acids, respectively. The deduced amino acids of these ALF*Pm*s showed 57-65 % homology with those of *T. tridentatus* and *L. polyhemus.* All LALF, TALF, and ALF*Pm* have two conserved cysteine residues and a highly conserved sequence of a positively charged and hydrophobic residues cluster within the disulfide loop (Aketagawa et al., 1986; Hoess et al., 1993; Supungul et al., 2002). This positive cluster is defined as putative LPS-binding site.

ALF*Pm*3 was the most abundant isoform and was found in both normal and *V. harveyi* infected shrimp haemocytes cDNA libraries, whereas the others were found only in the infected cDNA library. Analysis of mRNA expression level in *V. harveyi*-injected shrimp by semiquantitative RT-PCR show up-regulation of ALF*Pm*3 genes. Investigation on distribution of this genes reveals that haemocytes is the site of ALF and other AMPs of shrimp (Supungul et al., 2004).

A recombinant protein of synthetic LALF gene, termed endotoxin neutralizing protein (rENP), has been successfully expressed both in *Saccharomyces cerevisiae* (unpublished data) and *Pichia pastoris* (Paus et al., 2002), but not in *E. coli* or in Gram-positive bacteria, *Bacillus sp.* Expression and biological activity of native TALF has also been carried out in *Pichia pastoris* (Wang et al., 2001) and in *Baculovirus* expression system (Wang et al., 2002). Intensive studies of these recombinant proteins have focused on its potent biological activity like sepsis treatment properties (Andra et al., 2004; Ried et al., 1996; Vallespi et al., 2000). Few studies have been emphasized on the antimicrobial properties and spectrum of activity of ALF.

For ALF*Pm*3 identified in our laboratory, the first expression experiment of this protein in the baculovirus were performed by Anurakolan in 2001. The full-length and NH_2-terminal truncated derivative of the protein were produced but inclusion body proteins were obtained and purification of protein could not achieved. To solve the purification problem, histidine tag was added to the N-terminal of full-length and NH_2-terminal truncated anti-LPS factor proteins (Achakunwisut, 2003). Unfortunately, baculovirus expression system did not express the recombinant proteins of anti-LPS factor from both constructs. However, the protein was successfully expressed in the yeast *Pichia* expression system as 6XHis Tag NH_2-terminal truncated ALF. The obtained recombinant proteins were partially purified and subjected to preliminary test on antimicrobial activity. However, development of the method for protein expression and purification to obtain higher expression of purified recombinant protein is required for further characterization.

1.9 Differential Display PCR

Differential display PCR (DD-PCR) was firstly described by Liang and Pardee in 1992. It was the earliest technology for transcript profiling. DD-PCR has become a widely-used routine method for the discovery of differentially regulated genes because its simplicity, sensitivity, and ability to simultaneously compare multiple mRNA samples for the identification of both up- and down-regulated genes without prior knowledge of their sequences.

DD-PCR is an expression analysis method whereby mRNA from each sample is converted to cDNA. cDNA is then PCR-amplifed using a combination of oligo-dT and arbitrary primers. Oligo-dT primers have complementary sequences to the poly(A) tail of mRNA and the adjacent two nucleotides of the transcribed sequence. Under low stringency conditions, a secondary primer added in the reaction, resulted in amplification of double stranded cDNAs. The amplified cDNAs are labeled with isotope and separated on a gel to allow side-by-side comparison of mRNA expression profiles. The putative differentially expressed clones are further isolated, cloned, and sequenced to reveal identity (Figure 1.8).

However, the major drawback of this method is a high rate of false positives. Martin and Pardee (2000) reported that up to 50% false positives were determined. In addition, even if the amplified product is exactly cut out, the PCR product may be a mixture of products of similar size (Mathieu-Daude et al., 1996). This is due either to the presence of a non-differentially expressed band overlapping the unique band of interest or co-purification of neighboring band prior to the re-amplification step (Callard et al., 1994). In order to avoid false positive, independent confirmation of differential expression patterns is necessary.

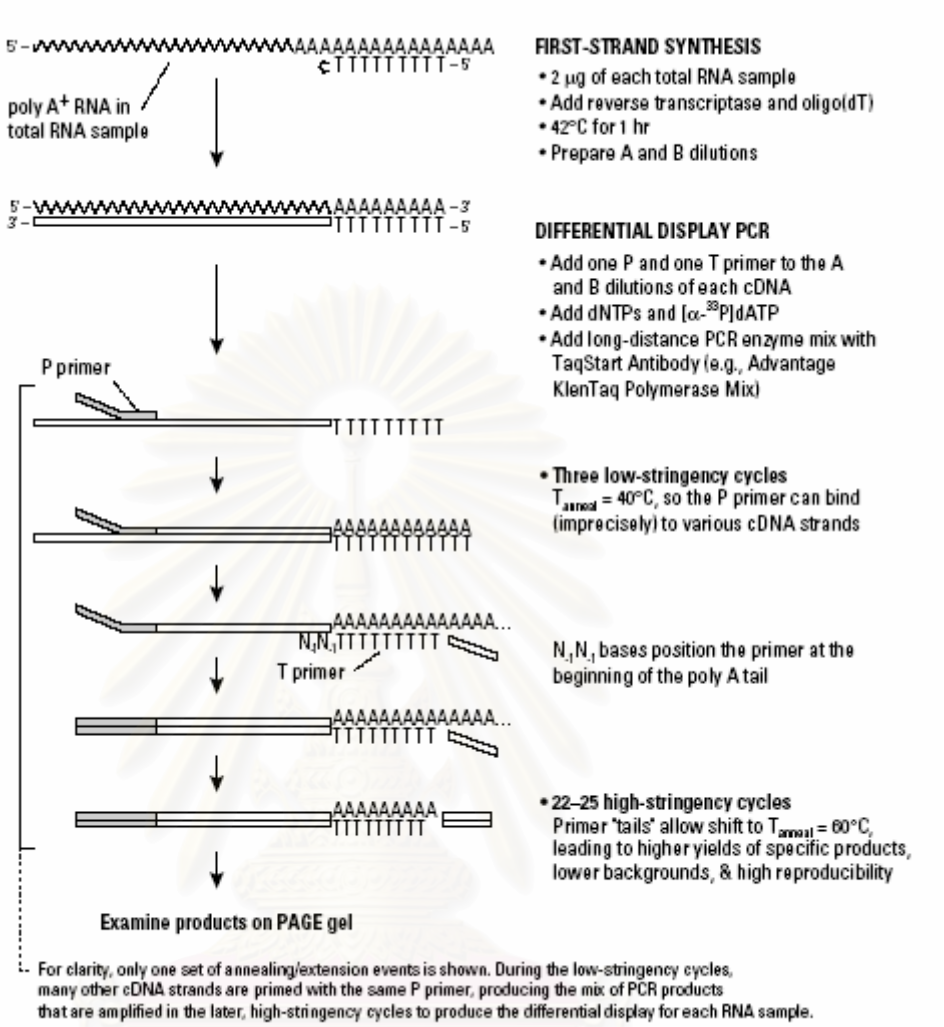


Figure 1.8 Detailed flow chart of the cDNA synthesis and PCR in the Differential Display protocol. This figure shows differential display PCR using one P primer and one T primer.

1.10 Real-time reverse transcription (RT) PCR

Reverse transcription-PCR (RT-PCR) is a method for studying gene expression and comparing levels of gene expression in different specimens especially when amounts of RNA are limitted. However, RT-PCR is just qualitative end-point analysis method which lacks precision for quantitative comparisons of gene expression. Real-time RT-PCR is currently the most sensitive method for the detection of low-abundance transcripts. Limitation of classical endpoint RT-PCR could be overcome by the kinetic or real-time RT-PCR assay. The truly linear part of the amplification can be easily observed with the developed fluorescence based PCR technique together with the specialized detector and software. Real-time RT-PCR can be used to compare and quantitate expression of selected genes in different biological samples. It is remarkably useful for confirming differential expression of candidate genes identified by other techniques, for example; DD-PCR, cDNA microarray, suppression-substractive hybridization (Astrofsky et al., 2002; de Lorgeril et al., 2005).

The amplified products can be detected by fluorescent dyes that are specific for double-stranded DNA (dsDNA) or by sequence-specific fluorescent oligonucleotide probes. The first dye used for this purposes was ethidium bromide. A dsDNA-specific dye frequently used in real-time PCR today is SYBR Green I. It is an asymmetric cyanine dye which binds sequence independently to the minor groove of dsDNA. The binding affinity is more than 100 times higher than that of ethidium bromide. It is suitable for monitoring accumulation of the product during PCR , without a separate assay to detect this product, because the fluorescence of the bound dye is more than 1,000 fold higher than that of free dye. However, the major drawback of SYBR Green is that it binds to nonspecific products like primer dimers and the desired PCR product with equally affinity. The fluorescence emitted from total dsDNA, both the desired amplicons as well as primer dimers, reveals interference of primer dimers to the target gene.

Quantitation of mRNA transcriptions by real-time RT-PCR can be either absolute using the standard curve method or relative using the comparative threshold method. In the standard curve method, a sample of known concentration is used to construct a standard curve. Since the cycle at which PCR enters log linear

amplification is directly proportional to the amont of starting template, one determines the concentration of an unknown sample by comparing it to such standard curve. Absolute quantification should be performed when determination of the absolute transcript copy number is required.

The comparative threshold method was used to report the changes in expression of interested genes relative to reference gene in a given treatment. Pfaffl (2001) reports a mathematical model for relative quantification determined from real-time PCR experiments without calibration curve. Expression of target genes was normalized to reference genes of housekeeping genes like glyceraldehyde-3-phosphate dehydrogenase (G3PDH or GAPDH), actins, tubulins, cyclophilin, 18S rRNA or 28S rRNA. In shrimp, elongation factor 1-alpha gene (EF 1α) was identified as constitutively expressed gene and was used as reference gene for real-time RT-PCR analysis (Astrofsky et al., 2002). The relative expression ratio (R) of a target gene is calculated as in equation **1** based on PCR efficiency (E) and the deviation of crossing points (CP) of an unknown sample versus a control, and expressed in comparison to a reference gene.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control-sample})}} \qquad \textbf{1}$$

E_{target} is the real-time PCR efficiency of target gene trancript;

E_{ref} is the real-time PCR efficiency of reference gene trancript;

$\Delta CP_{\text{target}}$ is the CP deviation of control-sample of the target gene transcript;

ΔCP_{ref} is the CP deviation of control-sample of the reference gene transcript;

In theory, the amount of amplified product will be a doubling of the amount of DNA at each cycle during exponential amplification where in the PCR efficiencies equal to 100%. The actual PCR efficiency of each amplified DNA may have a small different which can make a lot of differnce in the amount of the final product. To determine the PCR efficiency, CP cycles versus cDNA input were plotted to calculate the slope. The corresponding real-time PCR efficiences were calculated, according to $E = 10^{[-1/\text{slope}]}$ (Pfaffl, 2001).

1.11 Eukaryotic expression system

Efforts to elucidate the relationship between protein structure and biological function of interested protein and low abundance of those in the natural source resulted in attempt to produce the recombinant protein. Advances in molecular biology and biochemistry can fulfill this through the use of nonnative eukaryotic systems for the synthesis of recombinant protein that exhibits all the three-dimentional, posttranslational, and functional features of the native protein. The eukaryotic expression systems are able to perform many post-translational modifications including N- and O-linked glycosylation, phosphorylation, acylation, amidation, carboxymethylation, isoprenylation, signal peptide clevage and proteolytic clevage. At present, there are three main expression systems including yeast expression system, insect cell expression system and mammalian cell expression system. The mammalian cell expression system can be effective expression tools, but they can be plagued by difficulties in purifying recombinant proteins (Caputo et al., 1999), limitation on the size of the recombinant protein expressed, and mechanism of protein expression induction. Many of these obstacles have been bridged using expression system from insect and yeast cells. The baculovirus-mediated insect cell expression system is considered to be safe, powerful, but cell-lytic. The yeast hosts that can be used for expression studies include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Hansela polymorpha*, *Kluyveromyces lactis*, and *Yarrowia lipolytica*. The first three are the most widely used (Smith, 1998).

1.11.1 Baculovirus expression system

Baculoviruses belong to a diverse group of large double-stranded DNA virus that infect many different species of insect. Baculovirus strains are highly species specific and are not known to propagate in any non-invertebrate host. The Baculovirus genome is replicated and transcribed in the nuclei of infected host cells where the large Baculovirus DNA is package into rod-shaped nucleocapsids. Since the size of these nucleocapsids is flexible, this enables the expression of large proteins. In this system, baculovirus nonessential gene in the tissue culture life cycle was replaced by heterologous gene. Due to the size of Baculovirus genome is too

large to easily insert foreign genes, the transfer vectors were used for cloning the gene of interest before transferring it to Baculovirus DNA by co-transfection method.

The baculovirus expression system offer a number of advantages, including high expression level, limitless size of the expressed protein, efficient cleavage of signal peptides, post-translational modifications and corrected protein folding. The example of insect hosts of baculovirus were *Drosophila melanogaster, Bombyx mori, Aedes albopictus* (Baculovirus expression manual, Pharmingen).

1.11.2 *Pichia pastoris* expression system

Basically, wild-type yeasts are prototrophic, that is, they are nutritionally self-sufficient, capable of growing on minimal media. Classical genetic studies have created auxotrophic strains which require specific nutritional supplements to grow in minimal media. The nutritional requirements of the auxotrophic strains are the basis for selection of successfully transformed strains. By including a gene in the plasmid expression cassette that complements one or more defective genes in the host auxotroph, one can easily select recombinants on minimal media. Hence strains requiring histidine (HIS 4) will grow on minimal media if they habor a plasmid expressing the HIS4 gene (Smith, 1998).

The facultative methylotrophic yeast, *Pichia pastoris*, has been developed as a system for heterologous eukaryotic gene expression. The eukaryotic yeasts give advantages over alternative eukaryotic and prokaryotic systems. They do not harbor pyrogens, pathogens or viral inclusions. They can secrete proteins and modify them by post-translation modification process such as glycosylation, folding and proteolytic processing. Like the eukaryotic microbial production systems, yeasts combine the ease of genetic manipulation with rapid growth on inexpensive media to high cell densities and also they can be easily monitored and validated of products. It is faster, easier, and less expensive to use than other eukaryotic expression systems and generally gives higher expression levels.

P. pastoris, like other methylotrophic yeast species, uses methanol as a sole carbon source. The first step in metabolizing methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide (Cereghino and Cregg, 2000) by an enzyme alcohol oxidase. Alcohol oxidase has a poor affinity for O_2, and *Pichia pastoris*

compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is used to drive the recombinant protein expression in *Pichia*, so methanol can be used to induce the expression of recombinant protein. *Pichia pastoris* grows on a simple mineral media and does not secrete high amounts of endogenous protein. Therefore the heterologous protein secreted into the culture is relatively pure and purification is easier to accomplish (Faber et al., 1995).

The *P. pastoris* expression system has emerged as a powerful heterologous expression system for high level expression of variety of functionally active compounds. A number of proteins synthesized in *P. pastoris* are being tested for use as human pharmaceuticals in clinical trials as reveiwed by Cereghino and Cregg, 2000. A group of antimicrobial peptides normally toxic to bacteria have been successfully expressed in this system (Almeida et al., 2001; Cabral et al., 2003; Chen et al., 2004; Li et al., 2005).

1.12 *In situ* hybridization

In situ hybridization techniques was used to dectect specific nucleic acid sequences in morphologically preserved chromosomes, cells or tissue sections. Both radioactive and nonradioactive hybridization are available. However, working with nonradioactive system is preferable, probably because it is safer, easier to perform and longer probe-shelf life. There are two types of nonradioactive hybridizaiton methods: direct and indirect. Direct methods using fluorochromes directly coupled to nucleic acid probe so that probe-target hybrids can be visualized under microscope immediately after hybridizaiton. Indirect procedures require the probe to contain a detectable molecule, such as digoxigenin (DIG) and biotin, introduced chemically or enzymatically, that can be detected by specific antibody. For such methods, the presence of the label should not interfere with the hybridization reaction of the stability of the resulting hybrid.

The DIG is naturally extracted from the digitalis plants (Digitalis purpurea and Digitalis lanata). It was linked to the C-5 position of uridine nucleotides. The DIG-labeled uridine nucleotides were incorporated into nucleic acid probes enzymatically with DNA polymerases, RNA polymerases, and Terminal Transferase. Hybridized DIG-labeled probes may be detected with high affinity anti-digoxigenin (anti-DIG)

antibodies that are conjugated to alkaline phosphatase, peroxidase, fluorescein, rhodamine, AMCA, or colloidal gold because the anti-DIG antibody does not bind to other biological material. For unconjugated anti-DIG antibodies, detection by conguated secondary antibodies may be used instead. Detection sensitivity depends on the method used to visualize the conjugate. In this study, an anti-DIG antibody conjugated to alkaline phosphatase is visualized with colorimetric (NBT and BCIP) substrates. The diagram of *in situ* hybridization techniques was shown in Figure 1.9.

In order to detect the interested transcripts in the cells, RNA probes are generated by *in vitro* transcription from a linearized plasmids containing the template and a promoter for RNA polymerases. SP6, T3, or T7 RNA polymerase is commonly used to synthesize run-off transcripts which complementary to the DNA template. The sequence of probes begin from the promoter site to the restriction site used for linearization. The RNA probes are single-stranded and about 10 µg of probe were produced from about 1 µg of DNA template. *In situ* hybridization was widely applied for localization of many genes encoded for antimicrobial peptides (Kang et al., 2004; Munoz et al., 2002; Tanabe et al., 2004; Uzzell et al., 2003).

1.13 Immunohistochemistry or immunocytochemistry

The purpose of an immunohistochemistry technique is to determine if a particular protein is present in cells or tissues by means of antigen-antibody interactions. The expressed protein can be detected by direct labelling of the antibody, or by the use of a secondary method viewed under a microscope. The specificity of the results does not only depends on the method used but also the specificity of the antibody. The antibody specificity is best determined by immunoblot and or immunoprecipitation. Preabsorption controls, in which the antibody is mixed with the protein or peptide used to generate the antibody, are not always reliable in determining the specificity of the antibody for the protein in the tissue in immunohistochemistry (Burry, 2000).

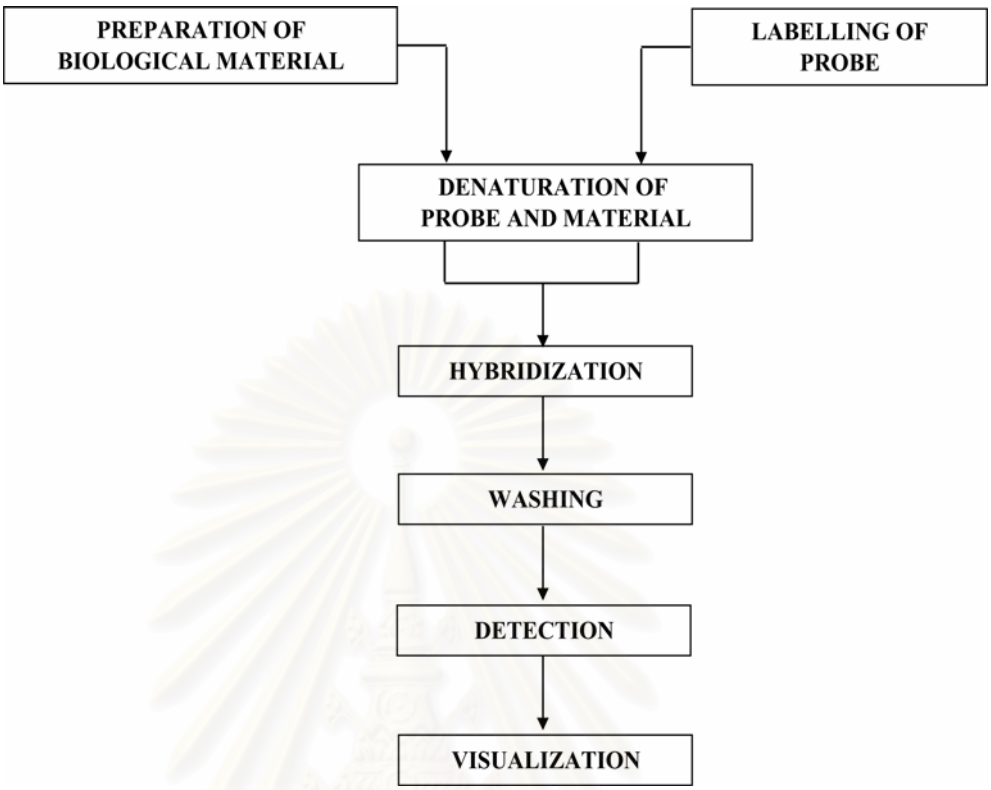


Figure 1.9 The diagram of *in situ* hybridization techniques.

Specificity of the immunocytochimical method is validated by negative controls to show that the labeling is specifically due to the primary antibody, in this case, the primary antibody was replaced with normal serum. The positive control is to use the sections of cells known to contain the protein for labeling or to use the experimental sections with several different antibodies prepared to the same protein to show that the same structure is labeled (Burry, 2000).

1.14 Objective of the thesis

Knowledge of shrimp immunity is needed for effective prevention and control of diseases. In this thesis, we aim to identify immune effectors that are responded to bacterial invasion in the haemocyte of normal and *V. harveyi* infected *P. monodon* by DD-PCR techniques. Additionally, an antimicrobial peptide ALF*Pm*3 which was strongly responded to *V. harveyi* infection was characterized. The cDNA homologue of ALF*Pm*3 was cloned and expressed in *P. pastoris* and purified to homogeneity. The purified recombinant ALF*Pm*3 (rALF*Pm*3) was further characterized for its antimicrobial activity and subjected to produce a specific polyclonol antibody used for detection of ALF*Pm*3 protein *in vivo*. Analysis of the ALF*Pm*3 expression level in shrimp haemocytes after injection of Gram-negative bacteria, *V. harveyi* was performed by *in situ* hybridization and real-time RT-PCR.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave Model # LS-2D (Rex all industries Co. Ltd.)

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

Balance: Satorius 1702 (Scientific Promotion Co.)

Cyto-centrifuge (cyto-tex centrifuge, Miles Scientific)

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Revco)

Gel dryer (Bio-RAD)

Gene Pulser (Bio-RAD)

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

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

High performance liquid chromatrography system (Waters)

- Waters 2996 Photodiode Array Detector
- Waters 616 Pump
- Waters 600S Controller

Hoefer SQ3 sequencer (Amersham Pharmacia)

Hybridization oven (Hybrid, USA)

iCycler iQ™ Real-Time Detection system (Bio-Rad)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

Laminar flow: Dwyer Mark II Model # 25 (Dwyer instruments)

CX31 Biological Microscope (Olympus)

Light microscope (Leica)

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

Minicentrifuge (Costar)

Nipro disposable syringe (Nissho)

Orbital Shaker (Gallenkamp)

Medical X-ray film (Kodax, USA)

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-l (Pharmacia)

pH meter Model # SA720 (Orion)

Pipette boy ACU (Integra biosciences)

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

Poly-L-lysine coated slide (O.Kindler GmbH&Co)

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

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

Refrigerated centrifuge Model # J2-21 (Beckman)

Refrigerated micro centrifuge Kubota 1300 (Kubota)

Rocker platform (Belleo)

Sep-Pak® Accell™ Plus CM catridges (Waters Associates)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

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

Stirring hot plate (Fisher Scientific)

SuperPac Sephasil C_8 column (250 × 4 mm, 5 µm) (Pharmacia)

Touch mixer Model # 232 (Fisher Scientific, USA)

Trans-Blot® SD (Bio-RAD Laboratories)

Transilluminator 2011 Macrovue (LKB)

Vacuum pump (Bio-RAD Laboratories)

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

2.1.2 Chemicals and reagents

Anti-Digoxigenin-AP Fab fragment (Roche, Germany)

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acetic anhydride (Sigma)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

Ammonium persulfate, $(NH_4)_2S_20_8$ (USB)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

BCIP(5-bromo-4-chloro-indolyl phosphate) (Roche, Germany)

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)

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

Diaminobenzidine (Sigma)

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

Ethidium bromide (Sigma)

Ethylene diamine tetraacetic acid (EDTA), disodium salt dihydrate (Fluka) Ficoll^{TM} - 400 (Amersham)

Fetal bovine serum (Gibco BRL)

Formaldehyde, CH_2O (BDH)

GeneRuler™ 100bp DNA Ladder

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine NH_2CH_2COOH (Scharlau)

Grace insect medium (Gibco BRL)

Hybond-P PVDF membrane (Amersham)

Hydrochloric acid, HCl (Merck)

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

Isopropanol, C_3H_7OH (Merck)

Lamda DNA/*Eco*RI+*Hin*dIII marker (Promega)

Levamisol (Sigma)

Methanol, CH_3OH (Merck)

2-mercaptoethanol, C_2H_6OS (Fluka)

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

0.22 μm millipore membrane filter (Millipore)

3-(N-morpholino)propanesulfonic acid (MOPS), $HO_3S(CH_2)_3(C_4H_8NO)$ (USB)

Nitroblue tetrazolium (NBT) (Roche, Germany)

NBT (nitroblue tetrazolium)/BCIP(5-bromo-4-chloro-indolyl phosphate) tablet (Sigma)

Neutral red (Fluka)

Nytrans® super charge nylon membrane (Schleicher & Schuell)

Phenol:chloroform:isoamyl alcohol (Sigma)

Potato dextrose broth (Difco)

Prestained protein molecular weight marker (Fermentus)

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, NaHCO3 (BDH)

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

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

Sodium hydroxide, NaOH (Eka Nobel)

RNA markers (Promega)

IQ™ SYBR® supermix (Bio-Rad)

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

TRIreagent (Molecular biology)

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

Triton® X-100 (Merck, Germany)

Tryptic soy broth (Difco)

Tween™-20 (Fluka)

Whatman 3 MM™ filter paper (Whatman)

2.1.3 Enzymes

Advantage KlenTaq Polymerase (Clontech)

DNA polymerase, Large (Klenow) fragment (Biolabs, UK)

DyNazyme II DNA™ polymerase (Finnzymes, Finland)

Eco RI (Biolabs, UK)

ISIS™ DNA polymerase (Qbiogene)

Not I (Biolabs, UK)

Pme I (Biolabs, UK)

Proteinase K (Sigma)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

SnaB I (Biolabs, UK)

T3 RNA polymerase (Roche, Germany)

T4 DNA ligase (Biolabs, UK)

Xba I (Biolabs, UK)

Xho I (Biolabs, UK)

2.1.4 Microorganisms

Escherichia coli strain JM 109

E. coli strain XL-I blue

E. coli strain 363

Salmonella thyphimurium

Klebsiella pneumoniae

Enterobacter cloacae

Erwinia carotovora

Aerococcus viridans

Micrococcus luteus

Bacillus megaterium

Enterococcus faecalis

Staphylococcus aureus
Staphylococcus haemolyticus
Vibrio harveyi 1526
V. harveyi
V. alginolyticus
V. penaeicida
V. anguillarum
Botrytis cinerea
Penicillium crustosum
Fusarium oxysporum

2.1.5 Kits

DIG RNA Labeling Kit (SP6/T7) (Roche, Germany)
Delta™ Differentia Display Kit (Clontech, USA)
Mini Quick Spin™ RNA column (Roche, Germany)
QIAquick™ Gel Extraction Kit (Qiagen, Germany)
QIAquick™ PCR Purification Kit (Qiagen, Germany)
SilverXpress® Silver Staining Kit (Invitrogen)
pGem®-T Easy vector system I (Promega)

2.1.6 Radioactive

[α-^{33}P]dATP* (1000–3000 Ci/mmole) (Amersham Biosciences Biotech, England)

2.2 Samples

Sub-adult *P. monodon* (approximately 3 month-old, 20 g of body weight) were perchased from local farms and seperated into 3 groups. The first group was the unchallenged or normal shrimp. The second group was *P. monodon* experimentally infected with 0.85% (w/v) NaCl. The last group was *P. monodon* infected with *V. harveyi* 1526. All groups were acclimatized in aquaria at the ambient temperature (28± 4°C) and at the salinity of 15 ppt for at least 1 day before used in the experiments.

2.3 Preparation of *Vibrio harveyi* infected shrimp (Supungul, 2002)

A single colony of *V. harveyi* 1526 (kindly provided by Charoenpokaphand Group of Companies) was inoculated in the tryptic soy broth (TSB) supplemented with 2%(w/v) NaCl at 30°C for 12-16 hours, The overnight culture was diluted (1:100) in the same medium and grown at 30°C for 2 hours. The culture was then diluted 1:100 with a sterile 0.85%(w/v) NaCl. The titer of this dilution was monitered by a plate count method in tryptic soy agar (TSA) supplemented with 2%(w/v)NaCl (modified from Austin, 1988). The 10^6 CFU diluted culture was intramuscularly injected into the forth abdominal segment, whereas the control group was injected with 100 μl of 0.85% (w/v) NaCl solution.

At 0, 6, 24, 40, 48, 72 hours post-injection, haemolymph was collected and shrimps were tested whether the infection was successful by culture the suspensions of hepatopancreas on TSA plates supplemented with 2% (w/v) NaCl and incubated at 30°C overnight. Colonies of *V. harveyi* 1526 from infected shrimps showed strong luminescence in the dark.

2.4 Haemocyte collection and total RNA preparation

Haemolymph was collected from the ventral sinus of each shrimp using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe pre-loaded with 200 μl of anti-coagulant (10% sodium citrate, w/v). Haemolymph was immediately centrifuged at 800xg for 10 minutes at 4°C to separate haemocytes from the plasma. The haemocyte pellet was resuspended in 1 ml of TRIreagent (Molecular biology) and briefly homogenized. The homogenate was stored at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. After that 200 μl of chloroform was added and vigorously shaken for 15 seconds. The resulting mixture was stored at room temperature for 2-5 minutes and centrifuged at 12,000xg for 15 minutes at 4°C. The colorless upper aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube. RNA was precipitated by the addition of 500 μl of isopropanol. The mixture was left at room temperature for 5-10 minutes and centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was removed. The RNA pellet was washed with 500 ml of 75% (v/v) ethanol. The RNA pellet was kept under 75% (v/v)

ethanol until used. When required, the samples were centrifuged at 12,000xg for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was briefly air-dried for 5-10 minutes. The total RNA was dissolved with an appropriate amount of diethy pyrocarbonate (DEPC)-treated water.

The concentration of total RNA was determined by measuring the OD at 260 nm and estimated in μg/ml using the following equation,

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

*A 1 OD unit at 260 nm corresponds to approximately 40 μg/ml of RNA (Sambrook et al., 1989)

2.5 Formaldehyde-agarose gel electrophoresis

A 1.0% (w/v) formaldehyde agarose gel was prepared using 1x MOPS buffer (diluted from a 10x MOPS buffer to 0.2 mM MOPS, 50mM NaOAc, 10 mM EDTA, pH 7.0 final concentration). The gel slurry was boiled until complete solubilization, and allowed to cool to 60°C. Formaldehyde (0.66M final concentration) and ethidium bromide (0.2 μg) were added to the gel and poured into a chamber set. The comb was inserted.

Ten micrograms of total RNA in 3.5 μl of DEPC-treated H_2O, 5 μl of formamide, 1.5 μl of 10x MOPS and 2 μl of formaldehyde were combined, mixed well and incubated at 65°C for 15 minutes. The mixture was immediately placed on ice. One-forth volume of the gel-loading buffer (50%, v/v glycerol; 1mM EDTA, pH 8.0, 0.5%, w/v bromphenol blue) was added to each sample. The sample was loaded to the 1.0% agarose gel containing formaldehyde and ethidium bromide. The RNA marker was used as a standard RNA marker. Electrophoresis was carried out in 1x MOPS buffer at 50 volts, until bromphenol blue migrated approximately ¾ of the gel length. The EtBr stained gel was visualized total RNA as fluorescent bands by a UV transilluminator (UVP Inc.).

2.6 DNase treatment of total RNA samples

Chromosomal DNA contamination in total RNA samples was removed by treating 25 μg of total RNA with 5 units of RQ1 RNase-free DNase (Promega) at

37°C for 1 hour. After the reaction volume was adjusted to 40 µl with DEPC-treated water, 250 µl of TRIreagent were added and vortexed for 5 seconds. Two hundred microliters of chloroform was then added and vigorously shaken for 15 seconds. The resulting mixture was stored at room temperature for 2-5 minutes and centrifuged at 12,000xg for 15 minutes at 4°C. The top layer was added to 1 volume of isopropanol and incubated for 10 minutes at room temperature. The resulting mixture was centrifuged at 12,000xg for 15 minutes at 4°C. The supernatant was removed. The RNA pellet was washed with 500 µl of 75% (v/v) ethanol. The supernatant was removed. The RNA pellet was briefly air-dried for 5-10 minutes. The DNase-treated total RNA was dissolved with an appropriate amount of diethy pyrocarbonate (DEPC)-treated water. The concentration of DNA-free total RNA was determined as described in 2.4.

2.7 Differential Display PCR

2.7.1 Samples

Sub-adult *P. monodon* were infected with 10^6 cfu of *V. harveyi* 1526 as described in 2.3. Haemolymph was collected from the vetral sinus of each infected and uninfected shrimp using 10% sodium citrate as an anti-coagutant at 40 hours after injection. Haemocytes were prepared by centrifugation of the haemolymph and used to prepare total RNA as described in 2.4.

2.7.2 First-stranded cDNAs synthesis

The first-stranded cDNAs were generated according to Delta® Differential Display Kit (Clontech) protocol. Two micrograms of DNA-free total RNA sample and 0.1 µM of oligo (dT) primers were mixed and then added sterile H_2O to a final volume of 5 µl. The reaction was incubated at 70°C for 3 minutes and immediately placed on ice for 2 minutes. After that, 2 µl of 5x reaction buffer, 2 µl of dNTP mix (5 mM each (dATP, dCTP, dTTP, dGTP), 1 µl of MMLV Reverse Transcriptase (200 units/ µl) were added and gently mixed. The reaction was incubated at 42°C for 60 minutes. Then, the reaction was incubated at 75°C for 10 minutes to terminate reverse

transcriptase activity. The cDNA was then diluted with sterile water to 1: 10 and 1:30 dilutions and used as template for differential display PCR (DD-PCR) reaction. All cDNA dilutions were stored at –20°C until ready for use.

2.7.3 Differential Display PCR

DD-PCR was performed using Delta® Differential Display Kit (Clontech). Forty-four combinations of primers, one of nine oligo(dT) T primers and one of 10 arbitrary P primers provided in the kit, were used for DD-PCR reactions. The sequences of PCR primers for DD-PCR were listed in Table 2.1. For each experiment display, 1 μl of each diluted cDNA sample, 1 μl of 20 μM P primer, and 1 μl of 20 μM T primer were mixed well with a 17 μl of master mix containing 2 μl of 10X KlenTaq PCR reaction buffer, 14.2 μl of Sterile H_2O, 0.2 μl of dNTP mix (5 mM each); final concentration 50 μM), 0.2 μl of [α-^{33}P]dATP* (1000–3000 Ci/mmole; 3.3μM; final concentration 50 nM) (Amersham Biosciences), 0.4 μl 50X Advantage KlenTaq Polymerase Mix. One drop of mineral oil was added on top of the PCR mixture and tightly capped the tubes. For each pair of primers, an H_2O control was prepared by using 1μl of H_2O instead of cDNA. The thermal cycling was performed on Perkin-Elmer GeneAmp PCR Systems 2400.

The cycling parameters were one cycle at 94°C for 5 min, 40°C for 5 min, 68°C for 5 min, two cycles at 94°C for 2 min, 40°C for 5 min, 68°C for 5 min, 25 cycles at 94°C for 1 min, 58°C for 1 min, 68°C for 2 min and a final extension at 68°C for 7 min. The differential display reactions were stored at –20°C.

2.7.4 Electrophoresis and autoradiography

A denaturing 6% polyacrylamide/8 M urea gel in 1X TBE buffer was poured onto preset glass plates with 0.4-mm spacers for 0.4-mm thick gels. The gel was prerun at 33 mA (constant current) for at least 20 min. For each reaction, combine 5 μl of the PCR mixture with 5 μl of loading buffer. A molecular weight marker, Lamda DNA/*Eco*RI+*Hin*dIII marker (Promega), was labeled with [α-^{33}P] dATP by end-repairing with a Klenow polymerase. All samples and a molecular weight marker

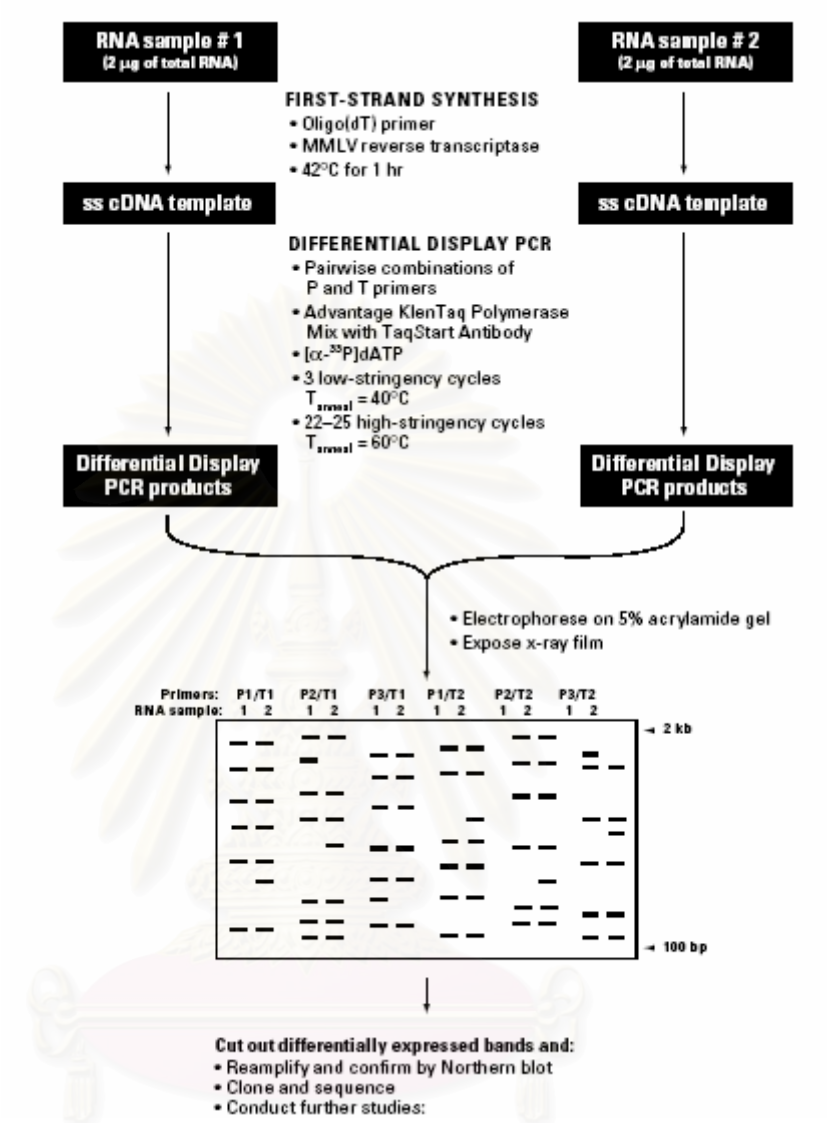


Figure 2.1 Overview of the Delta Differential Display protocol. First-strand cDNA were synthesized from total RNA and subjected to differential display PCR combinations of P and T primers. DD-PCR products were analyzed by polyacrylamide gel electrophoresis and autoradiography.

Table 2.1 Differential display primers[a] ; Oligo(dT) (T) primers and Arbitrary (P) primers

P1	5'-ATTAACCCTCACTAAATGCTGGGGA-3'
P2	5'-ATTAACCCTCACTAAATCGGTCATAG-3'
P3	5'-ATTAACCCTCACTAAATGCTGGTGG-3'
P4	5'-ATTAACCCTCACTAAATGCTGGTAG-3'
P5	5'-ATTAACCCTCACTAAAGATCTGACTG-3'
P6	5'-ATTAACCCTCACTAAATGCTGGGTG-3'
P7	5'-ATTAACCCTCACTAAATGCTGTATG-3'
P8	5'-ATTAACCCTCACTAAATGGAGCTGG-3'
P9	5'-ATTAACCCTCACTAAATGTGGCAGG-3'
P10	5'-ATTAACCCTCACTAAAGCACCGTCC-3'
T1	5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'
T2	5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'
T3	5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'
T4	5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'
T5	5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'
T6	5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'
T7	5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'
T8	5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'
T9	5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

[a] Obtained from Delta™ Differential Display Kit (Clonetech)

were denatured by incubating at 94°C for 2 min and immediately placed on ice. The wells of the gel were rinsed prior to loading of 2 μl samples.

Each H_2O control for the same primer combination was loaded adjacent to the corresponding set of displays. Electrophorese at 70W (constant power) for 4 hours. The electrophoresis apparatus were dismantled. The glass plates were allowed to cool down to room temperature before attempting to separate the glass from the gel. After removing the top notched plate, the gel was transferred to Whatman paper. Finally, the plastic wrap was placed carefully over the gel. The gel was dried under vacuum at 80°C for 1 hour. The gel was exposed to Medical X-ray film (Kodak) at –80°C overnight with an intensifying screen.

2.7.5 Identification of differentially expressed bands

DD-PCR patterns with visually different signal intensities between normal and infected shrimps were compared for each primer combination. To isolate differential expressed cDNA fragments, the autoradiograph was aligned on top of the dried gel (on Whatman paper). Tape was used for keeping the autoradiograph firmly and precisely aligned on top of the dried gel during the following steps. A sharp pin was used to mark the differentially expressed bands by poking holes through the film and the gel beneath. The regions of dried gels corresponding to the cDNAs were excised using a clean, sharp razor. Each fragment was placed in a separate 0.5-ml tube. Forty microliters of sterile distilled water were added to each tube and overlaid with 2–3 drops of mineral oil. cDNA fragment was extracted from the gels by incubating at 100°C for 10 min. The Whatman paper was carefully removed and discarded in the appropriate radioactive waste container.

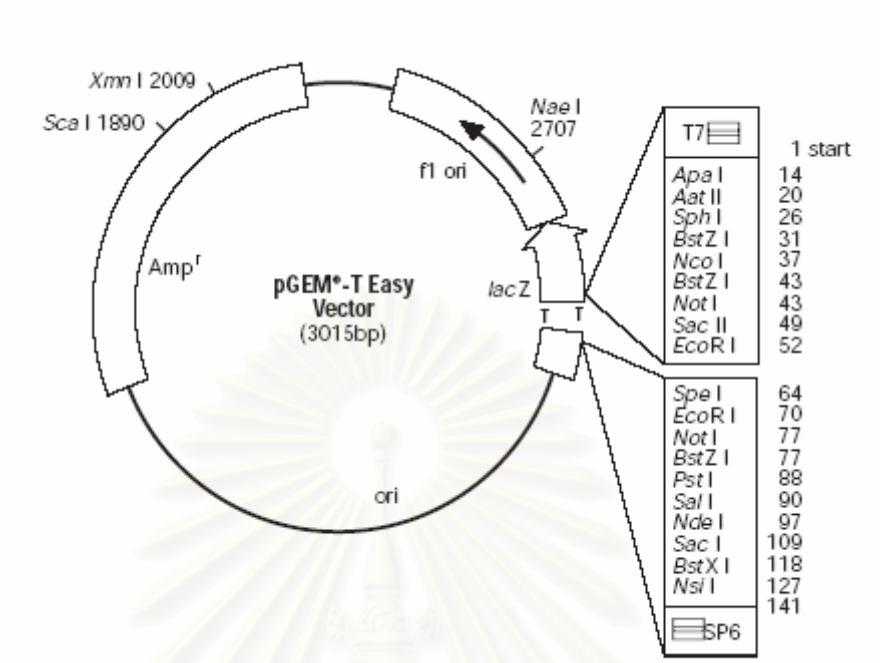
2.7.6 Reamplification of the band of interest

The eluted cDNA was reamplified with the identical pair of primers and condition used in DD-PCR reaction. The PCR reaction mix contained 7 μl of eluted cDNA, 5 μl of 10X PCR buffer, 0.5 μl of 5 mM dNTP, 2.5 μl of 20 μM P primer, 2.5 μl of 20 μM T primer, 1 μl of 50X polymerase mixture, 31.5 μl of sterile H_2O.

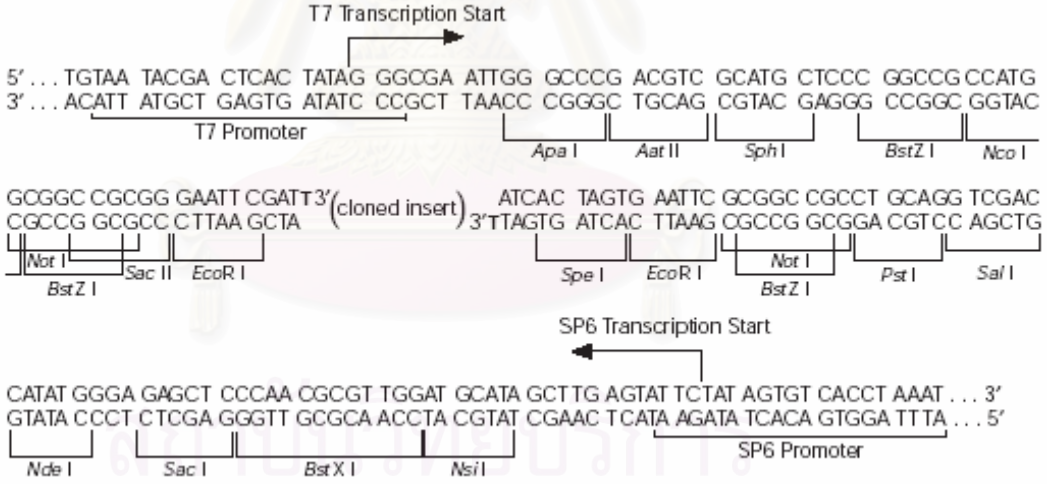
Amplified cDNA was electrophoresed on 1-2% agarose gel in 1x TBE buffer. The gel was stained with EtBr and determined the sized of product by visualization under UV light. The expected product was purified using QIAquick gel extraction kit (QIAGEN). The expected product was excised from the gel using a clean sharp scalpel and then determined the weight of the gel slice. Three volumes of buffer QG were added to 1 volume of gel and incubated at 50°C for 10 minutes or until the gel slice was completely dissolved. In case of 2% agarose gel, 6 volume of buffer QG was used. Afterthat, the clear solution was applied to the QIAquick column and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded. In order to remove all traces of agarose, the 0.5 ml of buffer QG was added to the column and centrifuged at 13,000 rpm for 1 minute. The column was washed by adding 0.75 ml of buffer PE and then centrifuged at 13,000 rpm for 1 minute after incubation for 2-5 minutes. The flow-through was discarded and the column was dried by centrifugation at 13,000 rpm for 1 minute. The column was transferred to a clean microcentrifuge tube. The DNA was eluted by appling 30 μl of buffer EB to the centered of membrane within the column. The column was then centrifuged at 13,000 rpm for 1 minute. The eluted DNA was kept at -20°C until used.

2.7.7 Preparation of competent cells

A single colony of *E. coli* XL-1 blue was cultured used as the starter inoculums in 10 ml of LB broth [1 % (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1% (w/v) NaCl] and incubated at 37°C with shaking at 250 rpm overnight. One percent of the microbial starter was inoculated into 1000 ml of LB broth [1 % (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, and 1 % (w/v) NaCl] and the culture was incubated 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 much 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



a)



b)

Figure 2.2 The circular map of the pGEM®-T Easy vector showing sequence reference points(a) and the linear map showing promotor and multiple cloning sites (b).

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 are good for at least 6 months under these conditions.

2.7.8 Cloning of differential display cDNA fragment

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

To calculate the appropriate amount of insert to include in the ligation reaction, use the following equation.

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

Two microliters of each ligation reaction were added to 40 µl of E. coli strain XL-I blue competent cells. The reaction was mixed and placed on ice for 1 minute. The reaction mixture were transferred to a cold elctroporation cuvette. The apparatus was set as followed; 25 F of the Gene pulser, 200 Ω of the Pulse controller unit, and 2.50 kV of the Gene pulser apparatus (Bio-RAD). After one pulse was applied at the above setting, one milliliter of SOC medium [2 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgS04, and 20 mM glucose] was added to the cuvette and quickly resuspended the cells with a pasture pipette. The cell suspension was transferred to a sterile test tube and incubated at 37°C with shaking at 250 rpm for 1 hour. One hundred microliters of each transformation was then plated onto a LB/ampicillin/IPTG/X-Gal plate.

2.7.9 Determination of insert sizes by colony PCR

Four to ten of recombinant clones were randomly selected for each cDNA and screened by colony PCR The pGEM-T Easy vector was 3,015 bp in length (Figure 2.2). This plasmid had unique restriction sites in the multiple cloning region flanked by T3 and SP6 RNA promoters, therefore T7 and SP6 primers can be used to analyzed insert sizes. These primer sets annealed at T7 and SP6 RNA promoter and amplified the insert cDNA. Colony PCR was performed in a 25 µl reaction volume containing 2.5µl of 2.5 mM of dNTP mix, 0.5 µl of each 50 µM T7 and SP6 primers, 2 units of DyNAzyme™ II DNA polymerase. For the DNA template, the single colony was picked using a sterile toothpick and resuspended in the reaction mixture. The cycling parameters were one cycle at 96°C for 2 minutes, 30 cycles at 96°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute and a final extension at 72°C for 5 min. The PCR products were analyzed by 1.2 % agarose gel electrophoresis. The clone containing an expected size of insert was selected and the recombinant plasmid was isolated.

2.7.10 Preparation of recombinant plasmid

The colonies were inoculated into 1.5 ml of low salt-LB broth (one colony per tube) and incubated at 37°C with shaking 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-HC1, 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. Two hundred microlitres 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 10,000 rpm for 10 minutes to separate cell debris. The supernatant was transferred into a new microcentrifuge tube, added with an equal volume of phenol:chloroform:isoamyl alcohol (25 : 24 : 1), mixed and spun at 10,000 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 10,000 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-HC1,pH 8.0 and 1mM EDTA, pH 8.0).

2.7.11 DNA sequencing and data analysis

The recombinant plasmid of each differentially expressed cDNA clone was identified by sequencing. DNA sequencing was carried out using a ThermoSequenase Fluorescent Labelled Primer Sequencing kit (Amersham Pharmacia Biotech) with M13 forward and reverse primers on an automated DNA sequencer LC4000 (Licor, Lincoln, Neb). Three microlitres (0.5-5µg) of plasmid DNA and two pmol of M13 fluorescent labeled primer were added into each 4 tubes; 2 µl of A-reagent (Tris-HCl, pH9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddATP, thermostable pyrophosphatase and ThermoSequence DNA polymerase), 2 µl of G-reagent (Tris-HCl, pH9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddGTP, thermostable pyrophosphatase and ThermoSequence DNA polymerase), 2 µl of C-reagent (Tris-HCl, pH9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddCTP, thermostable pyrophosphatase and ThermoSequence DNA polymerase), and 2 µl of T-reagent (Tris-HCl, pH9.5, $MgCl_2$, Tween 20, Nonidet P-40, 2-mercaptoethanol, dATP, dCTP, 7-deaza-dGTP, dTTP, ddTTP, thermostable pyrophosphatase and ThermoSequence DNA polymerase).

Each reaction was overlaid with light mineral oil and was amplified in a thermal cycling using a 96°C, 2 minutes initial denaturation followed by a 96°C, 30 seconds denaturation, a 60°C, 30 second annealing temperature, a 72°C, 1 minute extension for 25 cycles, and a 72°C, 5 minutes final extension. After amplification was complete, each A, C, G, and T-reaction was added with 5 µl of formamide loading dye (formamide, EDTA and funchsin) and 2.5 µl of each reaction were loaded onto 6% denaturing polyacrylamide gel (500 ml of gel mixture prepared from 250 g urea, 75 ml 40% acrylamide stock solution, 4 g N, N methylenebisacrylamide and 187.5 ml deionized water) and were electrophoresed on an automated DNA sequencer

(LC4000, LICOR) with 1x TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3) at 50 w for 8-16 hours.

Sequences of cDNAs were edited and compared with the sequence in the nucleotide sequence database of the GenBank (the National Center for Biotechnology Information; NCBI) using BLASTN and BLASTX programs (NCBI Advanced Blast Search, http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997). Significant probabilities and numbers of matched nucleotides/proteins are considered when E-values less than 10-4 and a match more than 100 nucleotides for BLASTN and a match more than 10 amino acid residues for BLASTX, respectively.

2.8 Quantitative real-time RT-PCR

The mRNA expression of the some differentially expressed genes identified by DD-PCR including serpin, profilin, lysozyme, interferon-related developmental regulator 1(IRDR), caspase 3B and glucose Transporter I, were confirmed by real-time RT-PCR.

2.8.1 Total RNA isolation and DNase treatment

Total RNA samples was extracted individually from haemocytes of *V. harveyi*- and saline-injected *P. mondon* at time 0, 6, 24, and 48 hour post-injection as described in section 2.4. At each time point, the total RNA from 10 individuals were pooled and subsequently treated with RQ1 RNase-free DNase (Promega) to remove genomic DNA contaminant as mention above. After purified the DNA-free total RNA by phenol chloroform extraction, DNA-free total RNA from each time point was subjected to first strand cDNA synthesis reaction.

2.8.2 First-stranded cDNA synthesis

The first-stranded cDNAs were generated from 1 μg of DNA-free total RNA sample and 0.5 μg of oligo (dT_{18}) primers and ImProm-II^{TM} Reverse Transcriptase System kit (Promega). The reaction was incubated at 70°C for 5 minutes and immediately placed on ice for 5 minutes. After that, 4 μl of 5x reaction buffer, 3.8 μl of 25 mM $MgCl_2$, 1 μl of dNTP mix (10 mM each), 20 units of Ribonuclease inhibitor

and 1 µl of ImProm-II™ Reverse Transcriptase were added and gently mixed. The total volume of reactions was adjusted to 20 µl with DNase free water. The reaction was incubated at 25°C for 5 minutes and at 42°C for 60 minutes. Then, the reaction was incubated at 70°C for 15 minutes to terminate reverse transcriptase activity. The cDNAs were stored at –20°C unitl use.

2.8.3 Real-time RT-PCR

The SYBR Green I real-time RT-PCR assay was carried out using the iCycler iQ™ Real-Time Detection system (Bio-Rad). The amplifications were performed in a 96-well plate in a 20 µl reaction volume containing 10 µl of 2X SYBR Green supermix (Bio-rad), the appropriate amount of each forward and reverse primer (The required final concentration of primers shows in Table 2.2), 1 µl of 10 diluted cDNA from each reverse transcription reaction as template and adjusted the reaction volume with sterile water . The thermal profile for SYBR Green real-time RT-PCR was 95°C for 3 minutes followed by 40 cycles of denaturation annealing and extension as indicated in Table 2.2. Fluorescent data were collected at the end of extension step. In a 96-well plate, each sample was conducted in triplicate. Sterile-water replaced template as the negative control. To determine of specificity of PCR amplification of each primer pair a melting curve cycling was done immediately after finishing the amplification. The reactions were incubated at 95°C for 1 minutes and subsequently 50°C for 1 minutes, followed by 80 repeats of heating for 10 seconds starting at 50°C with 0.5°C increments.

2.8.4 Data analysis of real-time RT-PCR

Fluorescence signal was analyzed by the data analysis software of iCycler iQ™ Real-Time Detection system (Bio-Rad) using PCR base line Subtracted curve fit method. For each sample, the cycle number at which the fluorescence crosses the arbitrary line called the threshold. The threshold should be in the line part of the reaction and higher than the background signal to ensure that the reactions cross the

Table 2.2 Primer pairs and condition for real-time PCR

Gene name	Primers	Final primer concentration (μm)	Denaturing (Temp/sec)	Annealing (Temp/sec)	Elongation (Temp/sec)
Caspase3B	Forward: TCTACCTCCATGACCGTTCTTG	150	95/10	56/20	72/10
	Reverse: TCCTGGTGAGCAACAACTGAG				
IFRD1	Forward: GGAAGGTCTGTCGGAGATGG	100	95/10	58/20	72/10
	Reverse: ACGGGTGAGAGGCTTACAAC				
GLUT1	Forward: GCGAAACGACGATGATTGTG	200	95/10	56/20	72/10
	Reverse: TGAAGAAGCGAGCGATGATG				
Lysozyme	Forward: GCTGCTGGTTGGGCTTCTG	100	95/10	58/15	72/10
	Reverse: TGCGGTTGCGGTTGATGG				
Profilin	Forward: GCTGGTGGAATCTGGAAACG	100	95/10	58/20	72/10
	Reverse: GCGGTGTTAGTCTTGGTGATG				
SERPINB3	Forward: CCCTCAAACGCCTCAAAGTC	100	95/10	62/15	72/10
	Reverse: GTCGGTCCACGGTGAAGG				
ALF*Pm*3	Forward: CCCACAGTGCCAGGCTCAA	500	95/10	58/20	72/10
	Reverse: TGCTGGCTTCTCCTCTGATG				
EF-1α	Forward: GGTGCTGGACAAGCTGAAGGC	500	95/10	58/20	72/10
	Reverse: CGTTCCGGTGATCATGTTCTTGATG				

line due to amplification rather than noise. This crossing point, CP, is also known as the threshold cycle or Ct value. The obtained Ct values were used to calculate the relative expression ratio (R).

The relative quantifiction analyses the amount of a target transcript relatively to an internal standard (elongation factor 1-alpha gene (EF 1α)) in the same sample of *V. harveyi* infected shrimp haemocytes. Moreover, the Ct values of *V. harveyi*-injected sample at each time point were normalized with saline-injected samples. A mathematical model described by Pfaffl was used to determine the relative expression ratio according to the equation:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{CP}_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta \text{CP}_{\text{ref}}(\text{control-sample})}} \quad \textbf{1}$$

E_{target} is the real-time PCR efficiency of target gene trancript;

E_{ref} is the real-time PCR efficiency of reference gene trancript;

ΔCP_{target} is the CP deviation of control (saline-injected) - sample (*V. harveyi*-injected) of the target gene transcript;
ΔCP_{ref} is the CP deviation of control (saline-injected) - sample (*V. harveyi*-injected) of the reference gene transcript.

2.8.5 Determination of PCR efficiency

As target and reference gene had different sequences and amplicon lengths, it was probable that they would show different PCR efficiency. PCR efficiency of each gene amplified with specific pair of primers were determined by constucting a standard curve. cDNAs was made from haemocytes of normal *P. monodon* by the same procedure as described above. This cDNA was used for creating all standard curves of both target and reference genes. These standard cDNAs was diluted in five steps from 5 or 10 to 5^5 to 10^5, respectively. The amplification was performed in triplicate including a negative control, used water as template, for each run. Three independent runs were performed in different days to ensure the reproducibility. At the end of each run, data were autometically analyzed by the system and amplification plots and a standard curve were obtained. A calibration curve plotting Ct values against input quantities (log scale) was constructed for both reference (EF-1α) and each target genes. In each plot, a linear graph should have an excellent correlation coefficient (certainly more than 0.990). PCR effieciency was equal to $10^{-1/slope}$. These efficiencies were taken into account in relative quantification.

2.9 Expression of anti-LPS factor type3 in the *Pichia pastoris* expression system

A full-length cDNA clone (sh 71) containing the sequence of the anti-lipolysaccharide factor type (ALF*Pm*3) cDNA, the most abundant isoform of ALF found in *P. monodon*, was isolated from haemocytes of nonstimulated shrimps by Supungul (2002). It was expressed by *Pichia pastoris* methylotrophic yeast system (Invitrogen).

2.9.1 Construction of expression vector pALF*Pm*K

The *Pichia* muti-copy expression vector, pPIC9K (Figure 2.3), was selected for ALF*Pm*3 expression. It carries kanamycin resistance gene of pPIC9K plasmid which confers resistance to G418 in *Pichia*. Spontaneous generation of multiple insertion events, which occur in *Pichia* at a frequency of 1-10%, can be identified by the level of resistance to G418.

Pichia transformants are selected on histidine deficient medium and screened for their level of resistance to G418. An increased level of resistance to G418 indicates multiple copies of the kanamycin resistance gene and of the gene of interest. The pPIC9K vector allows secreted expression of the gene of interest. Increasing the number of copies of the gene of interest in a recombinant *Pichia* strain may increase protein expression levels.

2.9.1.1 Primer design

The forward and reverse primers were designed for constructing an expression cassette. For the convenience of cloning, a SnaBI site was added to 5'-end of forward PCR primers, thereby introducing two additional amino acids (Tyr and Val) to the amino terminus of the ALF protein. Besides, NotI site was added to 3'-end of reverse primers after the stop codon. Primer sequences were:

ALFFwSnaBI : 5'-ATTACATACGTACAAGGGTGGGAGGCTGTG-3'

ALFRVNotI : 5'-AATTATTGCGGCCGCCTATGAGCTGAGCCACTGG-3'

The primers were used to amplify the mature gene in order to clone in-frame into the *Pichia* expression vector pPIC9K and then transform into TOP10 cells (Invitrogen), downstream of the sequence for the α-mating factor signal peptide from *Saccharomyces cerevisiae* and the Glu-Ala-Glu-Ala repeat sequence.

2.9.1.2 Amplification of ALF*Pm*3 gene by PCR

ALF*Pm*3 cDNA was amplified using ISIS^{TM} DNA polymerase (Qbiogene), and PCR profile as follow: heat denature at 94°C for 1 min, 30 cycles of amplification; denature at 94°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 45 sec, and the last additional extension at 72°C for 5 min. The PCR reaction was performed in the total 50 μl reaction, composed of 0.4 μg of plasmid containing ALF*Pm*3 gene, 1X reaction buffer, 2 μM of each forward and reverse

primers, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, and 1 unit of ISIS^{TM} DNA polymerase. After PCR amplification, the expected 324 bp PCR products were purified by phenol/chloroform extraction. The purified PCR products were concentrated by ethanol precipitation with 3 M NaOAc, pH 5.0 and absolute ethanol overnight at -80°C. Pellets of the PCR products were washed with cold 70% ethanol, air dried, and resuspended in 100 µl sterile water.

2.9.1.3 Restriction enzyme digestion of the purified PCR products and expression vector

The purified PCR products and pPIC9K vector were digested with *Sna*BI and *Not*I. First step, 2.5 µg of them was cut at 37°C for 3 hours with *SnaB*I in the reaction of 1X reaction buffer, 1X BSA, and 10 unit of *Sna*BI (New England Biolab). The reaction was stopped by heat the reaction at 80°C for 20 minutes. The equal volume of sterile water was then added to the reaction. In the second step, the reaction from first step was cut overnight with *Not*I at 37°C; 0.7X reaction buffer, 0.7X BSA, and 20 units of *Not*I (New England Biolab). The reaction was stopped by heat the reaction at 60°C for 20 minutes. The completely digested products were purified by QIAquick gel extraction kit (QIAGEN) as describe in 2.7.6.

2.9.1.4 Ligation

The *Sna*BI/*Not*I digested-PCR fragment and -pPIC9K were ligated prior to transform to *Pichia*. The ligation reaction contained 2 µl of 10x ligation buffer, 2 µl of digested PCR product, 8 µl of *Sna*BI/*Not*I digested pPIC9K vector, and 2 µl of T4 ligase (New England Biolab), which molar ratio of insert:vector was 7:1. The total volume was adjusted to 20 µl with sterile water. The ligation reaction was then incubated at 16°C overnight.

2.9.1.5 Transformation into *E. coli*

The ligation reaction was transformed to TOP10 cells (Invitrogen) by heat shock approach. Two microliters of ligated product was mixed with 25 µl of TOP10 competent cells (Invitrogen) and incubated on ice for 20-30 minutes. The mixture was

heated at 42°C for 30 seconds and immediately placed on ice for 2 minutes. The cells were resuspended in 125 µl of SOC medium and incubated at 37°C, 250 rpm, for 1 hour. The cell suspension was spreaded onto LB-amplicillin selected plates and incubated overnight at 37°C. Transformants grown on LB-amplicillin selected plates were picked randomly and cultured in LB-broth with amplicillin overnight.

2.9.1.6 Plasmid preparation

Plasmids were extracted by alkaline lysis method as describe in 2.7.10. Plasmids were cut with *Sna*BI/*Not*I as described in 2.8.1.3 for checking the cloning success. The cut product would be the 324 bps corresponding to ALF*Pm*3 gene insert. All reactions were verified by 1.7 % agarose gel electrophoresis staining with ethidium bromide.

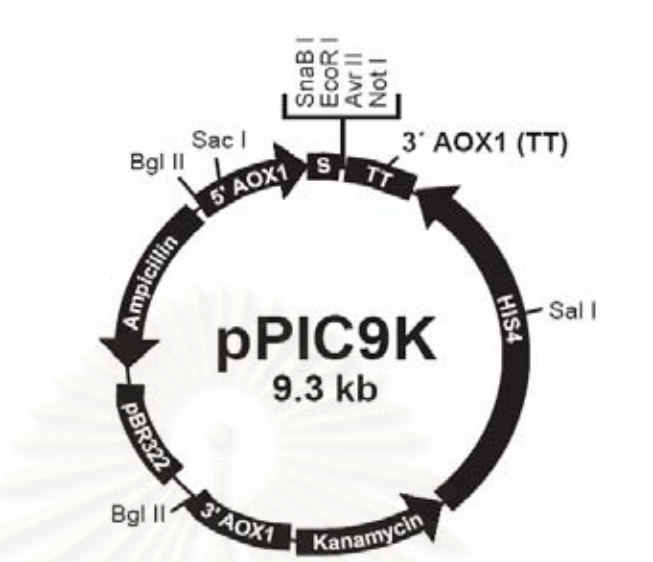
2.9.1.7 Confirmation the sequence of the construct plasmid

Few transformants were selected for sequencing in order to confirm the sequence and orientation of ALF*Pm*3 genes in the expression plasmid. The α-signal peptide and the 3'-AOX primers were used in sequencing reactions.

2.9.2 *Pichia* transformation

2.9.2.1 Preparation of electrocompetent cells

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



a)

b)

Figure 2.3 The circular map (a) and P_{AOX1} and multiple cloning site (b) of pPIC9K vector.

2.9.2.2 Preparation of the transforming DNA

The *Pichia* muti-copy expression vector, pPIC9K, with ALFPm3 gene cloned in the correct orientation for expression was linearized with *Sac*I for insertion at AOX1. When *Pichia* strain KM71 was transformed with the *Sac*I-linearized plasmid, Mut^S recombinants would be generated.

Both the pALF*Pm*K construct and the parent vector were digested by mixing 10 µg of plasmid, 10 units of *Sac*I, 1x reaction buffer, and incubating at 37°C for 12-16 hours. A small portion of the digest was analyzed by 1.0 % agarose gel electrophoresis to confirm complete digestion of the plasmids. The digest was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and the digested DNA was ethanol precipitated. DNA pellet was resuspended in 10 µl of water and stored at -20°C until ready to transform.

2.9.2.3 Transformation to *P. pastoris* by electroporation

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

2.9.2.4 Screening of yeast transformants

To screen transformants for G418 resistance, the transformants grown on MD plates were pooled by adding sterile water over each plate and running sterile spreader across the top of the agar to resuspend them. Pool transformants from all plates and determine cell density using a spectrophotomerter ($1OD_{600}$ = 5 x 10^7 cells/ml). Plate 10^5 cells on YPD plates containing G418 at a final concentration of 0, 0.25, 0.75, 1, 1.5, and 2 mg/ml. Incubate plates at 30°C and check daily. G418

resistant colonies take 2 to 5 days to appear. Resistant clones observed on each YPD-G418 plate were randomly selected. In order to purify putative G418 resistant clones, they were streaked for single colonies on YPD and then confirmed G418 resistance on the same concentration of YPD-G418 plates.

2.9.2.5 Determination of integration of ALF*Pm*3 gene into the *Pichia* genome by PCR

To test *Pichia* clones for insertion of ALF*Pm*3 genes, the first step was to prepare yeast genomic DNA by picking a single colony and resuspend in 10 µl of water. The cells were lysed by adding 5 µl of a 5 U/µl solution of lyticase (Sigma), incubating at 30°C for 10 minutes, and freezing at 80°C for 10 minutes. Five microliters of cell lysate were subjected to the master mix of hot start PCR reactions containing 1 µl of 10 µM of each α-factor and 3'-AOX primers, 5 µl of 25 mM $MgCl_2$, and 1 µl of 25 mM dNTP mix. Sterile distilled water was added to make the final volume to 49.5 µl. About 0.1 unit of Tag Polymerase was added after incubation the solution except the enzyme at 95°C for 5 minutes. The PCR are performed for 30 cycles as following parameters: denaturation at 95°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute. The resulting PCR product was analyzed on 1.2 % agarose gel to determine whether the DNA fragment was successfully amplified. The size of the DNA product from the amplification was a 482 bp, which is α-factor signal peptide with ALF*Pm*3 gene and 3'sequences of alcohol oxidase (AOX) gene, DNA fragment.

2.9.3 Expression of recombinant clones

Single colony of each clones were grown in YPD broth, at 30ºC overnight. Innoculate 100 ml of BMGY in a 1 liter flask with the starter. Grow at 30ºC at 300 rpm until the culture reach and OD_{600} = 4-6. Harvest the cells by centrifuging at 3500 rpm for 5 min at room temperature. To induce the expression the cell pellets were resuspended in 20 ml of BMMY medium. Add 100% methanol to a final volume concentration of 0.5% every 24 hours to maintain the induction. Collect 1 ml of the expression culture and centrifuge at 9,000 rpm for 2 min at RT at each time points (0,

1, 2, 3, 4, 5, 6 days). Store the supernatant and the cell pellets at -80ºC until ready to assay. Analysis for ALF expression by 15% Coomassie-stained and silver stained SDS PAGE.

2.9.4 Analysis of recombinant protein by SDS-PAGE

The SDS-PAGE system was performed according to the method of *Bollag et al.* The slab gel (10 x 10 x 0.75 cm) system consisted of 0.1% (w/v) SDS in 15 % (w/v) separating gel and 5 % (w/v) stacking gel. Tris-glycine buffer, pH 8.3 containing 0.1% (w/v) SDS was used as electrode buffer. The gel preparation was described in Appendix. The cells pellets of recombinant proteins were treated with sample buffer and boiled for 10 minutes before loading to the gel. The electrophoresis was performed at constant current of 20 mA per slab at room temperature from cathode towards anode. 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.

The SilverXpress® Silver Staining Kit (Invitrogen) was used to stain the gel. The gel was fixed in 200 ml of fixing solution for 10 minutes. The fixing solution was decanted and the gel was incubated in two changes of sensitizing solution for 10 minutes each. After decanting the sensitizing solution, the gel was rinsed twice with ultra pure water for 5 minutes each. The gel was then incubated in staining solution for 30 minutes. The gel was rinsed twice with ultra pure water as above. The gel was developed for 3-15 minutes in developing solution. The stopping solution was added directly to the gel when the desired staining intensity is reached. The stopping solution was decanted and the gel was washed three times in ultra pure water.

2.9.5 Detection of recombinant protein by Western blot

The recombinant protein was separated on 15 % SDS-PAGE gels as describe in section 2.5.2. Remove gel from slab and incubated for 10 min in tank-

blotting buffer (48 mM Tris pH 9.2, 39 mM glycine and 20% methanol). The PVDF membrane was activated in methanol for 3 minutes. Soak the activated membrane in tank-blotting buffer for 15 minutes. Wet 6 sheets of filter papers in tank-blotting buffer. Lay 3 sheets of them on sandwich model on the Trans-Blot® SD (Bio-Rad), and eliminate air bubbles with pipette, followed by the membrane, the gel, 3 sheets of filter paper, and finally the lid of the machine. (Figure 2.4).

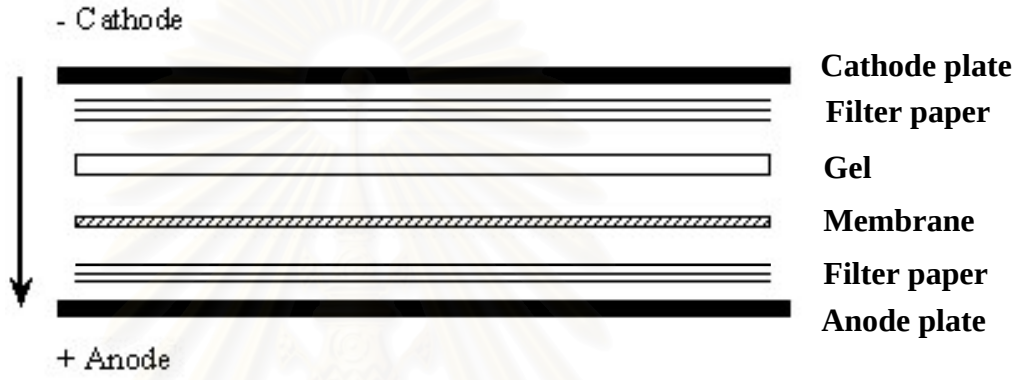


Figure 2.4 Western transfer cassette

Protein transfer was performed at constant electricity of 80 mA for 1 hour. After transfer, the membrane was stained in Ponceau S solution and then washed in ultrapure water. the marker bands were marked with pencil. The membrane was then incubated overnight in blocking buffer (5% (w/v) BSA, 0.05% (v/v) Tween 20 in PBS buffer) at room temperature. Wash membrane for 3 times for 10 min each time in PBS-Tween buffer (0.05% (v/v) Tween 20 in PBS buffer, pH 7.5) and incubate in mouse anti-ALF antibody 1/50 dilution diluted in 1% (w/v) BSA, 0.05% (v/v) Tween 20 in PBS buffer at 37ºC for 3 hours and then wash membrane 3 times for 10 min each time in PBS-Tween buffer at room temperature. After that incubate with secondary antibody, anti-mouse conjugated alkaline phosphatase solution 1/10,000 dilution diluted in the same buffer as primary antibody for 1 hour at room temperature. Wash 3 times for 10 minutes each time in PBS-Tween buffer at room temperature. One tablet of NBT (nitroblue tetrazolium)/BCIP(5-bromo-4-chloro-indolyl phosphate) (Sigma) was dissolved in 10 ml of ultrapure water. Detection was performed by diping the membrane in the NBT/BCIP solution until the positive band

was observed. Stop the chromogenic reaction by rinsing the membrane twice with water.

2.9.6 Purification of recombinant protein

The culture supernatant containing rALF*Pm*3 was first adjusted pH to 7 with 5N NaOH and then purified by weak cation exchange chromatography on a Sep-Pak® Accell™ Plus CM catridges (Waters Associates) equilibrated in 10 mM sodium phosphate, 200 mM NaCl, pH 7.0. Elution was performed in 3 steps by 0.5 M, 1M and 2M NaCl in 10 mM sodium phosphate, pH 7.0, respectively. The eluates were dialyzed overnight against sterile MilliQ water at 4ºC to eliminate salt. The purified fractions were then lyophilized and redissolved in sterile MilliQ water. The antimicrobial activity was performed against *E. coli* 363 as primary screening for rALF*Pm*3-containing fraction. The fractions were analyzed by 15% silver staining SDS-PAGE. Total protein concentration was determined by Micro BCA™ Protein Assay Reagent Kit (Pierce). The eluted fraction containing rALF*Pm*3 were lyophilized, redissolved in sterile water, and then acidified by trifluoroacetic acid. The acidified sample was subjected to reversed-phase HPLC on a SuperPac Sephasil C_8 column (250 × 4 mm, 5 µm, Pharmacia™) analytical C_8 column equilibrated in acidified water. The rALF*Pm*3 were purified by a linear biphasic gradient of 0-100% acetonitrile in acidified water over 60 minutes at a flow rate of 1 $ml.min^{-1}$. The HPLC fractions were subsequently lyophilized and resuspened in MilliQ water. The antimicrobial activity was performed against *E. coli* 363 as primary screening. The fractions were analyzed by 15% silver staining SDS-PAGE. The fractions with antimicrobial activity were then pooled.

2.9.7 Antimicrobial activity assay

The bacterial strains used in this study included Gram-negative bacteria, Escherichia coli 363, Salmonella thyphimurium, Klebsiella pneumoniae, Enterobacter cloacae, and Erwinia carotovora; Gram-positive bacteria, Aerococcus viridans, Micrococcus luteus, Bacillus megaterium, Staphylococcus aureus. In addition, the filamentous fungi Botrytis cinerea and Penicillium crustosum were used. Moreover,

marine pathogenic organisms for shrimps were also considered including Gram-negative bacteria of the Vibrio genus: V. harveyi, V. alginolyticus, V. penaeicida and V. anguillarum, and the filamentous fungus Fusarium oxysporum.

Antimicrobial activities and determination of minimum inhibitory concentration (MIC) values was determined by liquid growth inhibition assays as previously described (Destoumieux et al., 1999). Ten microliters of each diluted peptide, or sterile deionised water as a control, were incubated in a sterile micro titration plates with 100 μl of a suspension of mid-logarithmic growth phase culture of bacteria diluted in culture medium to OD_{600} = 0.001, or with 80 μl of fungal spores (final concentration 10^4 spores/ml) and 10 μl of water. Poor-broth nutrient medium (1% bactotryptone, 0.5% NaCl, pH 7.5) was used for standard bacterial strains cultures. For marine bacteria strains, they were grown in saline peptone water (1.5% peptone, 1.5% NaCl, pH 7.2). Antifungal assays were performed in potatoes dextrose broth (Difco) at half-strength supplemented with 10 μg/ml tetracycline. Bacteria were grown overnight under vigorous shaking at 25°C or 30°C according to the strains. Fungi were grown at 25°C in the dark without shaking for 48 h in a moist chamber. With the exception of fungal strains, microbial growth was controlled by measurement of the optical density at OD_{600} after incubation for 24 h. Inhibition of filamentous fungi growth was observed at microscopic level after 24 h and measured at 600 nm after 48 h. The MIC value was recorded as the range between the highest concentration of the peptide where bacterial growth was observed and the lowest concentration that caused 100% of inhibition bacterial growth.

2.9.8 Bacteriostatic/bactericidal activity assay

Four mililiters of the midlogarithmic-phase cultures of *E. coli* 363 and *B. megaterium* in poor-broth nutrient medium were incubated at 30°C in the presence of rALF*Pm*3 or water (control). The final concentration of the molecule tested was 10 times over the MIC value. Twenty microliter aliquots were removed at different time interval and spraded on the LB agar plate. If the culture medium is not clear, 10 folds serial dilution was made and 10^4 to 10^8 dilution fraction were then used for plating.

The number of colonies that can grow after incubation the plate for 24 h at 30°C were count. The number of colony forming units (cfu) was then determined.

2.10 Gene expression analysis using *in situ* hybridization

2.10.1 Tissue preparation for histology

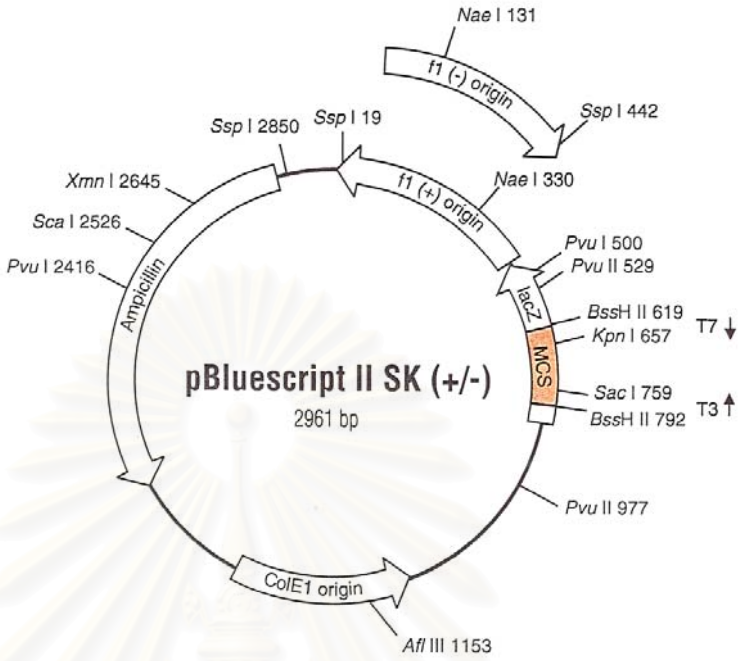
2.10.1.1 Haemocyte preparation

The shrimp haemolymph was collected from the ventral sinus located at the base of the first abdominal segment using a 27 G1//2 inch needle fitted onto a 1.0 ml syringe containing 500 μl of anticoagulant (Modified Alsever Solution (MAS): 27 mM sodium citrate, 336 nM NaCl, 115 mM glucose, and 9 mM EDTA, pH 7.0). The haemolymph was immediately centrifuged at 800xg at 4°C for 10 minutes to separate the haemocytes from plasma.

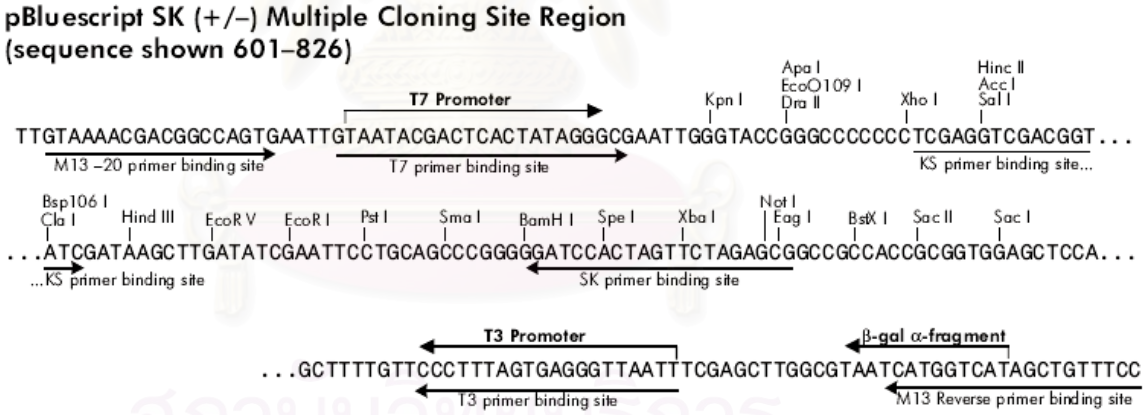
The supernatant was eliminated. The haemocytes were resuspended in freshly prepared ice-cold MAS containing 4% (w/v) paraformaldehyde. The resuspended haemocytes were incubated on ice for 10 minutes for fixation. After centrifugation as above, the haemocyte pellets were washed twice in MAS to eliminate plasma proteins. The pellets were resuspended in MAS. The total number of haemocyte was determined by using a haemocytometer. Using the cyto-centrifuge, the $2x10^5$ haemocytes were centrifuged onto the poly-L-lysine coated slide at 1,000xg for 5 minutes. The haemocytes were dried at room temperature for few minutes. The slide was stored at -20°C until used.

2.10.1.2 Tissue preparation

Tissues from juvenile shrimp were prepared by dissecting the animal to get cepharothorax. Immediately fix the dissected tissues in freshly prepared RNA friendly fixative solutions (220 ml formaldehyde 37%, 315 ml absolute ethanol, 115 ml Glacial acetic acid, 350 ml water) for 24 hours at 4 °C. Use an adequate supply of fixative; a rule of thumb is that a minimum of approximately 10 x their volume of fixative should be used for each specimen (eg. A shrimp of 10 ml volume would require 100 ml of fixative). Following fixation, the specimens should be transferred to



a)



b)

Figure 2.5 The circular map (a) and multiple cloning site (b) of pBluescript II SK (+/-) vector.

50% ethanol, where it can stored for an indefinite peroid. Slit the cuticle before transfer to 50% ethanol, paying particular attention not to cut deeply into the underlying tissue. Record a history of the specimen at number of specimen and the collection time post-infection, using soft-lead pencil on paper. Dehydrate tissues as following: 3 times in 70% ethanol room temperature for 20 minutes, 3 times in 90% ethanol room temperature for 20 minutes, 3 times in 100% ethanol room temperature for 20 minutes, and 3 times in 70% ethanol at room temperature overnight. The dehydrated fixed tissues were embedded in paraplast by incubating as following: 3 times in xylene for 20 minutes at room temperature, 12 hours (minimum) in xylene at room temperature, 12 hours in a solution (v/v) of xylene/Paraplast at 60°C, and 3 times in Paraplast 1 hour at 60°C. Prepare Paraplast blocks, cut 6 μm sections and mount sections on poly-L-lysine coated slide and stored them at -20°C until use.

2.10.2 Riboprobe preparation

The pBluescriptSK plasmid containing ALFPm3 gene cDNA was linearized by restriction enzyme digestion. The plasmid was digested with SalI to produce antisense probe for ALFPm3 in situ hybridization. The plasmid was digested with SacII to produce sense probe. One hundred microliters of linearized plasmids was adjusted volume to 400 l with sterile water. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed until formed by vortexing. They were centrifuged at 12,000 rpm for 10 minutes at 4°C. The aqous phase was added to equal volume of chloroform, mixed and centrifuged of as above. They were precipitated by adding 50 l of 3 M sodium acetate, 250 l of -20°C-cold absolute ethanol and incubated at –80°C for at least 1 hour. The mixture was centrifuged at 12,000 rpm for 20 minutes at 4°C, air-dried the pellet, and suspended in 20 l DEPC-treated water. The linearized plasmids were quantified on agarose gel electrophoresis.

Dioxigenin (DIG)-labeled probes were prepared by in vitro transcription using T7 polymerase for ALFPm3-sense probe and T3 polymerase ALFPm3-antisense probe. The reaction composed of 1 g of purified, linearized plasmid DNA, 2 l of 10x concentrated DIG RNA labeling mix (10 mM each ATP, CTP, and GTP, 6.5 mM

UTP, 3.5 mM DIG-UTP; pH 7.5), 2 μl of 10x concentrated transcription buffer (400 mM Tris-HCl, pH 8.0, 60 mM $MgCl_2$, 100 mM Dithiothreitol (DTT), and 20 mM spermidine), 40 units of T7 or T3 RNA polymerase. The DEPC-treated water was added to make a total reaction volume of 20 μl. The components were mixed and centrifuged briefly. The reaction was incubated at 37°C for 2 hours. Two units of DNaseI, Rnase-free were added to the tube and incubated at 37°C for 15 minutes. One microliter of 0.5 M EDTA, pH 8.0 was added to the tube to stop the polymerase reaction.

The labeled RNA transcript was purified by RNA mini Quick Spin column (Roche). The column was prepared by evenly resuspending the Sephadex metrix in the column buffer. The top cap was removed from the column and then snapped off the bottom tip. The excess buffer was removed by centrifugation at 3,000 rpm for 1 minute. The column was the placed in a clean, sterile 1.5 ml microcentrifuge tube and then slowly and carefully applied the sample to the center of the column bed. It was centrifuged at 3,000 rpm for 4 minutes at room temperature. The eluate was stored at -20°C until used. Each labeled RNA run-off transcript was quantified on agrose gel electrophoresis.

2.10.3 Prehybridization treatments

For the tissue sections, elimination of paraffin and hydration was carried out by consecutive baths in xylene; 2 baths of 15 minutes, 96% ethanol; 2 baths of 5 minutes, 70% ethanol; 2 baths of 5 minutes, 30% ethanol; 2 baths of 5 minutes, and 0.2M Tris-HCl pH7.4; 2 baths of 5 minutes. Quenching of paraformaldehyde, digestion with proteinase K, postfixation, acetylation and dehydration was performed by consecutive baths of 0.1M Glycine, 0.2M Tris pH 7.4 for 10 minutes, 0.1M Tris HCl pH 8 containing 0.05M EDTA for 5 minutes, and 0.1M Tris HCl pH 8 containing 0.05M EDTA supplemented with 1ug/ml of proteinase K, at 37°C for 15 minutes. The slides were rinsed with PBS (0.1 M phosphate buffer, pH 7.4 containing 0.9% NaCl) for 5 minutes.

For cytospin haemocytes, *in situ* hybridization protocol similar to those of tissue section, but the deparaffination, rehydration and Proteinase K treatment step

were not performed. The slides were incubated in 0.2 M Tris-HCl, pH7.4 twice for 5 minutes each and in 0.1 M glycine in 0.2 M Tris-HCl, pH 7.4 for 10 minutes. The cells were rinsed with PBS for 5 minutes.

2.10.4 Post-fixation

The slides were incubated in 4% paraformaldehyde in phosphate buffer containing 5 mM $MgCl_2$ for 15 minutes and rinsed with PBS for 5 minutes. The cells were incubated in 0.1M triethanolamine, pH 8.0 containing 0.25% acetic anhydride for 10 minutes. The cells were dehydrated in 30%, 70%, and 100% graded ethanol for 5 minutes each. The cells were dried at room temperature for at least 2 hours.

2.10.5 Riboprobe hybridization

DIG-labeled riboprobes (40-100 ng per slide) were diluted in 2X SSC containing 50% formamide, 10% dextran sulfate, 10X denhart's solution, 0.5 mg/ml tRNA from *E.coli*, 100 mM dithiothreitol, and 0.5 mg/ml salmon sperm DNA. The diluted DIG-labeled riboprobes were denatured at 55°C for 10 minutes and immediately placed on ice for 5 minutes. The riboprobes were dropped onto the slides. The hybridizaiton solution covered cells was covered by a glass slip. The slides were incubated overnight at 55°C for haemocytes, or at 58°C for the tissues in adequate humid chamber (1x SSC and 30% formamide). The cells were washed twice for 15 minutes with 2x SSC with shaking in a platform shaker. The cells were treated with 20 μg/ml RNase A in 2xSSC at 37°C for 30 minutes and consecutively rinsed twice for 10 minutes with 1x SSC supplemented with 0.07% 2-mercaptoethanol at room temperature, 0.5x SSC supplemented with 0.07% 2-mercaptoethanol for 10 minutes at room temperature, 0.1x SSC supplemented with 0.07% 2-mercaptoethanol for 10 minutes at room temperature and twice for 30 minutes at 55°C. The last solution with the slides was leaved on ice for 1 hour.

2.10.6 Riboprobe detection

The slides were washed in 0.1x SSC for 5 minutes and rinsed twice in TBS (0.1M Tris-HCl, 0.9%NaCl, pH 7.4) containing 0.05% of Triton X-100 for 5 minutes.

The slides were pre-incubated in blocking buffer (TBS supplemented with 1% normal sheep serum and 0.05% Triton X-100) for 20 minutes. Five hundred microliters of antibody solution (1/1000 diluted alkaline phosphatase-conjugated sheep anti-DIG antibody in blocking buffer) were added onto each slide and incubated overnight at room temperature in a humid chamber. After incubation, the slides were washed 3 times in TBS for 10 minutes each and 2 times in buffer B (0.1M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, and 0.9%NaCl) for 5 minutes each. Five hundred microliters of detection solution (375 μg/ml of NBT (nitroblue tetrazolium diluted in dimethyl formamide), 188 μg/ml of BCIP, and 1 mM of levamisole prepared in buffer B) were overlaid onto the slides and incubated in the dark at room temperature. The reaction should be monitored until optimal development. For ALF detection, the optimal development was observed within 2 hours. The slides were washed by TBS for 10 minutes to stop the reaction and rinsed briefly with distilled water. The slides were mounted with a mixture of (1:9) TBS: glycerol and stored at 4°C.

2.10.7 Control

Sense riboprobe and RNase-treatment antisense riboprobe were used as negative controls. For RNase-treatment antisense riboprobe, 10 μg/ml of RNase A was added onto the slide and incubated at 37°C for 30 minutes. The slide was then washed 3 times with TBS for 5 minutes each before proceeding to the hybridization step.

2.11 Immunohistochemistry

For the six micrometer-thick paraffin sections, elimination of paraffin and hydration was carried out by consecutive baths in xylene; 2 baths of 15 minutes, 96% ethanol; 2 baths of 5 minutes, 70% ethanol; 2 baths of 5 minutes, 30% ethanol; 2 baths of 5 minutes. The slides were equilibrated immediately for 1 hour at room temperature in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4.7H2O, and1.4 mM KH2PO4 pH 7.4). The slides were incubated for 10 minutes in new PBS and then preincubated with PBS containing 1% normal goat serum (NGS), 1% BSA, and 0.1% Triton X-100 (PBS/NGS/BSA/Triton X-100) for 1 hour at room temperature. Five

hundred microliters of rabbit anti-ALF polyclonal antibody purified IgG (4 μg/ml) diluted in (PBS/NGS/BSA/Triton X-100) were overlaid onto the section and incubated overnight at 4°C. They were washed 3 times for 5 minutes each with TBS. The secondary antibody, alkalinephosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch), diluted 1 : 2000 in PBS/NGS/BSA/Triton X-100, were incubated for 2 h at room temperature. After washing 2 times for 5 minutes each with TBS, they were equilibrated in 0.1M Tris-HCl, 100 mM NaCl, 50 mM MgCl2 pH 9.5 for 10 minutes. Immunodetection was performed by incubating the slides in the dark at room temperature with 500 l of detection solution (375 g/ml of NBT, 188 g/ml of BCIP, and 1 mM of levamisole prepared in 0.1M Tris-HCl, pH 9.5, 50 mM MgCl2, and 0.9%NaCl. The slides were washed by TBS for 10 minutes to stop the reaction and rinsed briefly with distilled water. The slides were mounted with a mixture of (1:9) TBS: glycerol and stored at 4°C. the slides were examined using a light microscope (Olympus).

Cytocentrifuged haemocytes were equilibrated for 10 min in TBS before permeabilization with 0.2% gelatine and 0.5% Triton X-100 in TBS for 10 min at room temperature. They were then washed for 3 times 10 minutes each in 0.1% Tween20 in PBS (PBS-T20). One hour preincubation was performed in the presence of 1% NGS and 1% BSA in PBS-T20 (PBS-T20/NGS/BSA) to block nonspecific antibody binding. Rabbit anti-ALF polyclonal antibody purified IgG (4 μg/mL) diluted in blocking solution, was applied for 12–16 h at 4°C in NaCl/Tris/NGS/BSA. After washing 5 minutes for 3 times in PBS-T20, cells were incubated for 1 hour at room temperature with 1:2000 alkalinephosphatase conjugated goat anti-rabbit IgG (Jackson Immunoresearch) diluted in blocking solution. The slides were washed 3 times for 10 min in PBS-T_{20}. Immunodection was also performed by using NBT/BCIP solution (Roche) as described above.

CHAPTER III

RESULTS

3.1 Haemocytes and total RNA preparation

Haemolymph was collected from normal, saline-injected and *Vibrio harveyi* infected shrimps. A virulent strain of *Vibrio harveyi* 1526 was used to challenge the juvenile *Penaeus monodon*. About 1 ml of haemolymph was obtained from each animal. Haemocytes were separated from haemolymph by centrifugation and resuspended in TRIreagent for total RNA preparation as described in section 2.4. The A_{260}/A_{280} ratios of the total RNA samples were 1.5-1.8 which were within the expected ratio for the acceptable quality of total RNA. The average quantity of total RNA was 17 µg per individual for normal shrimp and 14 µg per individual for *V. harveyi* infected shrimp, at which the 6 hour post-injected shrimps gave the lowest average quantities of total RNA, 10 µg per individual.

The total RNA samples were examined by electrophoresis on a denaturing formaldehyde/agarose/EtBr gel to check their integrity. Like that of normal haemocytes, the total RNA from haemocytes of *V. harveyi* infected shrimps at various time post-injection (0-72 hours) revealed a predominant band of 18S rRNA (1.9 kb) (Figure 3.1).

3.2 Identification of differentially expressed genes in *Penaeus monodon* haemocytes in response to *Vibrio harveyi* infection

3.2.1 Differential display PCR

Total RNA of normal and 40 hours-post *V. harveyi* infected *P. monodon* were isolated from pooled haemocytes of 5 individuals. They were treated with DNase I to eliminate DNA contamination. The DNA-free total RNA samples were subsequently

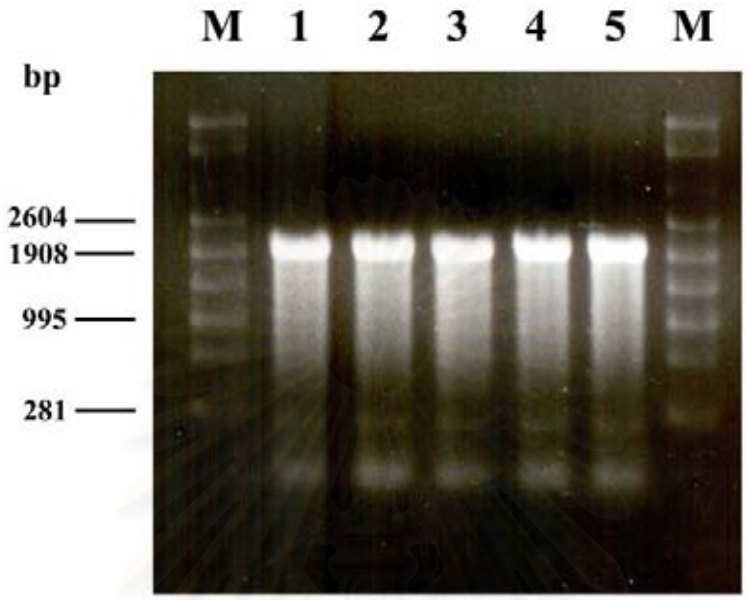


Figure 3.1 Total RNA from haemocytes of *V. harveyi* infected shrimps at various time post-injection (0-72 hours) electrophoresed on a 1% formaldehyde agarose gel.

Lane M : RNA marker
Lane 1 : 0 hour post-injection
Lane 2 : 6 hours post-injection
Lane 3 : 24 hours post-injection
Lane 4 : 48 hours post-injection
Lane 5 : 72 hours post-injection

used for the first strand cDNA synthesis and then subjected to differential display PCR (DD-PCR). The reactions were carried out using 44 combinations of primers (10 arbitrary primers in random combination with 9 oligo dT primers) in order to identify genes potentially involved in *V. harveyi* infection in shrimp. DD-PCR patterns of normal and *V. harveyi* infected *P. monodon* were compared for each primer combination and the genes, which responsed to *V. harveyi* challenge, were identified. Two dilutions, 1:10 and 1:30, of cDNA preparation were used in the DD-PCR. If a differential band of a cDNA determined in one dilution also observed in the other dilution, that cDNA band was selected for further analysis. The example of DD-PCR patterns are presented in Figure 3.2.

A total of 79 differentially expressed bands from 33 combinations of primers were observed. The level of expression of the cDNAs were evaluated by measuring the intensity of bands with gel documentation system (GeneCam FLEX1, Syngene). The average fold change in the expression level was calculated from the band intensity (Table 3.1). The differentially expressed bands could be divided into two groups: 48 bands showing increase in expression and 31 bands showing reduced expression. The approximate sizes of differentially expressed cDNA bands ranged from 400 to 3000 bp. Each band was cut from the dried gel, rehydrated, and reamplified with corresponding combination of primers. The reamplified products were checked for their sizes by agarose gel electrophoresis (Figure 3.3).

3.2.2 Sequence analysis

The cDNAs from 48 (61%) out of 79 differentially expressed bands were successfully extracted from the acrylamide gels, reamplified, and cloned as indicated in Table 3.1. Thirty bands were up-regulated, while the other 18 bands were down-regulated. Four to eight representative clones from each band were randomly selected and sequenced. A total of 267 clones were sequenced. The resulting sequences of the clones from each band were aligned to determine their sequence discrepancy. The sequence analysis of these clones showed that 21 (44%) out of 48 bands contained one sequence, while the remaining bands contained a mixture of two to four sequences. Twenty-one bands (44%) contained a mixture of two sequences. Five

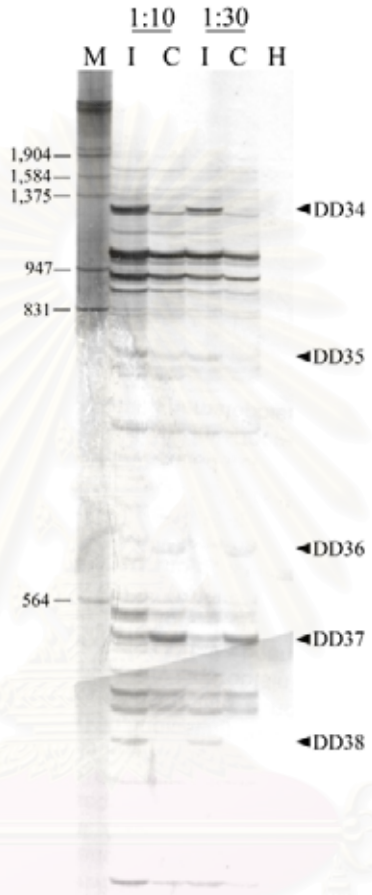


Figure 3.2 A differential display pattern of *V. harveyi* challenge shrimps. The cDNAs were amplified with P2 and T3 primers and separated on a 6% high-resolution polyacrylamide gel under denaturing (urea) conditions. The arrows indicate the differential bands.

Lane M : Lamda DNA/*Eco*RI+*Hin*dIII marker

Lane I : DD-PCR products from *V. harveyi* challenge shrimps

Lane C : DD-PCR products from unchallenged shrimps

Land H : DD-PCR products from negative control reaction (no template)

Table 3.1 Changes in mRNA level of differential display bands identified in *Vibrio harveyi* challenged shrimps

clone	primers used in differential display	size (bp)	fold change	clone	primers used in differential display	size (bp)	fold change
DD1	P2/T2	1200	+2.6	DD40	P6/T3	1500	-1.41
DD2	P2/T2	750	+1.55	**DD41**	P6/T3	800	-2.56
DD3	P2/T2	600	-4	DD42	P8/T2	700	-2.56
DD4	P2/T2	450	+2.33	**DD43**	P8/T2	640	+3.48
DD5	P2/T2	2300	+1.5	**DD44**	P8/T2	590	-2.38
DD6	P2/T2	600	-2.44	**DD45**	P8/T2	500	+2.11
DD7	P2/T2	750	+1.94	**DD46**	P10/T2	3000	-2.0
DD8	P3/T3	600	-1.96	**DD47**	P10/T2	1400	-2.04
DD9	P3/T3	500	+0.52	DD48	P10/T1	600	+1.95
DD10	P8/T8	550	+1.28	**DD49**	P10/T1	480	+2.84
DD11	P8/T8	2900	-3.70	**DD50**	P4/T3	750	+2.14
DD12	P8/T8	500	-3.13	**DD51**	P4/T3	740	+2.13
DD13	P8/T8	500	-4.55	DD52	P6/T4	2000	+1.31
DD14	P8/T1	1400	-1.92	DD53	P5/T4	550	+1.67
DD15	P8/T1	650	+2.79	**DD54**	P9/T4	580	-2.38
DD16	P3/T1	450	-2.94	**DD55**	P9/T4	530	+1.46
DD17	P3/T1	680	+3.36	DD56	P8/T4	1200	+2.12
DD18	P3/T1	550	-2.17	**DD57**	P8/T4	500	+1.26
DD19	P6/T6	1600	+1.91	DD58	P2/T4	1200	+1.6
DD20	P6/T6	800	-1.35	**DD59**	P2/T4	1100	+1.64
DD21	P10/T1	2000	-1.82	**DD60**	P2/T4	650	+2.08
DD22	P10/T1	700	+2.26	DD61	P1/T4	2300	+1.77
DD23	P1/T2	2000	-1.82	**DD62**	P1/T4	1500	+1.6
DD24	P1/T2	980	+4.53	DD63	P1/T4	880	+1.76
DD25	P1/T2	930	+2.03	**DD64**	P3/T4	550	-2.33
DD26	P1/T2	790	+1.35	DD65	P1/T3	1200	+2.8
DD27	P1/T2	750	+2.23	DD66	P9/T2	700	+2.86
DD28	P3/T2	750	+2.75	**DD67**	P5/T3	700	+2.49
DD29	P4/T2	1400	-1.82	**DD68**	P10/T3	520	-2
DD30	P5/T2	2400	+2.37	**DD69**	P9/T3	1400	+2.07
DD31	P5/T2	1100	-2.22	**DD70**	P9/T3	1200	+1.87
DD32	P5/T2	800	-2.13	DD71	P9/T3	1180	-1.82
DD33	P5/T2	400	-2.5	**DD72**	P9/T3	1000	-1.61
DD34	P2/T3	1250	+2.75	DD73	P9/T3	750	+1.62
DD35	P2/T3	780	+2.6	**DD74**	P9/T3	550	+1.96
DD36	P2/T3	630	-1.30	DD75	P7/T4	850	-2.22
DD37	P2/T3	500	-1.8	DD76	P7/T2	480	+12.08
DD38	P2/T3	480	+2.3	DD77	P8/T2	1502	-2.63
DD39	P5/T3	630	+2.54	**DD78**	P7/T1	850	+2.43
				DD79	P7/T1	650	-2.70

Remark: The clone names in bold indicate those that were successfully reamplified and cloned.

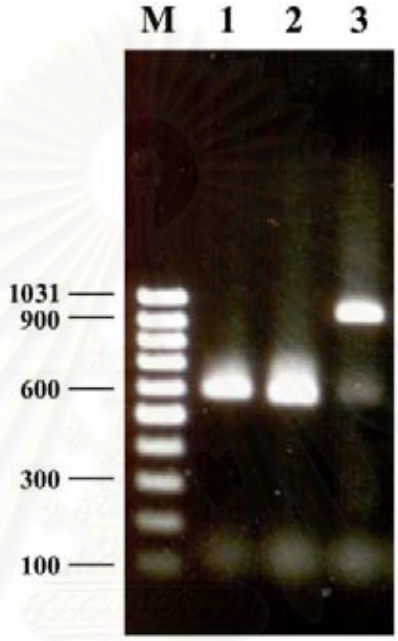


Figure 3.3 The reamplified products of differentially expressed cDNAs. The eluted DD-PCR bands were reamplified by the same primer pairs and subjected to 1.5% agarose gel electrophoresis.

Lane M : DNA ladder (100 bp marker)

Lane 1 : reamplified product of DD44

Lane 2 : reamplified product of DD54

Lane 3 : reamplified product of DD24

bands (10%) contained a mixture of three sequences. One band (2%) contained a mixture of four sequences.

Homology search against the GenBank database using the BLAST search program showed 85 clones (31%) derived from 24 different bands significantly matched the 28 GenBank database entries; 24 of which were known genes. These sequences were divided into 7 catagories according to the major function of their encoded proteins including gene expression and regulation, potential immune function, metabolism, structure and motility, signalling and communication, transport, and miscellaneous function, (Table 3.2). Among the known genes, 8 genes including balbiani ring protein3 precursor, argonaute protein, caspase 3B, serine protease inhibitor B3, Rc12 protein, glucose transporter 1, interferon-related developmental regulator 1, and huntingtin interacting protein E, were first found in the penaeid shrimp. A cDNA homologue to anti-lipopolysaccharide factor (ALF) was found among the up-regulated genes. Six genes that were cDNA homologue to caspase 3B, serine protease inhibitor B3, glucose transporter 1, interferon-related developmental regulator 1, lysozyme, and profilin, were further analyzed by real-time PCR in order to confirm their expression in response to *V. harveyi* challenge.

Anti-lipopolysaccharide factor (ALF)

The putative gene of anti-lipopolysaccharide factor (ALF), an anti-gram negative protein, were identified in this study from the 8 clones derived from 2 differentially expressed bands (DD17-2, DD17-4, DD17-5, DD64-2, DD64-3, DD64-4, DD64-5, and DD64-6). These clones exhibited 57% to 66% deduced amino acid homology with ALF of the atlantic horseshoe crab *Limulus polyphemus*. Comparing with the ALF*Pm*1-5 previously reported by Supungul et al., (2002), all of them encoded for protein similar to ALF*Pm*3 with 97 to 99% identity. All clones contained a complete open reading frame (ORF) of 342 bp or 114 deduced amino acids which was 9 amino acids shorter than the ALF*Pm*3. Due to the difference in length and high similarity to ALF*Pm*3, the clone sequences were carefully determined for the size of ORFs. Because these clones were amplified by PCR using P3 primer and T3 or T4 primers, it was speculated that the first amino acid Met (M) of the clones might come from the nucleotide sequences of primer. After comparing the nucleotide

sequences of P3 primer against that of the derived clones, it was found that the N-terminal amino acid “MRV” was deduced from the nucleotide “ATG CTG GTG” of P3 primer. Therefore, the start codon, ATG, obtained by PCR of the clones should not be the real start codon. These also infered that the complete ORF of these clones should be 123 amino acids as previously identified ALF*Pm*3. In addition, the deduced amino acid sequences of newly identified clones revealed variation of ALF*Pm*3 which could be divided into four isoforms (ALF*Pm*3a- ALF*Pm*3d). Of these, the designated ALF*Pm*3a (DD64-6) isoform was similar to the first isolate ALF*Pm*3 by Supungul et al., (2002). ALF*Pm*3b,-3c, and –3d possessed variation at residues 18, 47, and 122 based on the numbering of deduced amino acid of ALF*Pm*3a. A putative signal peptides of all ALF*Pm*3 isoforms were identical (Figure 3.4).

Caspase 3B

One clone, DD70-2, showed 49% homology to *Hydra vulgaris* caspase 3B, a member of ICE/CED-3 cysteine protease family, which played key roles in regulating cell death. Previous studies suggested their roles in the induction of apoptosis, possibly at the apex of the apoptotic pathway. Moreover, some data revealed that activation of these enzymes represented commitment to death (Webb et al., 1997). The deduced amino acids of caspase 3B homologue revealed two essential catalytic subunits: the p20 subunit and the p10 subunit, which are derived from the processing of proenzyme and required for active site formation. The p20 subunit contained a conserved catalytic residues, consisting of a pentapeptide **QACRG**, in the active site (Figure 3.5) (Fernandes-Alnemri et al., 1994).

Serine proteinase inhibitor B3 (SERPINB3)

The serine proteinase inhibitor B3 (SERPINB3) homologues derived from DD46-1, DD46-3, and DD46-5 clones showed 51% homology to *Rattus norvegicus*. All clones did not encode for the complete ORF but only 186 deduced amino acids at the C-terminus. The proteinase inhibitory effect of serpins normally require the reactive site loop (RSL) near their carboxyl terminus which can be divided functionally into two segments: a proximal (P15-P9) hinge region and a distal (P9-

Table 3.2. *Vibrio harveyi* responsive genes homologues identified in haemocytes of *P. monodon* by DD-PCR

Sequence homology	Closest species	Accession no. of closest species	Expected value	Positive	up or down*
Gene expression and regulation					
balbiani ring protein3 precursor	*Chironomus tentans*	Q03376	1.00E-05	50/111 (45%)	+
CG6671-PB (argonaute protein)	*Drosophila melanogaster*	NP_523734	8.00E-43	98/129 (75%)	+
ENSANGP00000015770(ADP-ribosylation factor1)	*Anopheles gambiae*	XP_320516	E-101	183/183 (100%)	-
glycyl-tRNA synthetase	*Homo sapiens*	P41250	4.00E-20	60/85 (70%)	+
Potential immune function					
anti-lipopolysaccharide factor	*Atlantic horseshoe crab*	A23931	4.00E-16	56/95 (58%)	+
caspase 3B	*Hydra vulgaris*	AAF98012	4.00E-22	107/217 (49%)	+
lysozyme	*Penaeus monodon*	AAN16375	9.00E-83	143/143 (100%)	+
serine protease inhibitor B3	*Rattus norvegicus*	NP_001008887	1.00E-23	110/184 (59%)	-
transglutaminase	*Penaeus monodon*	AAL78166	9.00E-29	64/64 (100%)	+
Metabolism					
CG7820-PA (CAH1)	*Drosophila melanogaster*	NP_523561	4.00E-52	123/199 (61%)	+
RE50216p (kynurenine aminotransferase)	*Drosophila melanogaster*	AAN71426	1.00E-35	94/125 (75%)	+
ENSANGP00000003832 (ligase family member)	*Anopheles gambiae*	XP_309685	6.00E-13	47/64 (73%)	+
phosphogluconate dehydrogenase	*Danio rerio*	AAQ91261	3.00E-70	143/165 (86%)	-

* + and - indicate up or down-regulation of each putative gene observed by DD-PCR technique.

Table 3.2 (continued)

Sequence homology	Closest species	Accession no. of closest species	Expected value	Positive	up or down*
Structure and motility					
LP06350p (alpha-spectrin)	*Drosophila melanogaster*	AAL39886	5.00E-67	137/154 (88%)	+
ENSANGP00000014553 (myosin like protein)	*Anopheles gambiae*	XP 314222	3.00E-63	141/172 (81%)	+
profilin	*Branchiostoma belcheri*	AAL75810	2.00E-20	72/111 (64%)	+
GA18989-PA (Rc12 protein)	*Drosophila melanogaster*	EAL27028	1.00E-22	77/124 (62%)	-
Signaling and communication					
asialoglyco protein receptor2 (rat hepatic lectin 2 RHL2)	*Rattus norvegicus*	NP_058885	3.00E-05	50/199 (42%)	+
calmodulin	*Metridium senile*	P02596	3.00E-17	44/44 (100%)	-
Transport					
autotransporter adhesin	*Magnetococcus sp. MC-1*	ZP_00288950	2.00E-13	70/134 (52%)	-
CG1086-PA (Glucose Transporter1)	*Drosophila melanogaster*	NP_728559	6.00E-28	90/152 (59%)	+

* + and - indicate up or down-regulation of each putative gene observed by DD-PCR technique.

Table 3.2 (continued)

Sequence homology	Closest species	Accession no. of closest species	Expected value	Positive	up or down*
Miscellaneous function					
interferon - related developmental regulator 1	*Homo sapiens*	AAH01272	3.00E-21	95/177 (55%)	+
putative type IV aminophospholipid transporting ATPase	*Mus musculus*	NP_700438	2.00E-53	128/192 (66%)	+
CG9523-PA (Huntingtin interacting protein E)	*Drosophila melanogaster*	NP_609026	4.00E-79	181/235 (77%)	+
Unidentified-similar to other cDNA/DNA					
ENSANGP00000016412	*Anopheles gambiae*	XP_306944	4.00E-53	133/201 (66%)	+
ENSANGP00000021966	*Anopheles gambiae*	XP_309498	2.00E-87	158/189 (83%)	+
ENSANGP00000022625	*Anopheles gambiae*	XP_308486	4.00E-29	92/156 (58%)	+
hypothetical protein MG03029.4	*Magnaporthe grisea*	EAA47786	6.00E-29	78/119 (65%)	-

* + and - indicate up or down-regulation of each putative gene observed by DD-PCR technique.

Figure 3.4 Multiple alignments of the deduced amino acid sequence of ALF*Pm*3 homologues obtained from DD-PCR and the ALF*Pm*3 previously identified by Supungul et al., 2004. * indicates amino acid identity and · and : indicates amino acid similarity. The putative signal peptides are underlined and and the clevage sites are indicated by an arrowhead. Based on the ALF*Pm*3 numbering, the open box indicates the variation at residue 47. The gray box indicates the difference of amino acid at residue 122. The black box shows a single amino acid change in the signal peptide sequences of DD64-2 clone.

P5') variable region. The latter region is critical for inhibition and defining target specificities. Cleavage of a specific peptide bond between residues designated P1 and P1' results in a large conformational rearrangement in the serpin and formation of a stable serpin-proteinase complex. The P1 residue, for most serpin-serine proteinase interactions, and the P2 residues, for SERPINB3, dictate the inhibitory activity of the molecule (Schick et al., 1998). The putative RSL (P15-P5') of the SERPINB3 homologues identified in this study were designated according to Schechter-Berger numbering scheme. Herein, a conserved Gly was P15, a consensus clevage site was between P1Ile and P1'Gly, and Arg was designated as P2 (Figure 3.6). However, the actual functional P1, P2 residues for this SERPINB3 homologue should be determined experimentally with different target proteinases (Schick et al., 1998).

Glucose transporter 1 (GLUT 1)

Glucose transporter 1 (GLUT 1) homologues were identifed from DD51-1, DD51-2, DD51-3, DD51-5, DD51-6 and DD51-8 in the present study with 59% homology to that of *Drosophila melanogaster*. The human GLUT 1 belongs to the Major Facilitator Superfamily (MFS). The homologues clones contained only a MFS domain but not the complete ORF (Figure 3.7). In mammals, GLUT is a family of structurally related glycoproteins composing of GLUT 1-5 gene members with variable tissue-specific expression, subcellular localization, and kinetics for glucose uptake. There is a report showing that GLUT 1 mRNA in rat is regulated by stress and is proposed as a stress protein like glucose-regulated protein 78 (GRP 78) (Wertherimer et al., 1991). GLUT isoforms are differentially expressed and regulated in human leukocytes. Previous study indicated that GLUT 1 protein in monocytes was increased 1.9 fold after lipopolysaccharide activation and high GLUT 1 and GLUT 3 expression could provide cellular fuel for the immune response (Fu et al., 2004).

Interferon-related developmental regulator 1 (IFRD1)

Three clones, DD78-2, DD78-3, and DD78-5 are interferon-related developmental regulator 1 (IFRD1) homologues with 55% homology to that of *Homo sapiens*. IFRD1 is the human homologue of the rat nerve-growth factor-inducible PC4 protein and murine homologue TIS7. The amino acid sequences of human, mouse,

and rat IFRD1 are highly conserved suggesting they may have a common function. The exact function of IFRD1 is unknown but it has been shown that PC4 is necessary for muscle differentiation and that it may have a role in signal transduction. Furthermore, the carboxy terminal region of human IFRD1 protein shows significant similarity to interferon-γ, a molecule known for its role in cellular diffentiation (Buanne et al., 1998). Moreover, The expression level of IFRD1 or nerve growth factor-inducible PC4 homologue is significantly induced in human peripheral blood cells by radiation (Amundson et al., 2000). The ORF of human IFRD1 protein contains 453 amino acid residues with three conserved domains (at positions 67-159, 291-347 and 414-448) which are characteristics of the family. Although, the complete ORF of this gene could not be obtained by the DD-PCR technique, the homologs contained 216 deduced amino acids which comprised the first domain of this protein family (Figure 3.8).

Lysozyme

Lysozyme cDNAs were identified from DD19-2, DD19-3, DD19-4, DD19-5, DD19-6, DD20-3, and DD20-4 as a differentially expressed gene in heamocytes of *V. harveyi* challenged *P. monodon*. Lysozyme genes have been identified in many penaeid species, such as *L. vannamei*, *P. monodon*, *M. japonicus*, *P. semisulcatus* and *L. stylirostris* (de Lorgeril et al., 2005; Rojtinnakorn et al., 2002; Sotelo-Mundo et al., 2003; Supungul et al., 2002). Lysozyme is an antimicrobial protein described in invertebrate immunity. The kuruma shrimp lysozyme shows lytic activity on infectious pathogens in various *Vibrio* strains (Hikima et al., 2003). However, the activity against *V. harveyi* has not been determined. Recently, analysis of lysozyme mRNA expression in haemocytes of surviving *L. stylirostris* infected with *V. penaeicida* revealed the decrease in its expression at 12 hour post-infection and increase at 96 hours. These results suggested the importance of this molecule in shrimp immunity. Figure 3.9 showed the deduced amino acid sequences alignment of lysozyme from selected species. Analysis of the amino acid sequence of black tiger shrimp lysozyme indicates that it belongs to the C-type family as described in *P. vannamei* and *Marsupenaeus japonicus*. They contained the conserved catalytic residues, glutamic acid and aspartic acid at position 51 and 68, respectively. Among

P. monodon lysozyme identified by EST and DD-PCR, one amino acid variation was observed.

Profilin

Two clones, DD17-1 and DD17-3, encoded for profilin. Profilin is a small actin-binding protein which has a major role in actin polymerization. Profilin also interacts with several ligands other than actin including poly-L-proline (PLP), phosphatidylinositol 4,5-bisphosphate (PIP_2), an actin-related protein complex, vasodilator-stimulated phosphoprotein (VASP)-like proteins, and formin-related proteins. Profilin also functions as hubs that control complex network of molecular interactions such as membrane trafficking, small GTPase signalling and nuclear activities. The profilin family is highly conserved in terms of the protein folds, whereas the homolgy of the primary sequences of different species are low (Witke, 2004). Alignment of *P. monodon* profilin with selected profilins showed that many of the conserved residues known to be involved in specific functions (Wilkes and Otto, 2000), e.g., PLP binding, actin binding, fold conservation, and conserved (+) patch, were predicted in the *P. monodon* sequences (Figure 3.10).

3.2.3 Confirmation of Differential display PCR by real-time RT-PCR

To verify the changes of expression level of some genes upon *V. harveyi* challenge observed by mRNA differential display and to detemine their expression level in haemocytes at various time post-injection, 6 differentially expressed genes including caspase 3B, lysozyme, glucose transporter1, interferon-related developmental regulator 1 (IFRD 1), profilin, and SERPINB3 were further evalutated by real-time RT-PCR. In this study, the housekeeping gene, elongation factor alpha-1 was used as a reference gene. cDNAs of pooled total RNA of 10 individuals at each time point (0, 6, 24, 48 hour post-injection (hpi) from both saline- and *V. harveyi*-injected haemocytes were used for real-time RT-PCR reaction.

To determine the amplification effiiciency of selected genes, serial dilutions of pooled cDNA of normal animal were made (5 to 5^5 dilutions for caspase 3B, GLUT 1, and IFRD 1; 1 and 5 to 5^4 dilutions for SERPINB3; 10 to 10^5 dilutions for lysozyme,

Figure 3.5 Alignment of selected caspase deduced amino acid sequences. The putative caspase 3B (DD70-2) of *P. monodon* (Pm caspase3b) obtained from DD-PCR was compared to caspase 3B of *Hydra vulgaris* (Hv caspase3b, accession no. AAF98012), caspase 8 of *Ictalurus punctatus* (Ip caspase8, accession no. AAT37512), and caspase 8 of *Mus musculus* (Mm caspase8, accession no. NP_033942).* indicates amino acid identity and · as well as : indicate amino acid similarity. A conserved catalytic residues are underlined. Two catalytic domains: the p20 subunit and the p10 subunit, were indicated by the gray and the black boxes, respectively.

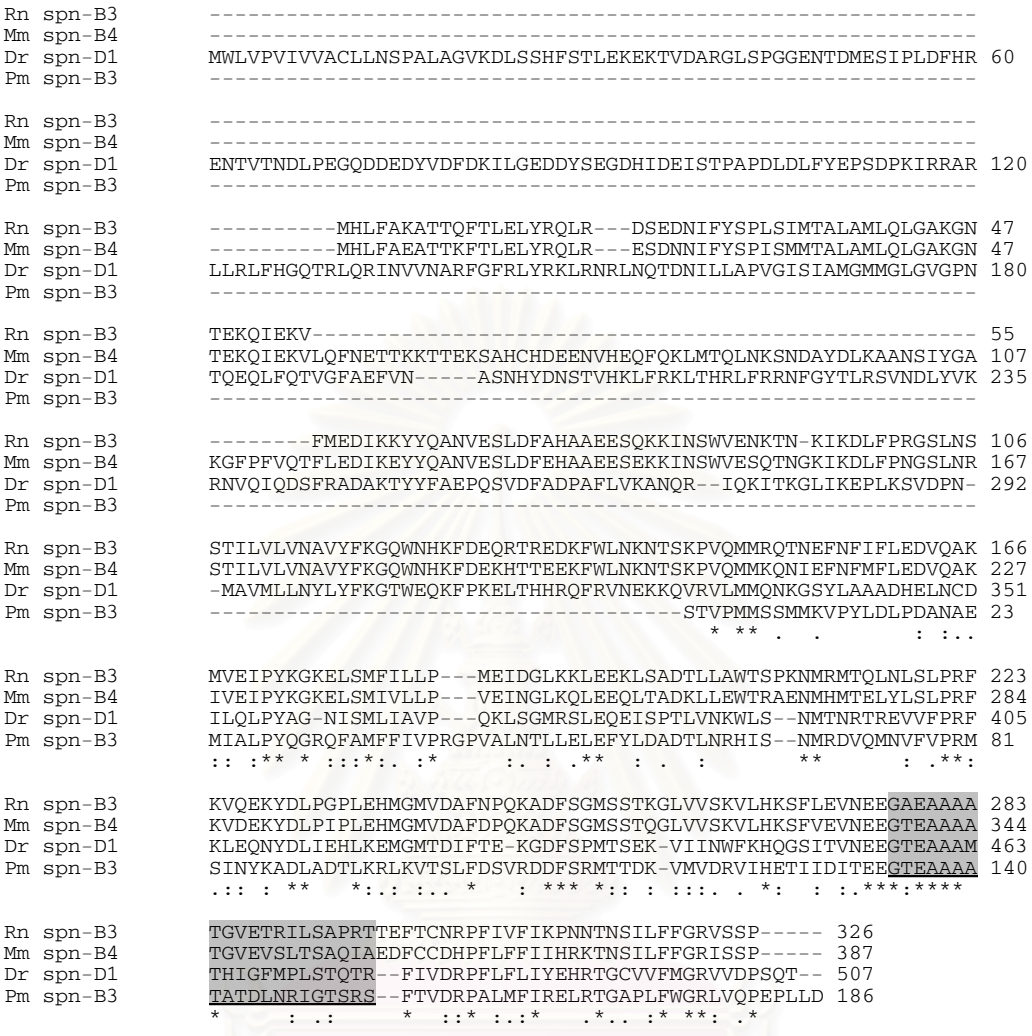


Figure 3.6 Alignment of selected SERPIN deduced amino acid sequences. The putative SERPINB3 (DD46-1) of *P. monodon* (Pm spn-B3) obtained from DD-PCR was compared to SERPIND1 of *Danio rerio* (zebrafish) (Dr spn-D1, accession no. NP_878300), SERPINB3 of *R. norvegicus* (Rn spn-B3, accession no. NP_001008887), and SERPINB4 of *M. musculus* (Mm spn-B4, accession no. NP_033152). * indicates amino acid identity and · as well as : indicate amino acid similarity. The predicted RSL of SERPINB3 (P15-P5') is underlined.

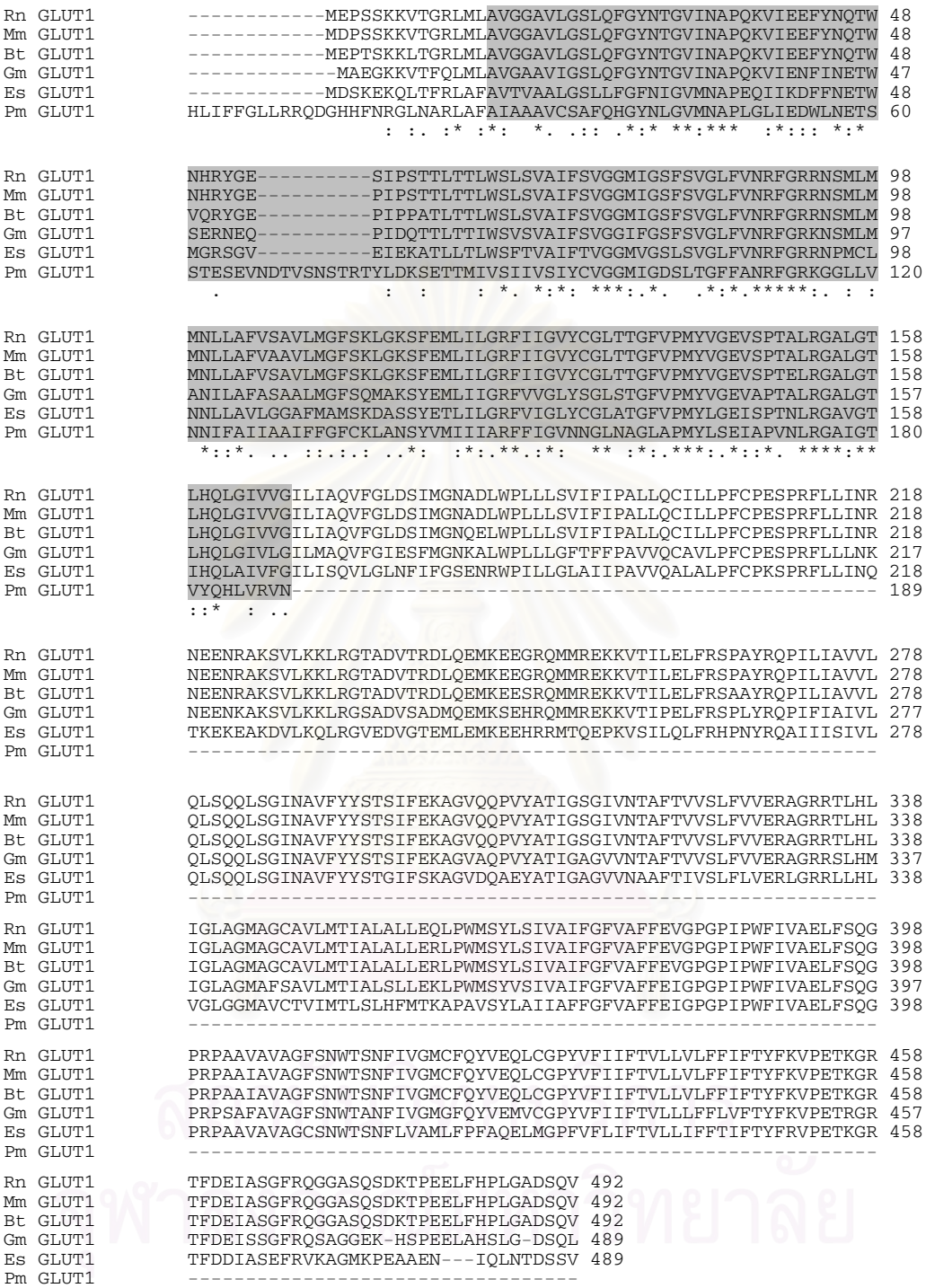


Figure 3.7 Deduced amino acid sequence alignment of selected GLUT1. The putative GLUT1 (DD51-1) of *P. monodon* (Pm GLUT1) obtained from DD-PCR was compared to GLUT1 of *Gadus morhua* (Atlantic cod) (Gm GLUT1, accession no. AAS17880), *R. norvegicus* (Rn GLUT1, accession no. AAH61873), *M.*

musculus (Mm GLUT1, accession no. AAH55340) and *Eptatretus stoutii* (Pacific hagfish) (Es GLUT1, accession no. AAL27090). * indicates amino acid identity and · as well as : indicate amino acid similarity. The underlined sequences are MFS domains are highlighted in gray.

```
Hs IFRD1          MPKNKKRNTPHRGSSAGGGGSGAAAATAATAGGQHRNVQPFSDEDASIETMSHCSGYSDP 60
Mm IFRD1          MPKNKKRNAPHRG--GGGGGGSGAATSAATAGGPHRTVQPFSDEDASIETMSHCSGYSDP 58
Rn IFRD1          MPKNKKRNAPHRG--GGGGGGSGAATSAATTGGPHRTVQPFSDEDASIETMSHCSGYSDP 58
Pm IFRD1          ------------------EDKPAAWLTAAAA-------LPVADD----DTASITSDKTDS 31
                                    .  .*  :**::        *.:*:    :* *  *. :*.
Hs IFRD1          SSFAEDGPEVLDEEGTQEDLEYKLKGLIDLTLDKSAKTRQAALEGIKNALASKMLYEFIL 120
Mm IFRD1          SSFAEDGPEVLDEEGTQEDLEYKLKGLIDLTLDKSAKTRQAALEGLKNALSSKVLYEFVL 118
Rn IFRD1          SSFAEDGPEVLDEEGTQEDLEYKLKGLIDLTLDKSAKTRQAALEGVKNALSSKVLYEFVL 118
Pm IFRD1          TAQITDGEEGLS---EMETYEQKVREAMDLALEKSVQTRTNALTALTTAFQKRVMTQFLL 88
                  ::   ** * *.     *  * *::  :**:*:**.:**  ** .:..*: .::: :*:*
Hs IFRD1          ERRMTLTDSIERCLKKGKSDEQRAAAALASVLCIQLGPGIESEEILKTLGPILKKIICDG 180
Mm IFRD1          ERRMTLTDSIERCLKKGKSDEQRAAAAVASVLCIQLGPGFESEEILKTLGPILKKIICDG 178
Rn IFRD1          ERRMTLTDSIERCLKKGKSDGQRAAAALASVLCIQLGPGLESEEILKTLGPILKKIICDG 178
Pm IFRD1          DNYQTITDLVERSLRKGRGPEQVAAARLATMLVVSLSPVAEAEKVFKTLNPILTMLVTDP 148
                  :.  *:** :**.*:**:.  * *** :*::* :.*.*  *:*:::***.***. :: *
Hs IFRD1          SASMQARQTCATCFGVCCFIATDDITELYSTLECLENIFTKSYLKEKDTTVICSTPNTVL 240
Mm IFRD1          AASIQARQTCATCFGVCCFIATDDITELYSTLECFENIFTKSYLKEKDTNVTCSTPNTVL 238
Rn IFRD1          TASIQARQTCATCFGVCCFIATDDITELYSTLECLEGIFTKSYLKEKDTNVPCSTPNTVL 238
Pm IFRD1          SAAVAARQDGALAMASSSFSACHDLADVTAAMNTLHSLFAGSLPKGNGELPNHPPAVSAM 208
                  :*:: ***  * .:. ..* * .*:::: :::: :..:*: *  * :.     ... :.:
Hs IFRD1          HISSLLAWTLLLTICPINEVKKKLEMHFHKLPSLLSCDDVNMRIAAGESLALLFELARGI 300
Mm IFRD1          HISSLLAWTLLLTICPINEVKKKLELHFHKLPSLLSCDDVNMRIAAGESLALLFELARGM 298
Rn IFRD1          HISSLLAWTLLLTICPISEVKKKLELHFHKLPSLLSCDDVNMRIAAGESLALLFELARGM 298
Pm IFRD1          HTAFSEG---------------------------------------------------- 215
                  * :   .
Hs IFRD1          ESDFFYEDMESLTQMLRALATDGNKHRAKVDKRKQRSVFRDVLRAVEERDFPTETIKFGP 360
Mm IFRD1          ESDFFYEDMDSLTQMLRALATDGNKHRAKVDKRKQRSVFRDVLRAVEERDFPTETVKFGP 358
Rn IFRD1          ESDFFYEDMDSLTQMLRALATDGNKHRAKVDKRKQRSVFRDVLRAVEERDFPTETVKFGP 358
Pm IFRD1          ------------------------------------------------------------

Hs IFRD1          ERMYIDCWVKKHTYDTFKEVLGSGMQYHLQSNEFLRNVFELGPPVMLDAATLKTMKISRF 420
Mm IFRD1          ERMYIDSWVKKHTYDTFKEVLGSGMQYHLQTNEFLRNVFELGPPVMLDAATLKTMKISRF 418
Rn IFRD1          ERMYIDSWVKKHTYDTFKEALGSGMQYHLQTNEFLRNVFELGPPVMLDAATLKTMKIPRF 418
Pm IFRD1          ------------------------------------------------------------

Hs IFRD1          ERHLYNSAAFKARTKARSKCRDKRADVGEFF 451
Mm IFRD1          ERHLYNSAAFKARTKARSKCRDKRADVGEFL 449
Rn IFRD1          ERHLYNSAAFKARTKARSKCRDKRADVGEFF 449
Pm IFRD1          --------------------------------
```

Figure 3.8 Deduced amino acid sequence alignment of selected IFRD1. The putative IFRD1 (DD78-2) of *P. monodon* (Pm IFRD1) obtained from DD-PCR was compared to IFRD1 of *H. sapiens* (Hs IFRD1, accession no. AAH01272), *M. musculus* (Mm IFRD1, accession no.AAH43723), and *R. norvegicus* (Rn IFRD1, accession no. NP_062115). * indicates amino acid identity and · and : indicates amino acid similarity. The first conserved domain of the protein family is highlighted in gray.

```
Pm lysozyme      MRVLPLALLVGLLAVSDAKVFRKCEFAELLETRYYLSRNDIKNWVCIAEFESSFNTAAIN 60
DD20-4           ------ALLVGLLAVSDAKVFRKCEFAELLETRYYLSRNDIKNWVCIAEFESSFNTAAIN 54
Mr lysozyme      MRVLPLALLVGLLAVSDAKVFRKCEFAELLETRYYLSRNDIKNWVCIAEFESSFNTAAIN 60
Lv lysozyme      MRVLPLALLGALLAASDAKVFGKCEFAELLKRDYYLSNDDIKNWVCIAEFESSFNTAAIN 60
Mj lysozyme      MRVLPLLLLVALVGASEAKIFRKCEFAELLERRYRLSREDIKNWVCIAEYESSFNTGAIN 60
                        ** .*:..*:**:* ********:  * **.:**********:******.***

Pm lysozyme      RNRNRSTDYGIFQINNKYWCGSDYGKNVCGIPCSDLMSDDITAAVRCAETVRRDTERYKG 120
DD20-4           RNRNRSTDYGIFQINNKYWCGSDYGKNVCGIPCSDLMSDDITAAVRCAETVRRDTERYMG 114
Mr lysozyme      RNRNRSTDYGIFQINNKHWCGSDYGKNVCGIPCSDLMSDDITAAVRCAETVRRDTERYKG 120
Lv lysozyme      RNRNRSTDYGIFQINNKYWCGSDYGKNVCKIPCSDLMSDDITEALRCAETIRRDTERFRG 120
Mj lysozyme      RNRNRSVDYGIFQINNKYWCDSSYGKNACGIPCSDLMSDDITAALRCAEAVRRDTEGFRG 120
                 ******.**********:**.*.****.* ************ *:****::***** : *

Pm lysozyme      RGKGYTAWVAYNSKCKNRDLDQYMAECWSRGSNSIFPF 158
DD20-4           RGKGYTAWVAYNSKCKNRDLDQYMAECWSRGSNSIFPF 152
Mr lysozyme      RGKGYTAWVAYNSKCKNRDLDQYMAECWSRGSNSVFPF 158
Lv lysozyme      RGKGYSAWVAYNSKCKNRDLDQYMAECWSHGSNSVFPF 158
Mj lysozyme      RGNGYTAWVAYNNKCKNRDLDRYMADCWSRPSNSIYPF 158
                 **:**:******.********:***:***: ***::**
```

Figure 3.9 Alignment of selected lysozyme deduced amino acid sequences. Catalytic residues are indicated by gray regions. A putative lysozyme (DD20-4) of *P. monodon* obtained from DD-PCR and GenBank database (accession no. AAN16375) are compared to those of *L. vannamei* (accession no.AAL23948), *Marsupenaeus japonicus* (accession no. BAC57467), and *Macrobrachium rosenbergii* (giant freshwater prawn) (accession no. AAP13577). * indicates amino acid identity and · and : indicates amino acid similarity. The black box indicates a single amino acid difference between the lysozymes of *P. monodon*.

Figure 3.10 Alignment of profilin deduced amino acid sequences. The putative profilin (DD17-1 and DD17-3) obtained from DD-PCR was compared to a full length clone from *P. monodon* haemocyte EST library (Pm profilin) and to those of *L. vannamei* (Lv profilin, accession no. BE188423), *Drosophila pseudoobscura* (Dp profilin, accession no. EAL34274), and *Branchiostoma belcheri* (Bb profilin, accession no. AAL75808). * indicates amino acid identity and · and : indicates amino acid similarity. The gray and black boxes indicate amino acid residues involved in PLP binding and fold conservation, respectively. The bold letters represent the conserved (+) patch. The underlined residues function as the actin binding residues.

profilin and EF-1α). Real time PCR efficiency was calculated from the slope, obtained from the curve plotted between five dilutions of cDNA (log scale) of normal animal with threshold cycle (Ct) (Figure 3.11), using the equation, $E = 10^{[-1/slope]}$. The real-time PCR efficiencies shown in Table 3.3 revealed unequal of efficiencies between them, and imperfect amplification (efficiencies were not 2.00) except for IFRD 1 gene. Therefore, relative expression ratios of certain gene were calculated using equation 1 to correct differences in efficiency. The specificity of real-time PCR results was assessed by dissociation curve analysis. Dissociation curve of each gene showed the single peak at expected melting temperature indicating that the intended gene was amplified and there are no non-specific amplification or primer-dimers (Figure 3.12).

The mRNA expression levels of each gene were determined by normalizing the Ct values of the pooled sample of *V. harveyi*-infected shrimp haemocytes with saline-infected shrimp haemocytes. The expression ratios of certain gene at each time point after infection were calculated relative to EF-1α and shown in Figure 3.13. For the selected genes, based on ratios at 0 hour post-injection (hpi), we found that all of them were up-regulated at 6 hpi with 8.29, 6.82, 3.58, 2.29, 2.12, and 1.93 folds for GLUT 1, caspase 3B, IFRD 1, lysozyme, profilin, and SERPINB3, respectively. Comparison of the fold changes of each gene at 48 hpi and at 0 hpi revealed significantly increasing in their expression levels. The fold changes at 48 hpi of GLUT 1, caspase 3B, IFRD 1, lysozyme, profilin, and SERPINB3 were 2.68, 2.71, 4.03, 4.48, 2.45, and 2.97, respectively. Figure 3.13 showed the expression levels of each gene at various time after microbial challenge suggesting that caspase 3B gene and GLUT 1 gene responsed to the invading at the early phase, while SERPINB3 transcripts increased gradually form 6 to 48 hpi In addition, lysozyme, profilin and IFRD 1 mRNAs expression rised at 6 hpi and fall down at 24 hpi before climbed up again at 48 hpi. The real-time PCR results, therefore, evaluated the expression levels of each transcript and indicated that the selected genes were differentially expressed in haemocytes of *P. monodon* upon microbial infection.

Due to the average fold change in the expression level of DD-PCR was calculated from the band intensity of 40 hpi- and normal shrimp haemocytes. The

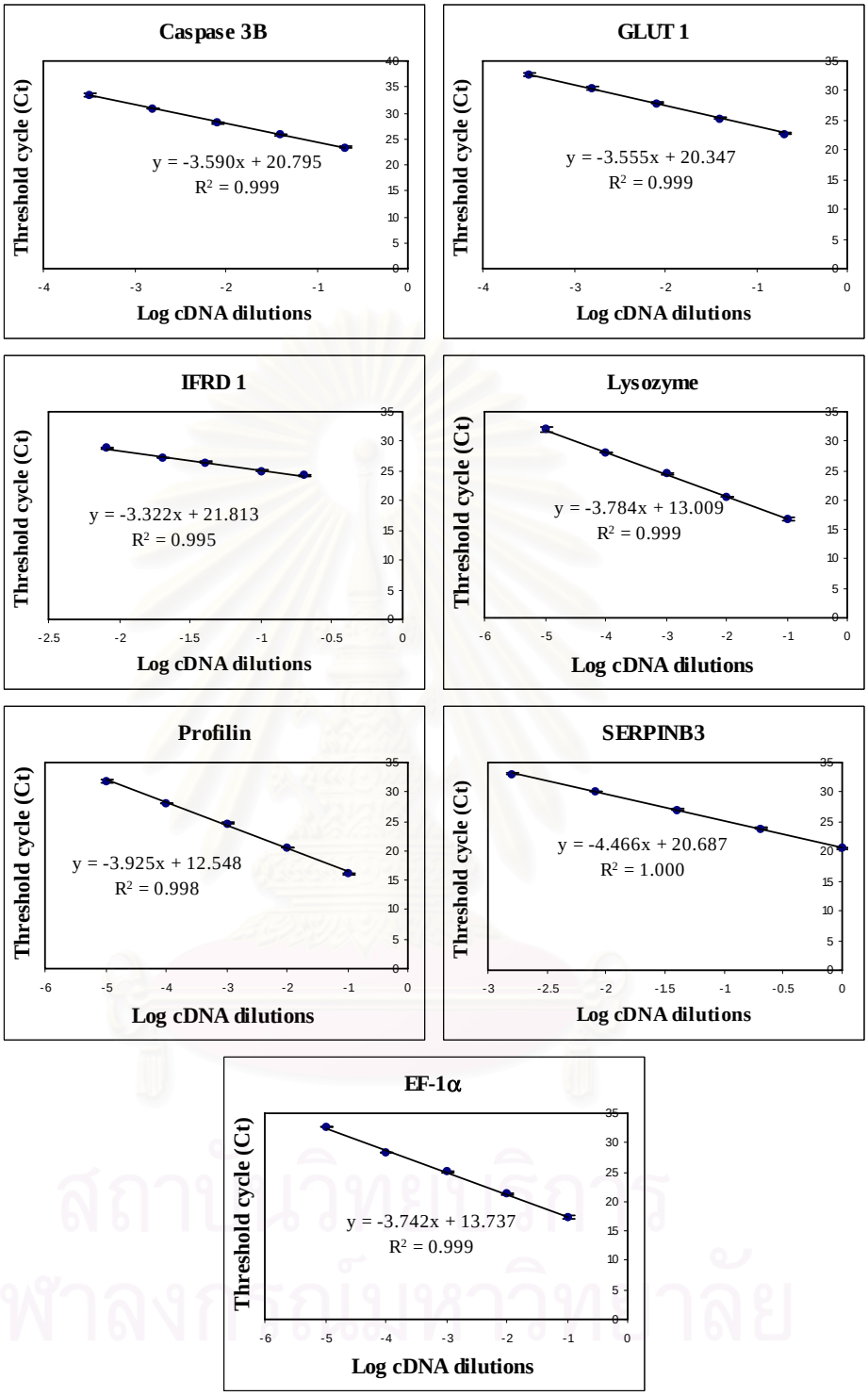


Figure 3.11 Amplification efficiency curves of target genes; caspase 3B, GLUT 1, IFRD 1, lysozyme, profilin, and SERPINB3, and reference gene EF-1α.

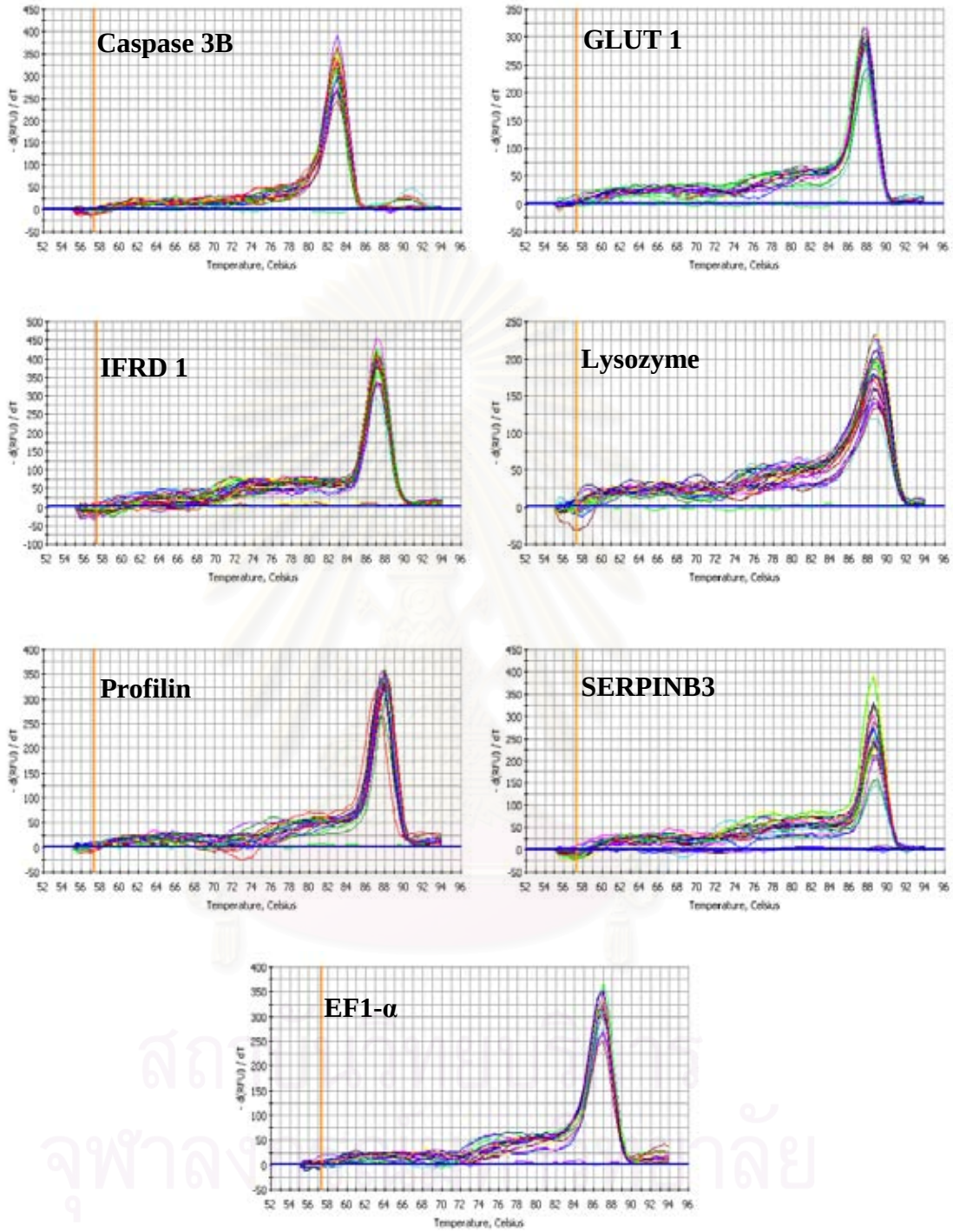


Figure 3.12 The dissociation curves of target genes; caspase 3B, GLUT 1, IFRD 1, lysozyme, profilin, and SERPINB3, and reference gene EF-1α.

results are the representative data of changes in gene expression at late phase of infection, therefore, for comparison DD-PCR results with real-time PCR, fold changes of gene expression of each gene at 48 hpi based on at 0 hpi were calculated from relative expression ratios obtained from real-time PCR results. The validated results in Table 3.4 shown that all selected genes were up-regulated upon *V. harveyi* challenge. These indicated that some results from DD-PCR were not agree with those from real-time PCR such as the results of lysozyme and SERPINB3.

Table 3.3 The PCR efficiency for each amplified gene and melting temperature of its product

Gene name	Product size (bp)	PCR efficiency	Melting temperature(˚C)
Caspase3B	169	1.90	83.0
IFRD1	263	2.00	87.0
GLUT1	210	1.91	88.0
Lysozyme	188	1.83	88.5
Profilin	252	1.87	88.0
SERPINB3	157	1.67	88.5
EF-1α	132	1.85	87.0

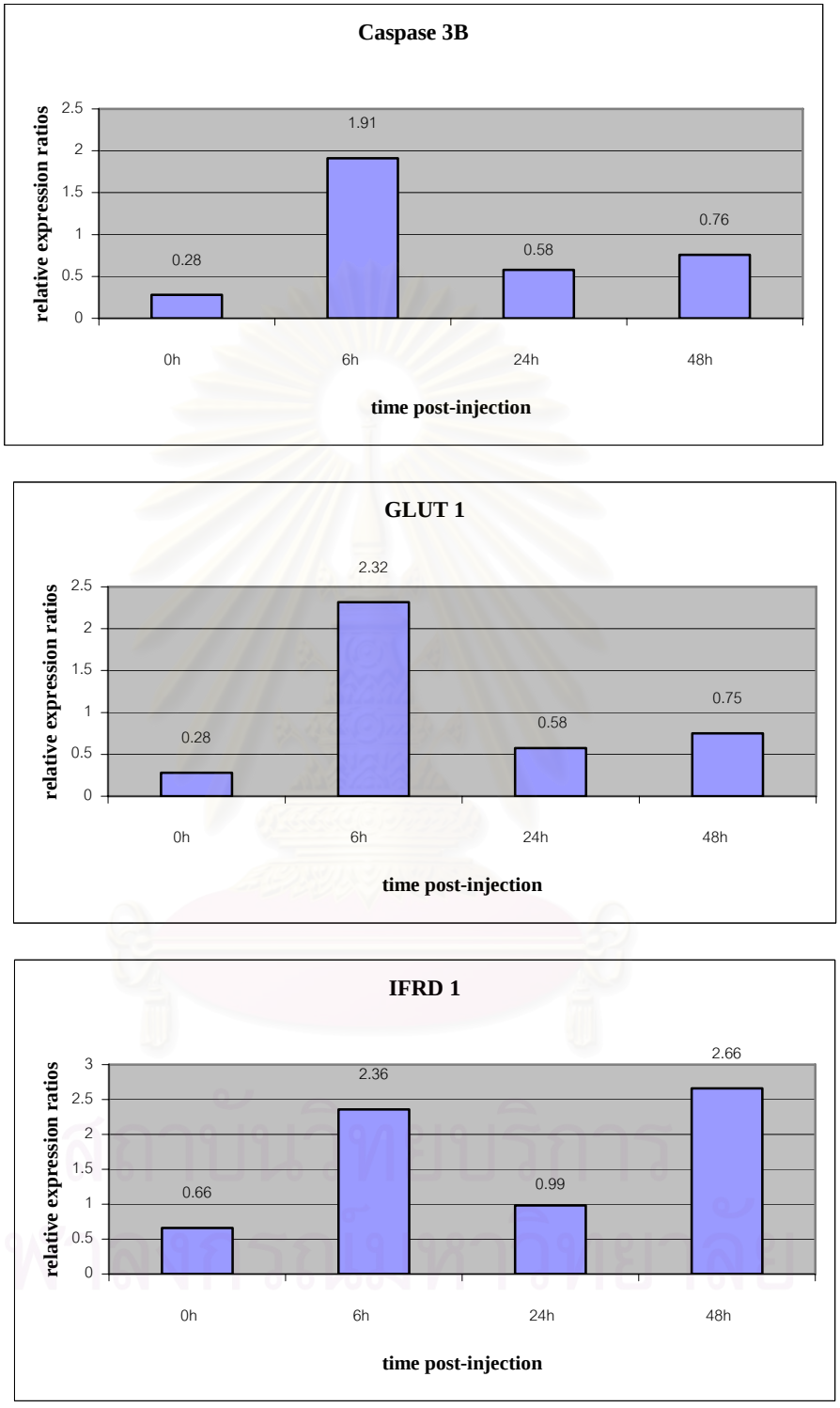


Figure 3.13 Verification of differential expressed genes using real-time PCR.

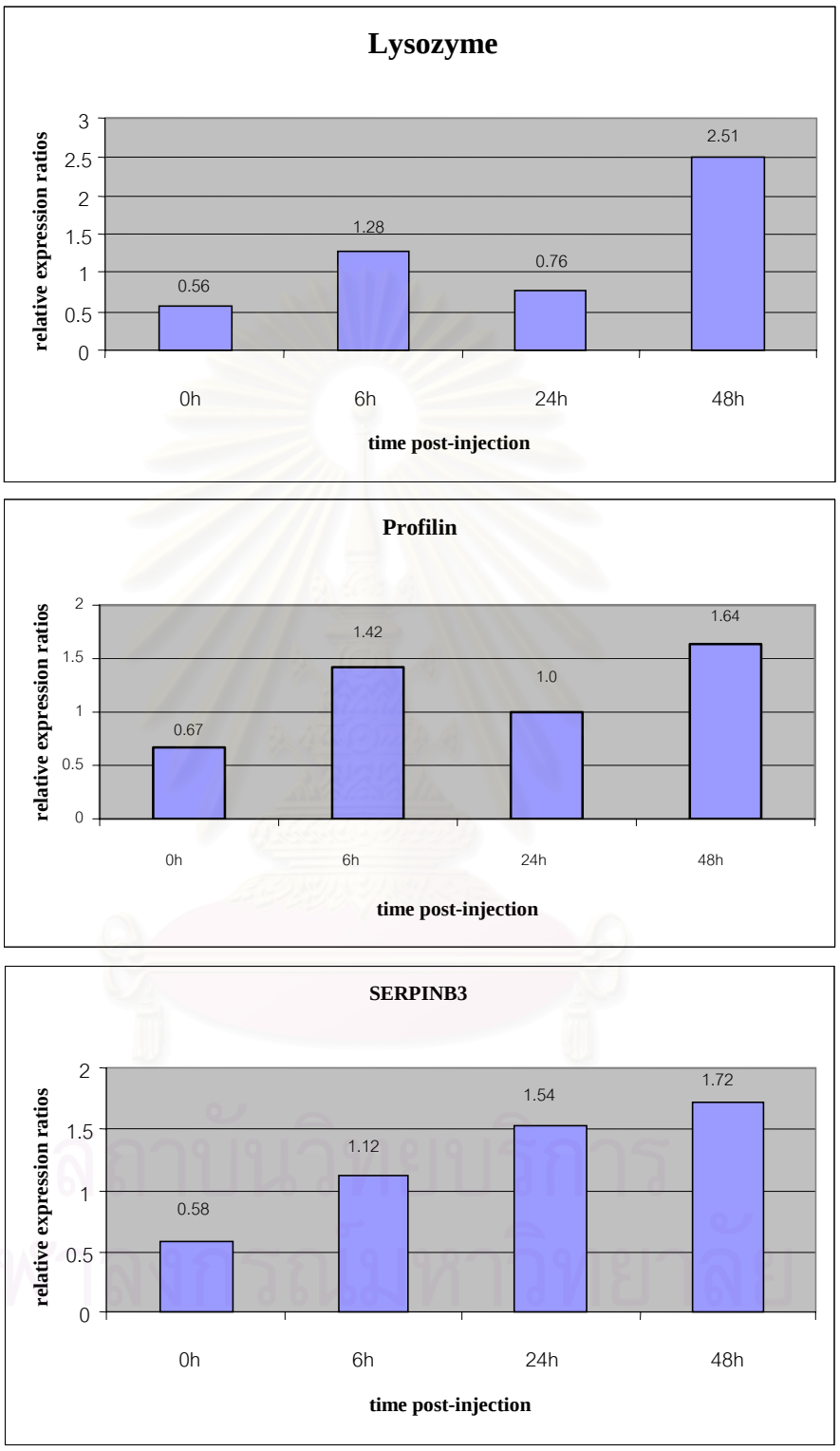


Figure 3.13 Verification of differential expressed genes using real-time PCR. (continued)

Table 3.4 Validation of DD-PCR results by real-time PCR

Gene name	fold change		expression of gene upon microbial challenge[c]
	DD-PCR[a]	Real time PCR[b]	
Caspase3B	+1.87	+2.71	up
IFRD1	+2.43	+4.03	up
GLUT1	+2.13	+2.68	up
Lysozyme	+1.91/-1.35*	+4.48	up
Profilin	+3.36	+2.45	up
SERPINB3	-2.0	+2.97	up

[a]Fold change of differentially expressed band corresponded to each gene at 40 hour post-injection.

[b]Fold chage of relative expression ratios of *V. harveyi* injected sample at 48 hour post-injection to that of 0 hour post-injection derived from real-time PCR results.

[c]The expression of gene upon microbial challenge assigned according to the more reliable real-time PCR result.

*a putative lysozyme cDNA obtained from 2 differentially expressed bands,

DD19 and DD20, which had a different fold change.

3.3 Expression of anti-LPS factor type3 in the *Pichia pastoris* expression system

Anti-LPS factor (ALF), an antimicrobial peptide, were previously identified only in the horseshoe crabs. In shrimp, *P. monodon*, five different isoforms of ALF genes were identified. ALF type3 or ALF*Pm*3 gene was identified by EST approach in both libraries of *V. harveyi* infected and uninfected *P. monodon* haemocytes with the highest redundancy. Moreover, in this study, ALF*Pm*3 transcripts were identified as differentially expressed genes which significantly responded to *V. harveyi* infection. These finding led us to focus on ALF*Pm*3 which should be an important antimicrobial effector in shrimp immunity. To further characterize the function of this effector, sufficient amount of the recombinant molecule is needed. The *P. pastoris* expression system was therefore selected for production of recombinant ALF*Pm*3.

3.3.1 Preparation of anti-LPS factor type 3 gene

The region encoding for ALF mature protein was amplified from ALF*Pm*3 cDNA, clone sh71 from normal shrimp EST library. The PCR product was separated on 1.5% agarose gel to determine the size of a specific 324 bp fragment (Figure 3.14). Primers used for amplification would incorporate 5' *Sna*BI and 3' *Not*I cleavage sites to the gene. The amplified product was further used for construction of expression plasmid. The deduced N-terminal amino acid sequences of the recombinant protein would be YVQGWEAVAAAV which Y and V came from cloning vector sequences (Figure 3.15). The predicted molecular weight of the recombinant protein is about 11.3 kDa.

3.3.2 Preparation of expression vector

The amplified products were cut with *SnaB*I and *Not* I, respectively, and then ligated further into the *SnaB*I/*Not*I-digested pPIC9K. The ligated product was transformed to *E. coli* TOP10 cells. Transformants were randomly picked. To confirm the cloning success, plasmids were extracted and then cut with *Bgl*II, *Not*I, and *Bam*HI. The expected product contained four fragments of 5639, 2403, 937 and 597 bp for recombinat plasmid, and of 5639, 2403, 937 and 350 bp for pPIC9K vector. Figure 3.16 showed the digested products of recombinat plasmid and pPIC9K ran on 1.7% agarose gel electrophoresis. The recombinant plasmid was then subjected to sequencing to verify the sequences of the clone gene. The sequencing result of constucted plasmid, pALF*Pm*K, confirmed its correct orientation. The pALF*Pm*K plasmid contained the coding sequence for mature ALF*Pm*3 with additional Tyr and Val at NH_2-terminus, fused in-frame with the α-factor signal and double Glu-Ala repeats (Figure 3.15).

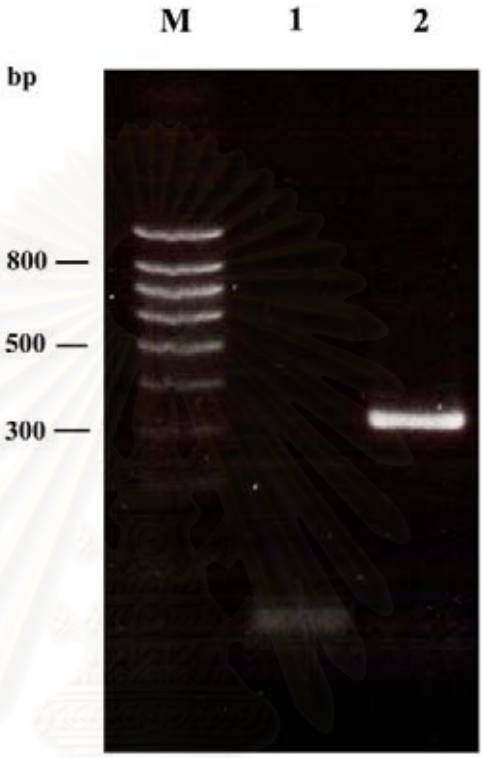


Figure 3.14 Agarose gel electrophoresis of ALF*Pm*3 gene amplified by PCR. The PCR product was run on a 1.5 % agarose gel at 100 volts for 1 hour.

Lane M : Standard DNA ladder (100 bp marker)

Lane 1 : negative control

Lane 2 : amplified ALF*Pm*3 gene products

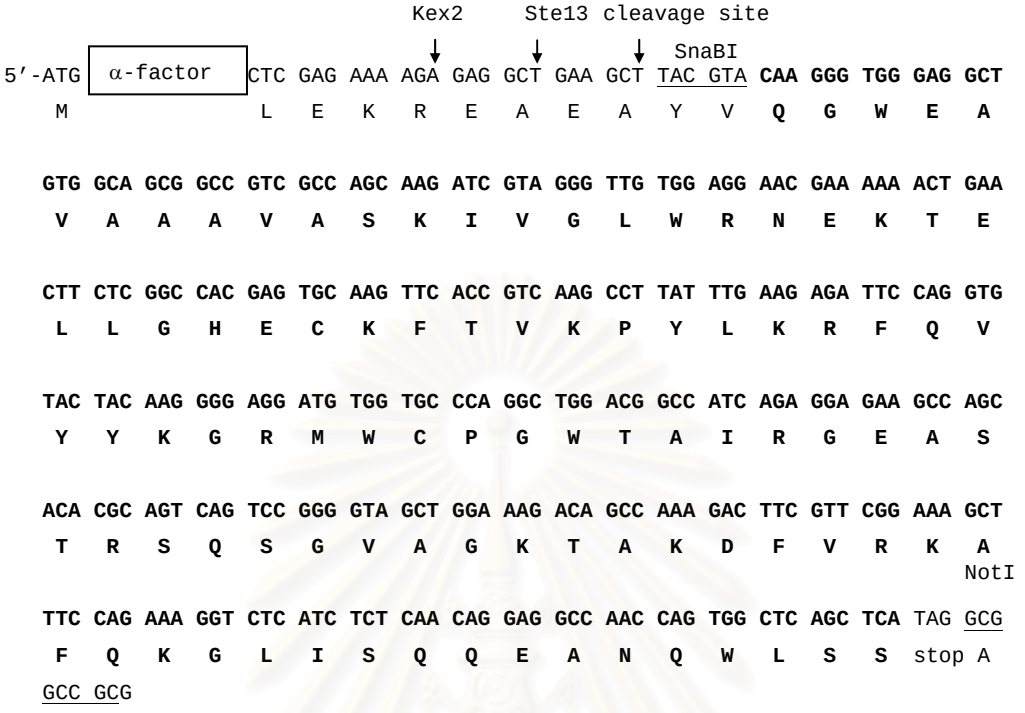


Figure 3.15 Restriction map of the expression vector pALF*Pm*K. The mature peptide of ALF*Pm*3 gene, shown in bold, was inserted between *Sna*B I and *Not* I sites, underlined, of *SnaB*I/*Not*I digested pPIC9K vector, which in-frame with α-factor secretion signal sequence and two Glu-Ala repeats. Arrows above the nucleotide sequence indicate the Kex2 and Ste13 clevage sites necessary for proteolytic processing of the fusion protein formed by the α-factor secretion signal and ALF*Pm*3. Nucleotide (upper) and deduced amino acid sequences (lower) are shown.

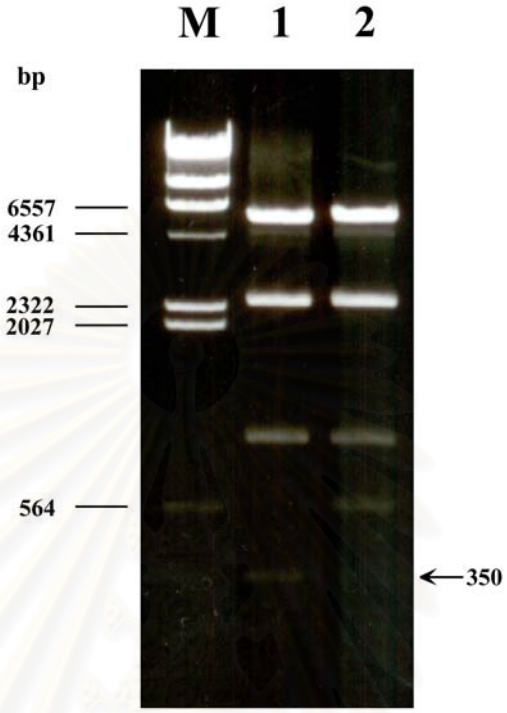


Figure 3.16 Verification of expression plasmid containing ALF*Pm*3 gene

Lane M : DNA marker *(λDNA/Hind* III)

Lane 1 : plasmid pPIC9K/*Bgl*II/*Bam*HI/*Not*I

Lane 2 : plasmid pALF*Pm*3K/*Bgl*II/*Bam*HI/*Not*I

3.3.3 Transformation of recombinant plasmid with ALF*Pm*3 gene into yeast cells

The pALF*Pm*K plasmid were linearized with *Sac*I and then transformed to *P. pastoris* KM71 by electroporation to generate the recombinant ALF*Pm*3 (rALF*Pm*3). Transformants were screened on YPD containing various concentration of G418-sulfate. Resistant clones were observed on 0.25, 0.75, and 1 mg/ml YPD-G418-sulfate plates. Three rALF*Pm*3 clones were randomly picked from plates at each concentration of G418-sulfate for expression analysis. The presence of ALF*Pm*3 expression cassette in *P. pastoris* transformants was confirmed by PCR amplification. The parental plasmid pPIC9K will produce a 195 bp PCR product, therefore, the expected sizes of PCR product of clones containing ALF*Pm*3 gene are 498 bp (195 bp + 303 bp). The results indicated that all selected transforming clones have ALF*Pm*3 genes integrated into *P. pastoris* genome (Figure 3.17). Although the number of copies of pALF*Pm*k constructs integrated to *Pichia* genome has not yet been determined, the transformants which can grow on YPD plates supplemented with G418-sulfate at 1 mg/ml, were considered as high-copy-number transformants.

3.3.4 Expression of *Pichia* clones

Single colony of each clone were grown in YPD broth, at 30ºC overnight. The starter was innoculated in BMGY media to increase biomass then cells were harvested and concentrated to induce expression in BMMY media. Methanol 100% was added to a final concentration of 0.5% (w/v) every 24 hours to maintain the induction. Using α-factor signal, rALF*Pm*3 was secreted into the culture medium. The signal peptide was proteolytically removed by KEX2 and STE13 proteases in the *P. pastoris* secretory pathway (Cereghino and Cregg, 2000). One milliliter of each expression culture was collected at each time point (0, 1, 2, 3, 4, 5, 6 days) to analyze supernatant for secreted recombinant protein. The silver stained SDS-PAGE of culture supernatant of the transformed *Pichia* clones revealed major band of approximately 14 kDa, which is larger than the expected size (11.3 kDa) for ALF*Pm*3 peptide but this band was not present in the culture supernatant of *P. pastoris* transformed with

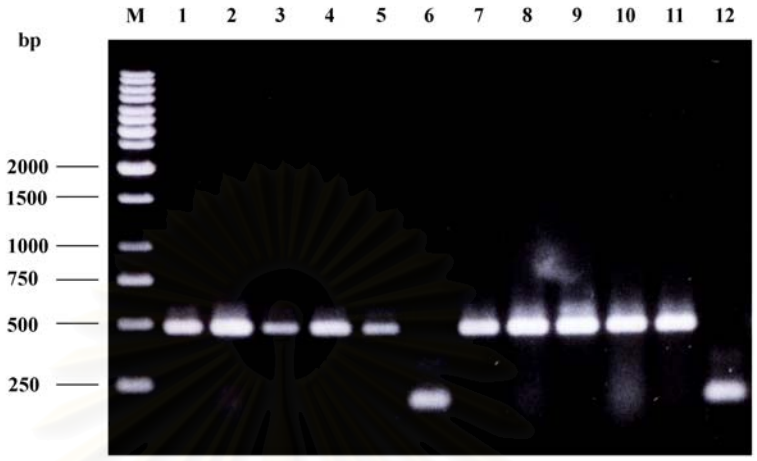


Figure 3.17 Screening of *Pichia* transformant clones containing ALF*Pm*3 genes integrated into *P. pastoris* genome by Colony PCR.

Lane M : 1 kb DNA Ladder

Lanes 1-5 : PCR product of 0.75 mg/ml G418 resistant *Pichia* transformants

Lanes 7-11 : PCR product of 1 mg/ml G418 resistant *Pichia* transformants

Lanes 6 and 12 : PCR product of *Pichia* clone transformed with parental plasmid pPIC9K

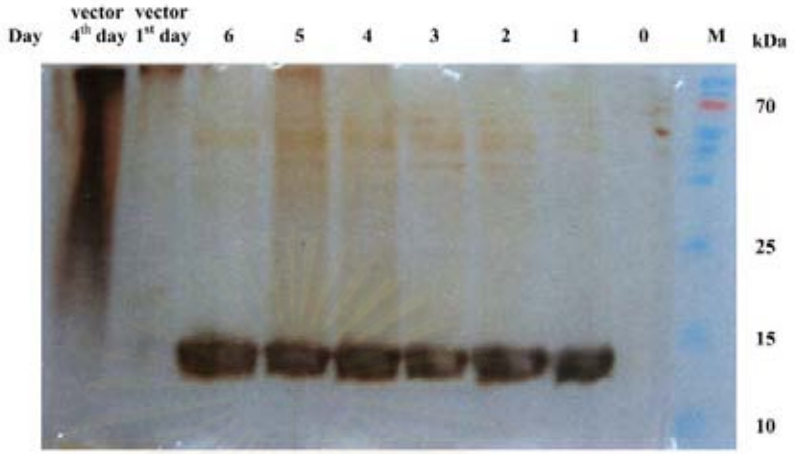


Figure 3.18 The silver stained 15% SDS-PAGE analysis of culture medium of ALFK9 *Pichia* clone with respective to cultivation time. ALFK9 was cultivated in BMGY medium and induced the expression of protein with 0.5% methanol. The supernatant was sampling every 24 hours during 6 days.

pPIC9K parental vector (Figure 3.18). However, no obvious protein band could be observed in other *Pichia* transformants. The clone which showed a highest expression level of rALF*Pm*3, namely ALFK9, was selected for a large-scale production in order to get enough material for purification and characterization (Figure 3.18). The preliminary antimicrobial assay of the culture supernatant were performed against gram-negative bacteria, *E. coli* 363, and gram-positive bacteria, *Bacillus megaterium* and *Micrococcus luteus*, using liquid broth assay. The rALF*Pm*3 could inhibit growth of all tested strains. Comparing to *B. megaterium* and *M. luteus*, inhibition of growth of *E. coli* 363 required lower amount of crude rALF*Pm*3. These results indicated that the functional rALF*Pm*3 were produced.

3.3.5 Detection of recombinant protein in crude supernatant by Western blot analysis

To identify whether the specific band corresponding to the rALF*Pm*3 protein expressed by ALFK9 clone, Western blot analysis was performed. The crude supernatant from ALFK9 was electrophoresed on 15%SDS-PAGE, and subsequently transferred to nylon membrane. The rALF*Pm*3 protein was hybridized with mouse anti-synthetic ALF peptide polyclonal antibody and second antibody, goat anti-mouse IgG linked with alkalinephosphatase. The rALF*Pm*3 protein was detected with colorimetric method using NBT/BCIP as substrate (Figure 3.19). The size of the specific band is about 14 kDa which is the same as a major band observed in Figure 3.18. This result confirmed the expression of rALF*Pm*3.

3.4 Purification of recombinant ALF*Pm*3 (rALF*Pm*3)

ALFK9 *Pichia* transformant was selected by G418-sulfate resistace screening and small scale cultured flask production. Large-scale production was carried out in fermenter by our collaborator, Mickaël Marcos, and Helene Boze (ENSAM/INRA, France). Supernatant was seperated by centrifugation for further purification. Weak cation-exchange catridge and reverse-phase HPLC purification C_8 analytic column were applied for rALF*Pm*3 purification. Supernatant of ALFK9 from a 5 L bioreactor

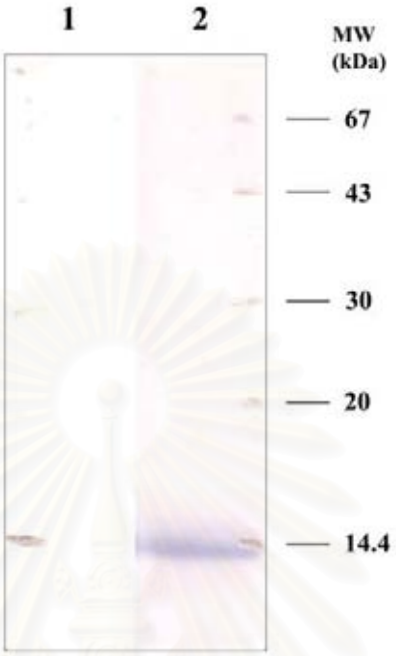


Figure 3.19 Western blot analysis of ALF*Pm*3 expressed by ALFK9 clone. A culture supernatant of ALFK9 clone was subjected to 15 % SDS-PAGE, blotted to PVDF membrane and incubated with mouse anti-synthetic ALF peptide polyclonal antibody and then goat-anti-mouse antibody conjugated with alkalinephosphatase. Detection was achieved by colorimetric method using NBT/BCIP as substrate.

Lane 1 :ALFK9 supernatant probed only with anti-mouse antibody conjugated with alkalinephosphatase (negative control)

Lane 2 : ALFK9 supernatant probed with anti-synthetic ALF peptide polyclonal antibody and anti-mouse antibody conjugated with alkalinephosphatase

containing *r*ALFP*m3* at the concentration of 262 $mg.L^{-1}$ with the specific production of 4.7 $mg_{rALFPm3}.g_{Biomass}^{-1}$, was adjusted pH from 3.0 to 7.0 with 5N NaOH, and subjected to weak cation-exchange CM Sep-Pak®catridge (Waters) equilibrated with 10 mM sodium phosphate, 200 mM NaCl, pH 7.0. The rALF*Pm*3 was eluted with 10 mM sodium phosphate, 1 M NaCl, pH 7.0. Eluted fraction was then dialysed overnight against sterile MilliQ water at 4ºC, and lyophilized and kept at -20ºC. The lyophilized powder were resuspended in 0.046% TFA and purified by gradient elution of 2-98% acetonitrile on HPLC C8 analytic column (Waters). According to the chromatogram (Figure 3.20), the major peak is at the retention time of 30.9 minutes, and the HPLC fractions corresponding to the under peak area were collected., pooled, lyophilized, and resuspened in Milli Q water for analyzing on SDS-PAGE and preliminary testing for antimicrobial activity against *E. coli* 363. From the 15% silver stained SDS-PAGE, one band of 14.4 kDa was detected in the pooled fraction number 3 (Figure 3.21). In addition, this fraction had an antibacterial activity against *E. coli* 363. These results indicated that the expected peak was the purified rALF*Pm*3. The purified rALF*Pm*3 were checked by Western blot analysis to confirmed the existent of rALF*Pm*3 (Figure 3.22). The disadvantage of these purification method is that quantity of purified protein is very low, however, the protein is pure enough for antibody production and measurement of MIC value of this molecule. The lyophilized rALF*Pm*3 was also used for N-terminal sequencing to check protein product.

3.5 Characterization of rALF*Pm*3 product

The purified rALF*Pm*3 was further characterized by N-terminal sequencing and Mass spectrometry. Mass measurement by MALDI-TOF mass spectrometry could identify two molecules with molecular mass of 11314.7±0.43 Da and 11514.83±0.54 Da, respectively. The major rALF*Pm*3 product is the molecule with lower molecular mass. Its mass corresponded to the expected calculated average mass (11314.96 Da) of rALF*Pm*3 including additional Tyr and Val residues. The N-terminal amino acid sequences of the major protein product, YVQGWEAVAAAV, revealed the identical sequence to the expected mature rALF*Pm*3 with additional Tyr and Val residues brought by the cloning step. With respected to the minor product of

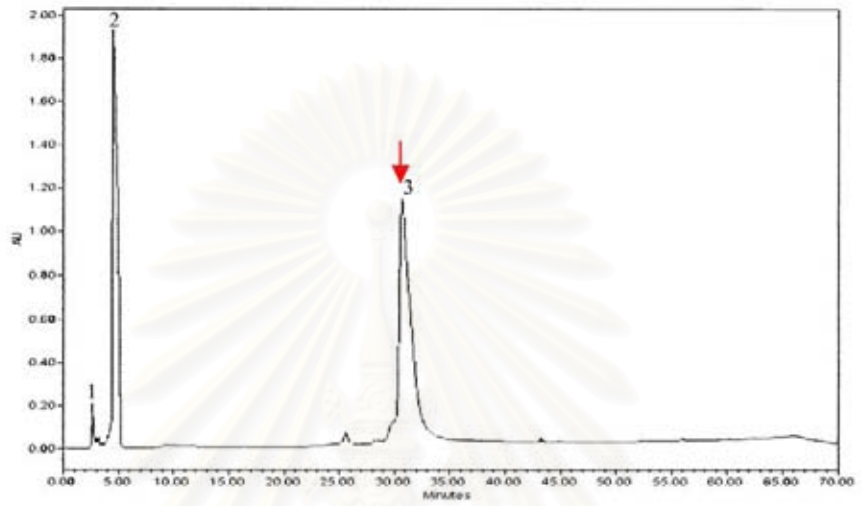


Figure 3.20 HPLC Chromatogram of cation-exchange Sep-Pak®catridge purified fraction subjected to Reverse-phase C8 analytic column. The peak that has the antimicrobial agianst *E.coli* 363 are indicated by arrow.

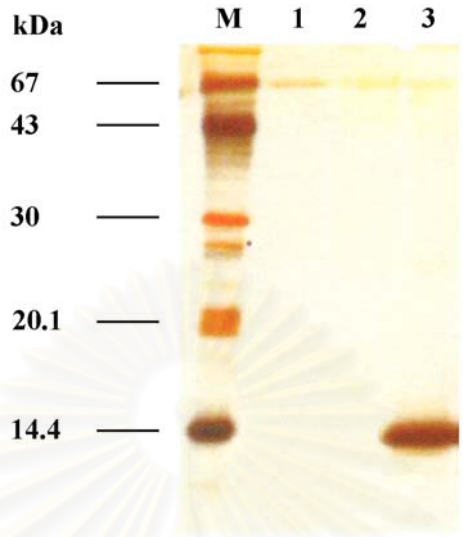


Figure 3.21 SDS-PAGE of rALF*Pm*3 produced in culture medium by *P. pastoris* after HPLC purification.

Lane M : Protein marker

Lane 1 : Pool of the first peak fractions

Lane 2 : Pool of the second peak fractions

Lane 3 : Pool of the third peak fractions

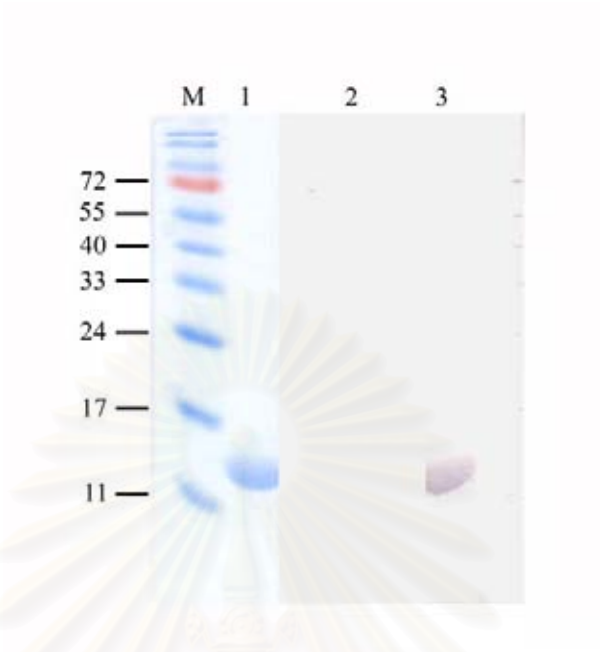


Figure 3.22 Western blot analysis of purified rALF*Pm*3. The protein was separated on 15% SDS-PAGE and transferred into PVDF membrane. The membrane was incubated with rabbit anti-rALF*Pm*3 antibody followed by goat-anti-rabbit IgG conjugated with alkalinephosphatase and detected by colorimetric method using NBT/BCIP substrate.

Lane M : Protein marker

Lane 1 : coomassie stained gel of the purified rALF*Pm*3

Lane 2 : purified rALF*Pm*3 probed only with anti-mouse antibody conjugated with alkalinephosphatase (negative control)

Lane 3 : purified rALF*Pm*3 probed with anti-synthetic ALF peptide polyclonal antibody and anti-mouse antibody conjugated with alkalinephosphatase

11514.83 Da, which represented about 15% of the amount of rALF*Pm*3 produced, the N-terminal amino acid sequence is EAYVQGWEAVAAA. These data implied that the mass excess of 200.66 Da for this minor product was due to the presence of extra Glu and Ala residues at the N-terminus. The calculated pI of rALF*Pm*3 is 9.9, therefore, at the biological pH the peptide should possess the net positive charge.

3.6 Antimicrobial activity of rALF*Pm*3

The purified rALF*Pm*3 protein was examined for antimicrobial activity spectrum against various strains of Gram-negative and -positive bacteria and also against filamentous fungi. The MIC values of rALF*Pm*3 are reported in Table 3.5. The broad range of antimicrobial activity were determined. A strong growth inhibitory effect against almost all tested strains of microorganisms was observed. rALF*Pm*3 was highly effective against all the Gram-positive bacteria, especially on *Bacillus megaterium* (MIC = 0.19-0.39 µM), while *Staphylococcus aureus* is an exceptional strain which remained unaffected at the highest range of contration tested, 50-100 µM. Activities against Gram-negative bacteria were also high. These were revealed by very low MIC values on *E. Coli* 363 (MIC = 0.095-0.19 µM) and inhibiton of growth of *Vibrio* strains tested with low MIC values, such as 0.39-0.78 µM against *V. alginolyticus* and 0.78-1.56 µM against *V. harveyi*, pathogen for shrimps. However, weak activity was observed against other pathogenic *V. penaeicida* (MIC 25-50 µM). In addition, rALF*Pm*3 also exhibited the antimicrobial activity against filamentous fungi, the shrimp pathogen *Fusarium oxysporum* (MIC = 1.56-3.12 µM) and *Penicillium crustosum* (MIC = 12.5-25 µM).

3.7 Determination of growth inhibition mechanism

To determine whether growth inhibition effect of rALF*Pm*3 to bacterial strains tested are bacteriostatic or bacteriocidal, the most sensitive Gram-negative bacteria: *E. coli 363* and Gram–positive bacteria: *B. megaterium* were subjected to kinetic experiments. The rALF*Pm*3 was incubated at various time intervals with the bacteria at a concentration of 10-fold higher than their MIC values. In this manner,

Table 3.5 Range of activity of rALF*Pm*3 against various strains of microorganisms using liquid growth inhibition assays.

Microorganisms	MIC* value (μM) of rALF*Pm*3
Gram (+) bacteria	
Aerococcus viridans	1.56-3.12
Bacillus megaterium	0.19-0.39
Micrococcus luteus	1.56-3.12
Staphylococcus aureus	50-100
Gram (-) bacteria	
Enterobacter cloacae	3.12-6.25
Erwinia carotovora	1.56-3.12
Escherichia coli 363	0.095-0.19
Klebsiella pneumoniae	3.12-6.25
Salmonella thyphimurium	6.25-12.5
Vibrio alginolyticus	0.39-0.78
Vibrio anguillarum	0.78-1.56
Vibrio harveyi	0.78-1.56
Vibrio penaeicida	25-50
Filamentous fungi	
Fusarium oxysporum	1.56-3.12
Botrytis cinerea	3.12-6.25
Penicillium crustosum	12.5-25

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

Table 3.6 Bacteriostatic/bacteriocidal effect of rALF*Pm*3 on *E. coli* 363 and *B. megaterium*. The concentration of the rALF*Pm*3 used in the study was 10 times over the MIC value.

Time of incubation	CFU/ml ($x10^4$)	
	Control	rALF*Pm*3
E. coli 363		
1-5 min	2.6	0
15 min	2.0	0
30 min	6.0	0
1 h	3.8	0
2 h	39	0
6 h	260	0
12 h	76,000	0
24 h	180,000,000	0
B. megaterium		
1-5 min	2.1	0
15 min	1.66	0
30 min	1.0	0
1 h	0.8	0
2 h	8.8	0
6 h	255	0
12 h	41,000	0
24 h	285,000	0

bacterial killing is time-dependent effect. There are no viable cells of both *E. coli* 363 and *B. megaterium*, could be observed within 5 minutes after direct contact with rALF*Pm*3 (Table 3.6). These results indicate that rALF*Pm*3 could immediately kill both of bacterial strains tested. The results implied that the antibacterial action of rALF*Pm*3 was relevant of strong bacteriocidal not bacteriostatic effect.

3.8 Localization of ALF*Pm*3 transcripts and protein

3.8.1 Localization of ALF*Pm*3 transcripts in normal shrimp

To characterize the involvement of ALF*Pm*3 in the defense reaction, localization and distribution of ALF*Pm*3 mRNAs were determined by *in situ* hybridization (ISH). The *P. monodon* tissue samples were probed with DIG labelled ALF*Pm*3 antisense and sense riboprobes and detected with alkaline phosphatase-conjugated anti-DIG antibodies. The sense probe was used as a control for signal specificity; hybridization using sense probe gave no positive signal in tissue sections and in cytocentrifuged cells. In addition, case of RNAse A pretreatment of the sections was performed prior to hybridization to assure that the signal was indeed from the hybridization probe.

Hybridization signals of ALF*Pm*3 mRNAs could be detected in the cytocentrifuged haemocytes (Figure 3.23) of normal shrimp in which ALF*Pm*3 was expressed in small amount. In the tissue sections, expression of ALF*Pm*3 transcripts was seen distributed as a small amount of positive labeling cells all over the cepharothorax especially in heart, lymphoid organ, interconnecting spaces of gills, muscle, and vessels of hepatopancreas (Figure 3.24). Few positive cells were also observed in the hematopoitic tissue. Among the tissues observed in the section, the positive signal could not be detected in the antennal gland. According to their shape, these cells appeared to be infiltrating haemocytes. Some positive haemocytes were detected in the afferent and efferent vessels of primary and secondary gill filaments. Under this investigation, clusters of positive haemocytes were also observed in the hemal sinus of gills. Few positive haemocytes were found in the hepatic artery and the heart. Some positive haemocytes were observed in the lymphoid organ and hematopoietic tissue.

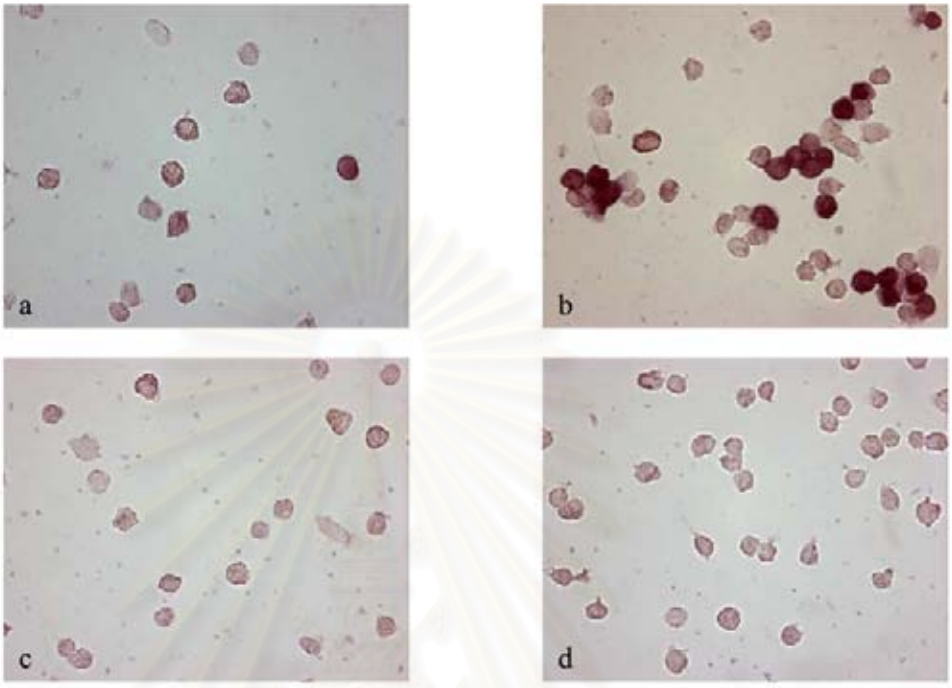


Figure 3.23 ***In situ*** **hybridization of haemocytes of unchallenged shrimps (a and c) and 6 hour post** ***V. harveyi*** **infection (b and d) probed with DIG labelled ALF*Pm*3-antisense riboprobe (a and b) and –sense riboprobe (c and d).**

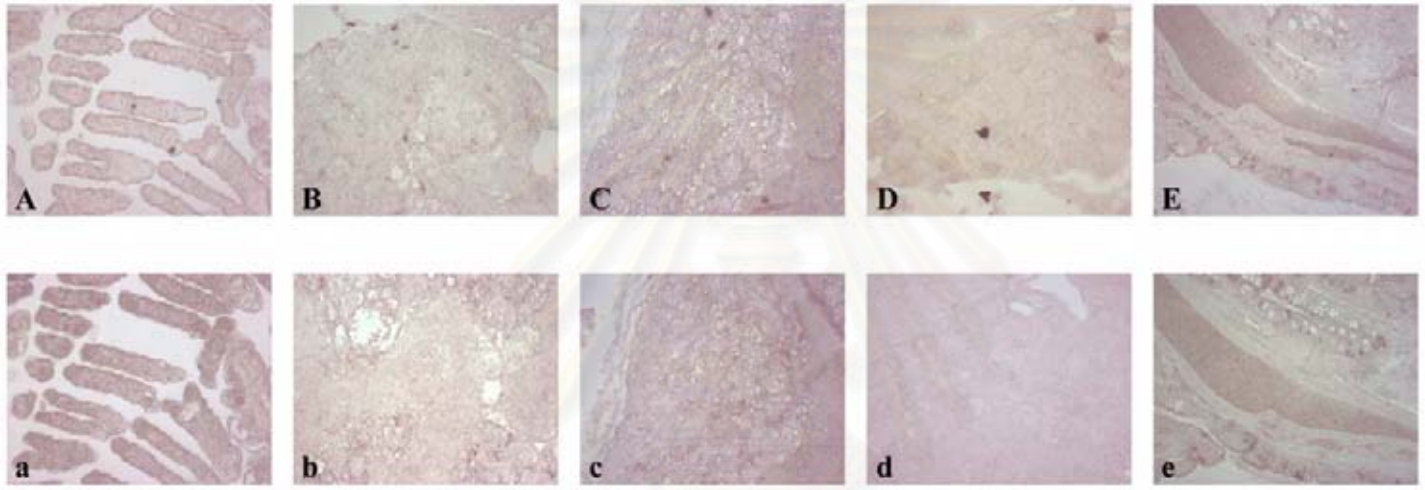


Figure 3.24 *In situ* hybridization of cepharothorax of unchallenged shrimp probed with DIG labelled ALF*Pm*3-antisense riboprobe (A-E) and –sense riboprobe (a-e).

A and a : gills

B and b : lymphoid organ

C and c : heart

D and d : hematopoietic tissues

E and e : hepatopancreas

3.8.2 Effect of microbial invasion on ALF*Pm*3 expressed circulating haemocytes

To investigate how changes in the population of ALF*Pm*3 expressed circulating haemocytes associated with microbial infection, time-course analysis of total haemocyte number and percentages of haemocytes expressing ALF*Pm*3 was carried out by *in situ* hybridization. Circulating haemocytes of each individual shrimp were collected at various times post-injection (0, 6, 24, 48 and 72 hr). They were fixed, counted and cytocentrifuged on the slides for determination of ALF*Pm*3 expressed cells. After shrimps were infected with *V. harveyi*, the circulating haemocyte populations sharply decreased from $1.6\times10^6 \pm 1.6\times10^6$ cells/ml at normal level to $0.5\times10^6 \pm 1.2 \times 10^6$ cells/ml at 6 hpi, and increased at 24 hpi ($1.4\times10^6 \pm 1.7\times10^6$ cells/ml), respectively. They returned to the basal level after 48 hpi, and slightly decreased at 72 hpi ($1.2\times10^6 \pm 1.1\times10^6$ cells/ml) (Figure 3.25).

According to *in situ* hybridiztion method, the positive haemocytes at each time point were counted in order to calculate the percentage of positive haemocytes based on the total number of populations. The highest percentage of positive haemocytes was 27 ± 8% at 6 hpi which was sharply increased compared to that of normal animal (5 ± 5%). After 6 hours of infection, the positive circulating haemocytes investigated at 24 hpi was greatly reduced to 4 ± 7%. At 48 and 72 hpi, a little increase in percentage of positive haemocyte was observed (1 ± 2% and 5 ± 6%, respectively) (Figure 3.25). These revealed that ALF*Pm*3 mRNAs were synthesized in circulating haemocytes of normal and infected shrimps and they were highest expressed at 6 hpi. The expression level of ALF*Pm*3 transcripts in haemocytes was further verifed by real-time RT PCR.

3.8.3 Localization of ALF*Pm*3 transcripts in tissues of infected shrimps

Expression of ALF*Pm*3 genes at various time after microbial injection were also determined by *in situ* hybridization within shrimp tissues. In shrimp stimulated by injection of *V. harveyi*, at 6 hours (Figure 3.26), the number of haemocytes expressing ALF*Pm*3 were increased dramatically in shrimp tissues with higher hybridization signals than those observed in non-stimulated animals (Figure 3.25).

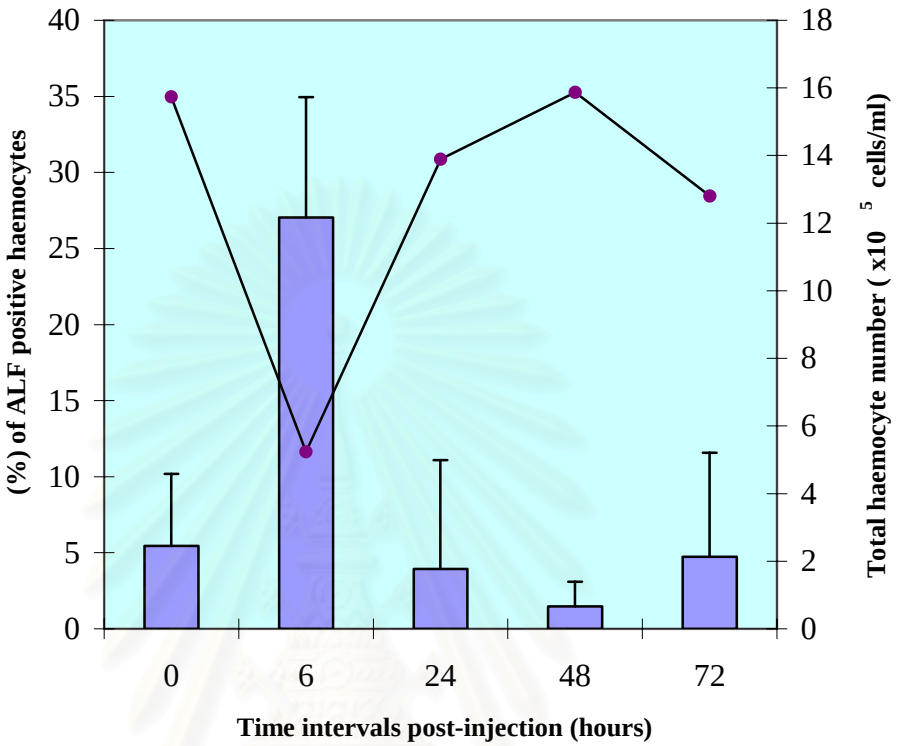


Figure 3.25 Time-course analysis of total circulating haemocytes number (line) and percentage of ALF*Pm*3 expressing haemocytes (bar) after *V. haeveyi* challenge. *In situ* hybridization was performed by incubating cytospin haemocytes with DIG-labeled riboprobes and detected the hybridization signal by colorimetric method using NBT/BCIP solution as substrate.

Figure 3.26-A1 showed that a lot of positive haemocytes were found in the vessels of the central axis, primary filaments and secondary filaments of gills. A large number of positive haemocytes were distributed throughout the hepatopancreas (Figure 3.26-B1) and also in the aorta of the heart. Large amounts of positive haemocytes were found in the antennal gland (Figure 3.26-C1). In the muscle (Figure 3.26-E1), the positive haemocytes were also observed. The positive haemocytes were found in the 2nd maxilliped hematopoietic tissue (Figure 3.26-F1) and the adjacent organ, the mandibular pulp (MDP) (Figure 3.26-G1). It was observed that the positive haemocytes were located in the blood vessels as groups. Within the stomach, the haemocytes expressing ALF*Pm*3 were observed in the layer of spongy connective tissue (Figure 3.26-D1).

At 24 hpi, no positive haemocytes could be detected in the gills (Figure 3.26-A2). However, a few haemocytes expressing ALF*Pm*3 were observed at the rim of the hepatopancreas (Figure 3.26-B2). The morphology of the hepatopancreatic cells of the infected animals changed comparing to that of normal animals and these cells gave very strong signal. In the reproductive organs located near the hepatopancreas, the positive haemocytes or positive cells were not found. Almost every cells of hematopoietic tissue but not the haemocytes at the maxilliped were positive (Figure 3.26-F2). In the ventral region to hepatopancreas, a lot of haemocytes which gave positive signal were found in the line of epithilial cells in many parts, i.e. maxilla, maxilliped. The positive haemocytes also infiltrated in many organs, for example, mandibular pulp (Figure 3.26-G2), maxilla, maxilliped, and muscle (Figure 3.26-E2). It should be noted that there are no positive haemocytes observed in the gills.

For the animal fixed at 48 hpi (Figure 3.26-A3 to G3), a small number of positive haemocytes were found in hepatopancreas, hematopoietic tissue, muscle, MDP, antennal gland, muscle and spongy connective tissue of stomach. No positive haemocytes could be detected in reproductive organ and gills.

At 72 hpi, there were much higher positive haemocytes found in the cepharothorax parts of animal than that of 48 hpi. In the antennal gland (Figure 3.26-C4), even if it had a lot of positive haemocytes, it contained smaller amount of positive cells than at 6 hpi. The positive haemocytes appeared to distribute all over the hepatopancreas (Figure 3.26-B4). A lot of haemocytes expressing ALF*Pm*3 were

located in the afferent and eferent vessels and interconnecting spaces of secondary gill filaments (Figure 3.26-A4). In addition, similar results were observed in the primary gill filament. Like at 6 hpi, a significant number of positive haemocytes were found in the spongy connective tissues located in the stomach (Figure 3.26-D4). There were bunches of positive haemocytes detected in the muscle (Figure 3.26-E4). In the reproductive organs, some positive haemocytes infiltrating in the tissue were also found. Some positve haemocytes were observed in 2nd maxilliped haematopoietic tissue (Figure 3.26-F4). A number of ALF*Pm*3 expressed haemocytes were revealed (Figure 3.26-G4).

The hybridization signal corresponding to the number of ALF*Pm*3 expressing cells in the cepharothorax of normal and *V. harveyi* infected shrimp were compared in each observed organ and summarized in Table 3.7.

3.8.4 Localization of ALF*Pm*3 protein

A specific polyclonal antibody was raised against rALF*Pm*3 in rabbits and used for localization of the native ALF*Pm*3 in shrimp by immunohistochemistry. The alkalinephosphatase conjugated secondary antibody was used to facilitate immunodetection by colorimetry using NBT/BCIP solution. Immunodetection showed that the different parts of the cytocentrifuged hemocyte population reacted to the specific anti-ALF*Pm3* antibody with various intensity (Figure 3.27). Sections of the second abdominal segments (site of injection) of normal and *V. harveyi* injected shrimps at 0, 6 and 24 hours were also analyzed (Figure 3.28). The ALF*Pm*3 producing haemocytes and the released peptides were localized at the site of injection. In the untreated animals, few ALF*Pm*3-positive haemocytes were observed. On the contrary, at 6 and 24 hpi, strong ALF*Pm*3 immunoreactivity was detected around the injection sites with increasing number of haemocytes containing ALF*Pm*3. This revealed the presence of released peptides and accumation of free peptide in the muscle around the injection site. It should be noted that the haemocytes producing ALF*Pm*3 were localized predominantly at the injury muscle not the adjacent one.

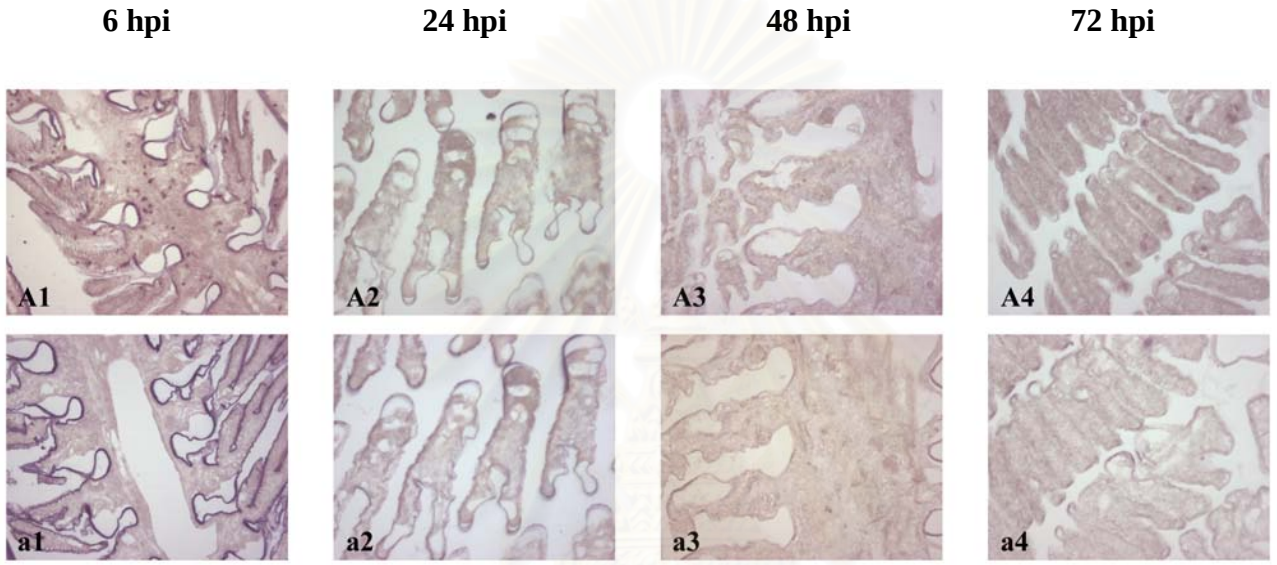


Figure 3.26 Detection of ALF*Pm*3 mRNA expression in the cepharothorax of *V. harveyi* infected shrimp at 6, 24, 48 and 72 hour post injection. The tissue sections were probed with ALF*Pm*3- antisense (A-G) and sense (a-g) riboprobes. The observed organs included gills (A1-4 and a1-4), hepatopancreas (B1-4 and b1-4), antennal gland (C1-4 and c1-4), stomach (D1-4 and d1-4), muscle (E1-4 and e1-4), hematopoietic tissue (F1-4 and f1-4), and mandibular pulp (G1-4 and g1-4).

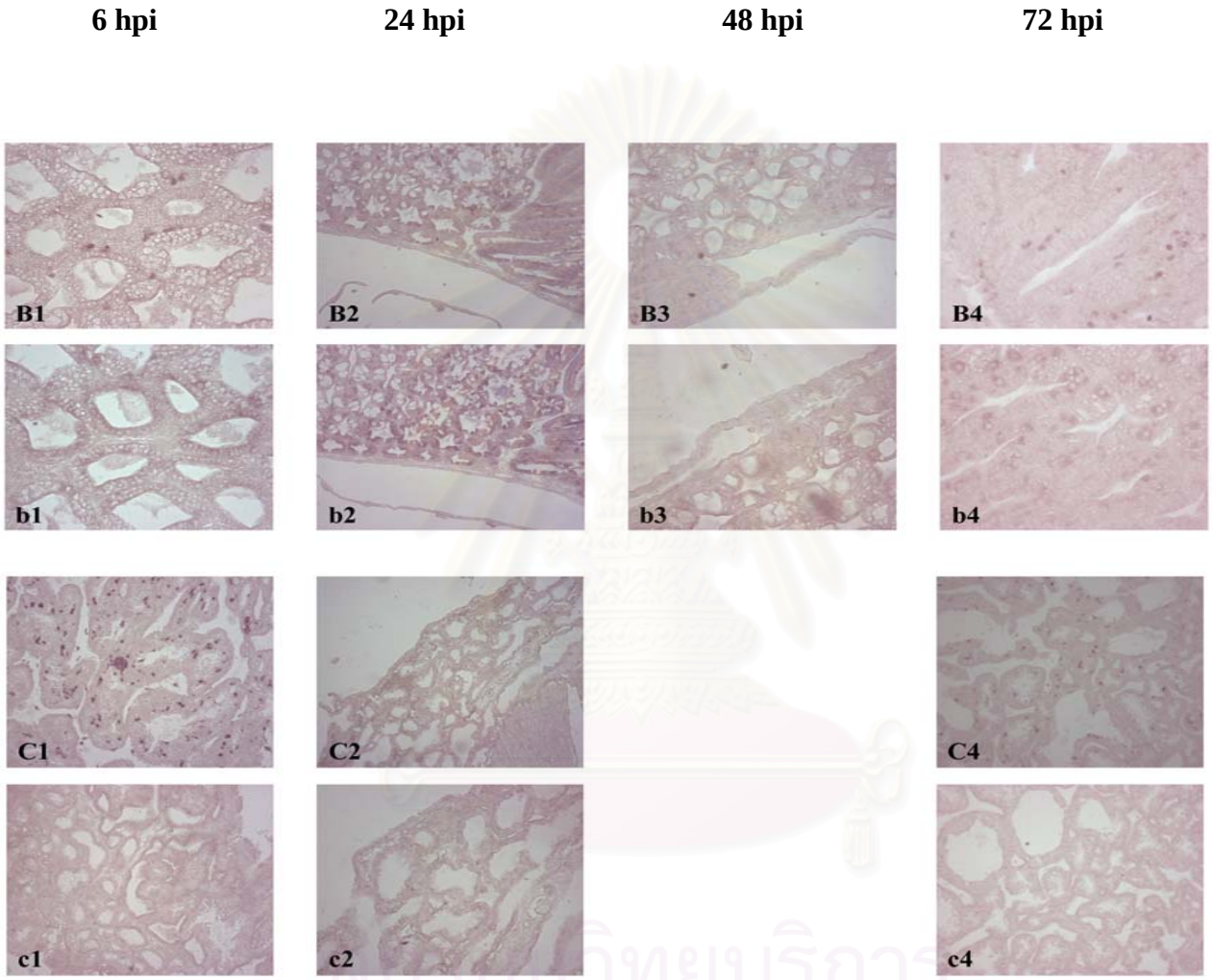


Figure 3.26 Detection of ALF*Pm*3 mRNA expression in the cepharothorax of *V. harveyi* infected shrimp at 6, 24, 48 and 72 hours. (continued)

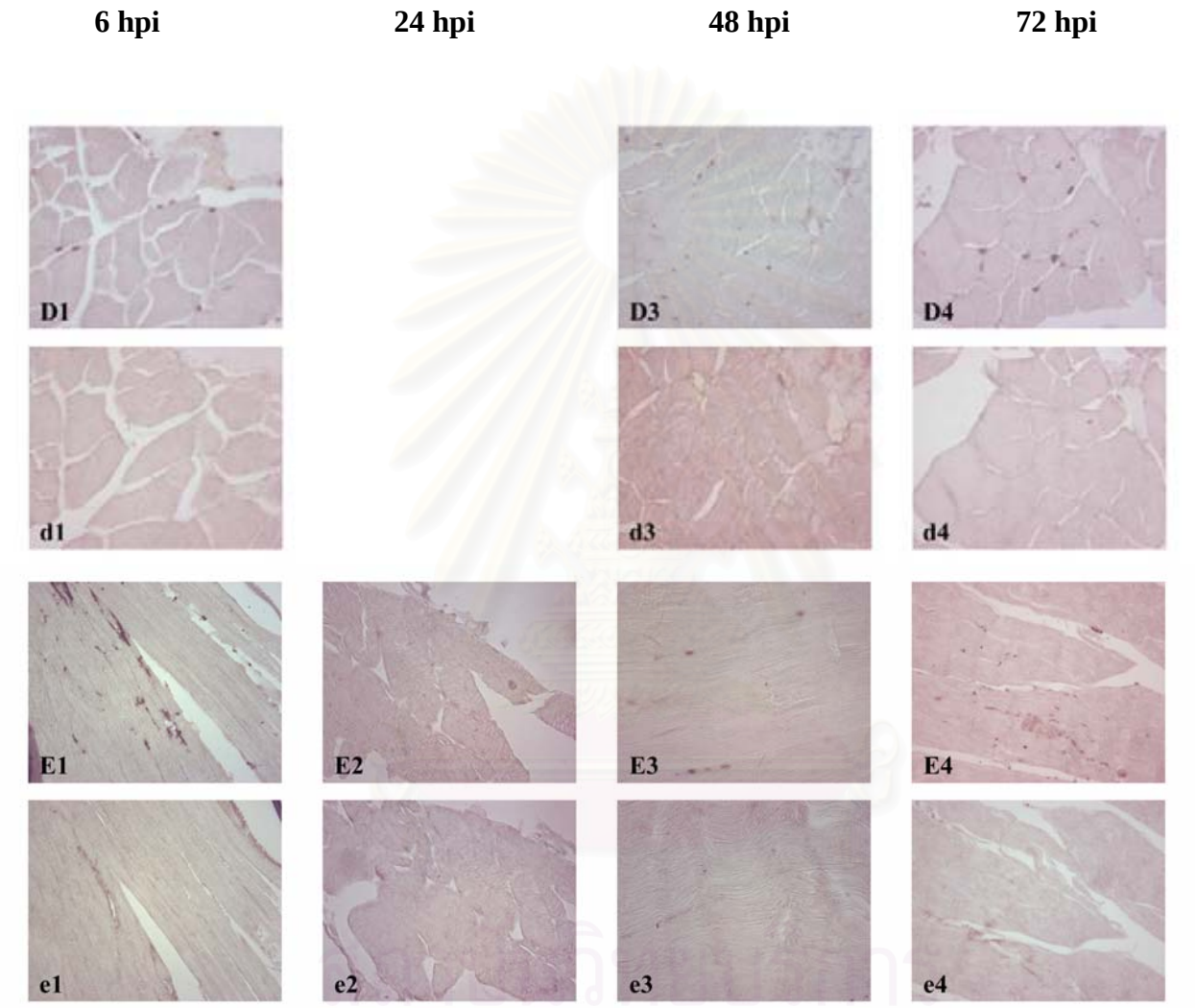


Figure 3.26 Detection of ALF*Pm*3 mRNA expression in the cepharothorax of *V. harveyi* infected shrimp at 6, 24, 48 and 72 hours. (continued)

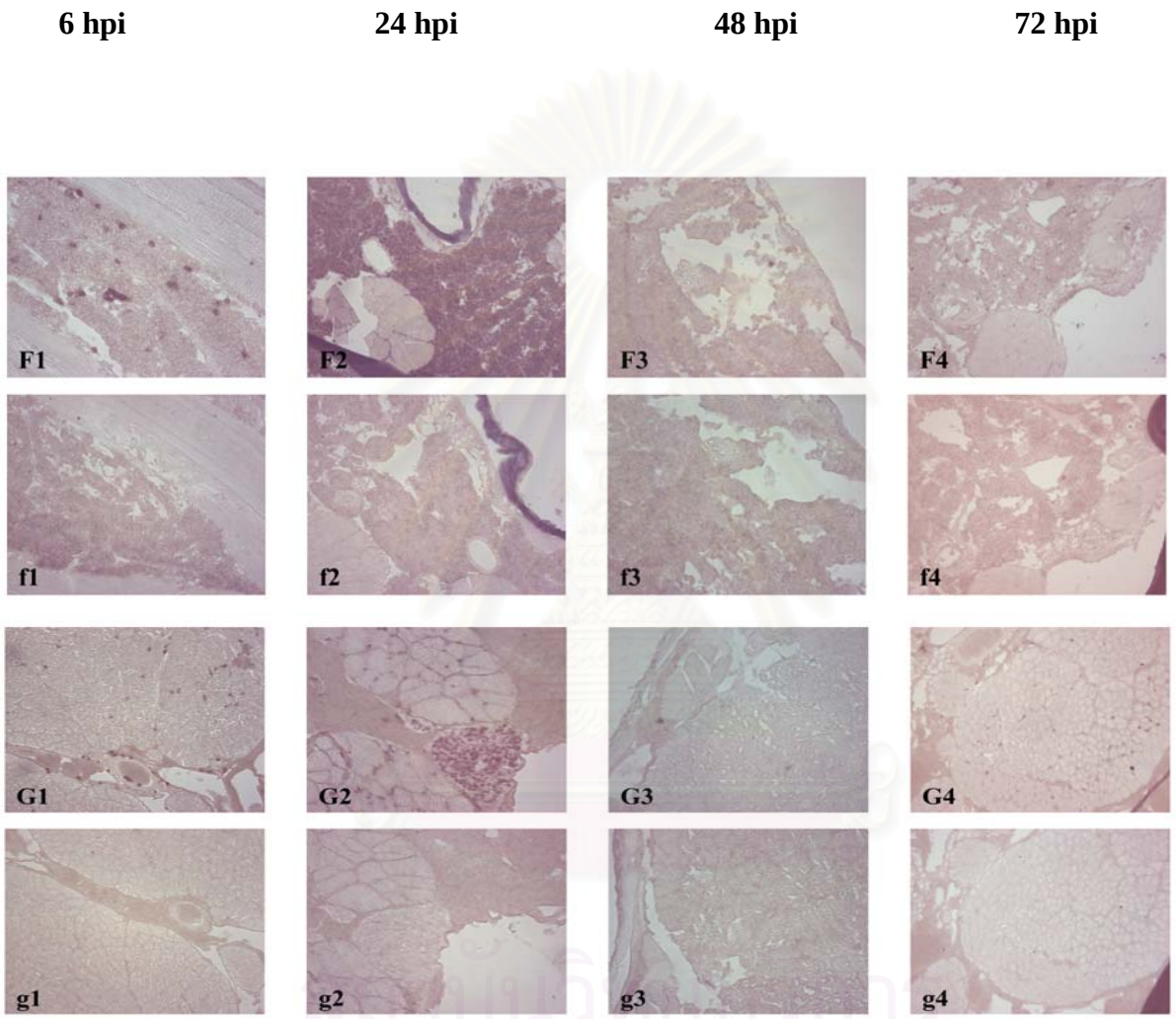


Figure 3.26 Detection of ALF*Pm*3 mRNA expression in the cepharothorax of *V. harveyi* infected shrimp at 6, 24, 48 and 72 hours. (continued)

Table 3.7 Summary of the *in situ* hybridization results of *V. harveyi* injected *P. monodon*

ALF*Pm*3 organ	**Normal**	**Time post-*Vibrio harveyi* injection (hours)***			
		6	**24**	**48**	**72**
gill	+	++++	-	-	+++
hepatopancreas	+	+++	+	+	+++
muscle	+	++	-	+	++++
reproductive system	+	++	-	+	+++
antennal gland	+	+++++	++	+	++++
heart	+	nd	+++	-	nd
lymphoid organ	+	nd	++++	nd	nd
hematopoietic tissue	+/-	+++	++++	+/-	++

*The signal intensity of hybridization is expressed as + to +++++. +/- indicates very low level of hybridization signal.

nd means not determined

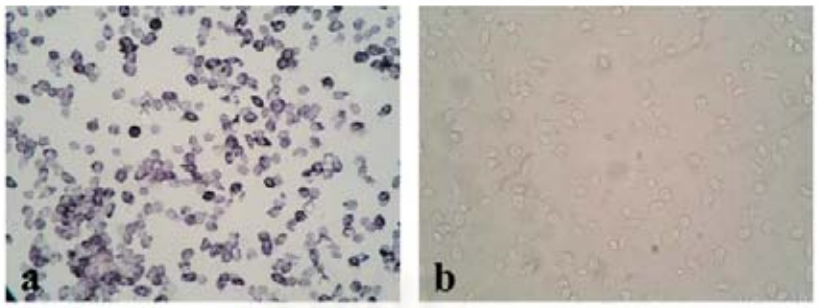


Figure 3.27 Immunodetection of ALF*Pm*3 in haemocytes of *V. harveyi* infected shrimp at 6 hours. Hemocytes were cytocentrifuged on slides and incubated with anti-ALF*Pm*3 IgG (a) , without specific anti-ALF*Pm*3 IgG as negative control (b), followed by incubation with alkalinephosphatase-conjugated anti-rabbit IgG and detection by using NBT/BCIP substrate.

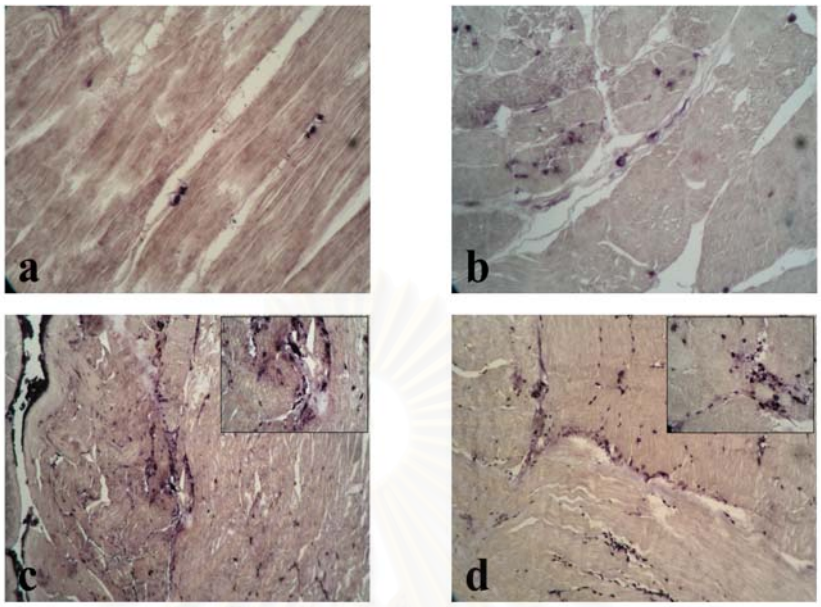


Figure 3.28 Immunodetection of ALF*Pm*3 at the injection site of normal shrimp (a), and those of *V. harveyi* infected shrimp at 0 hour (b), 6 hours (c), and 24 hours (d). The tissue section of injection site were incubated with anti-ALF*Pm*3 IgG followed by alkaline phosphatase-conjugated anti-rabbit IgG.

3.9 Expression analysis of ALF*Pm*3 mRNAs by real-time RT-PCR

The level of expression of ALF*Pm*3 transcripts in haemocytes of shrimp challenged with *V. harveyi* was investigated by quantitative real-time PCR assay. The method of relative expression analysis were performed as described in section 2.8. The PCR efficiency of ALF*Pm*3 equals to 1.96 and the single peak was obtained from melting curve analysis of PCR products (Figure 3.29). The expression of ALF*Pm*3 transcript in *V. harveyi* infected sample was normalized with saline injected samples and the gene expression ratios of ALF*Pm*3 expression were relative to EF-1α. Microbial challenge resulted in up-regulation of ALF*Pm*3 gene at the first phase of response as indicated by the increase in relative expression ratios increased dramatically from 0.58 (0 hpi) to 3.99 (6 hpi) or about 7 folds increase (Figure 3.30). The relative expression ratios of ALF*Pm*3 transcripts then declined dramatically to 2.02 and 1.14 at 24 and 48 hpi, respectively.

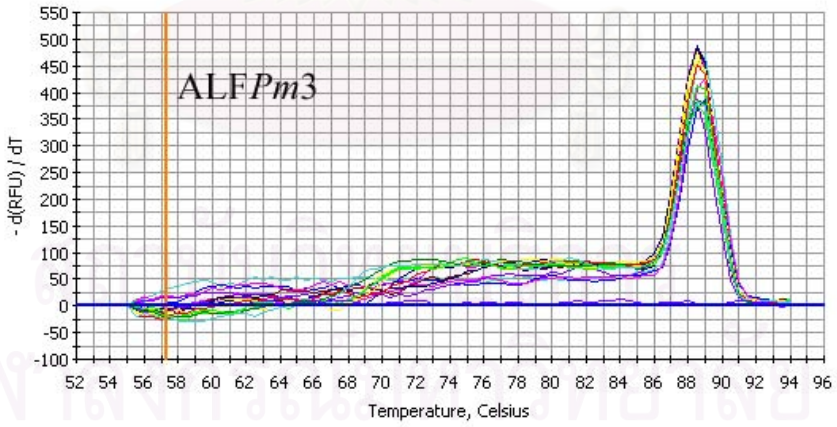


Figure 3.29 The dissociation curve of PCR product of ALF*Pm*3.

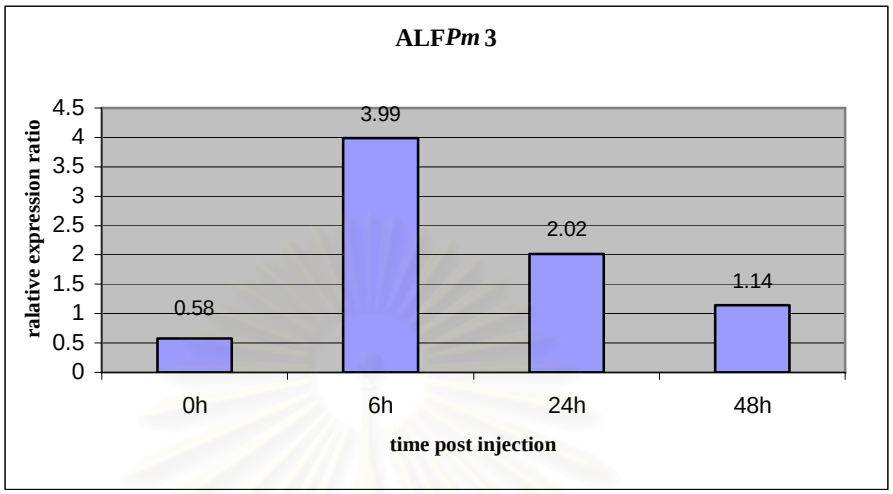


Figure 3.30 Time-course analysis of ALF*Pm*3 transcripts in *P. monodon* haemocytes upon *V. harveyi* challenge using real-time PCR.

CHAPTER IV

DISCUSSION

The problem of infectious disease outbreaks in cultured shrimp was still uncontrollable. To establish the effective strategies for disease prevention and control, the understanding in shrimp immunity is thus important. Identification of genes involved in the immune response is the first step in the understanding the immune system. The effect of microbial invasion on shrimp gene expression was elucidated in the haemocytes which are the main site of immune response in shrimp (Astrofsky et al., 2002; de Lorgeril et al., 2005; Supungul et al., 2004). In the present study, the *Vibrio harveyi* responsive genes were identified by differential display PCR. Theoretically, 240 primer combinations (80 arbitrary primers in combination with 3-one base anchored oligo dT primers) could detect 96% [1-$(0.96)^n$ where n is the number of arbitrary primers] of the expressed genes in any particular cell type (Astrofsky et al., 2002). Therefore, in theory, about 34% of the expressed genes in haemocytes of *V. harveyi* infected *P. monodon* were observed when using 9 different arbitrary primers. According to DD-PCR, about 56% of differentially display bands were the mixture of products of similar size obtained from one cDNA band (Mathieu-Daude et al., 1996). These might be arisen from the co-migration of other non-homologous cDNAs or a technical error while excising bands from the gel. Some of them should be the artifacts that might result in error of identification of differential display cDNAs. Mixture of sequences were also detected in differentially expressed genes from white spot virus infected *L. stylirostris* (Astrofsky et al., 2002). The sequence analysis showed that high number of unknown sequences (69%) was obtained. There are two possibility that resulted in high number of this uninformative data. Firstly, DD-PCR generally amplified 3'-untranslated region (UTR) of mRNA which can not be identified and predicted the gene function (Jurecic and Belmont, 2000). Secondly, a limited number of sequences is available for shrimp in the GenBank database, even many EST projects have been conducted in many shrimp species (Gross et al., 2001; Rojtinnakorn et al., 2002; Supungul, 2002). It was

indicated in many studies that it is possible to identify novel transcripts using DD-PCR (Luo et al., 2003; Watanabe et al., 2000). Interestingly, among the candidate differentially expressed cDNAs, novel genes including balbiani ring protein3 precursor, argonaute protein, caspase 3B, SERPINB3, Rc12 protein, glucose transporter 1, interferon-related developmental regulator 1 (IFRD 1), and huntingtin interacting protein E, were identified in shrimp. Due to a large number of clones identified in this study, only the interested differentially expressed genes including caspase 3B, lysozme, glucose transporter1, IFRD 1, profilin, and SERPINB3. were selected to confirm for their expression levels at various time after injection by real-time RT-PCR.

DD-PCR relies on the RT-PCR amplification of cDNAs complementary to 3'-terminal mRNA sequences (Kornmann et al., 2001), thus, the cDNAs identified in this study were not complete genes. To access the complete nucleotide sequences of such gene, other molecular biology techniques such as RACE or screening of cDNA library should be performed base on the sequence of DD-PCR clones. The level of expression differences measured by real-time PCR for many genes agreed with the results of respective DD-PCR method excepted for SERPINB3. The disagreement may be due to the lack of specificity in the primers designed to discriminate gene family members at the level of primary screening by DD-PCR. Designing specific primers for validation of DD-PCR bands could be limited by the lack of information on sequence relatedness to gene families and regulation of novel transcripts. The strength of real-time PCR assay as a secondary validation procedure lies in its potential to quantify relative change in expression of large numbers of known and novel genes with limited RNA in a rapid and precise manner (Li et al., 2004); (Rajeevan et al., 2001).

The putative GLUT 1 gene in haemocytes of *P. monodon* was identified as the up-regulated transcript after *V. harveyi* injection. Real-time RT-PCR analysis revealed that the highest level of expression of GLUT 1 was at 6 hpi. The level of GLUT 1 decreased significantly at 24 and 48 hpi. Like DD-PCR result, at 48 hpi, the expression remained higher than at 0 hpi. Since GLUT 1 may also functions as a glucose transporter in shrimp, high level of GLUT 1 transcript at 6 hpi implied that during the immune response, the haemocytes required more energy from glucose for

cellular immune processes than in the normal state. This also infered that innate immune response in shrimp is very active at the first phase of response. The animals usually return to the normal stage after 24-48 hour.

Apoptosis is a cellular antiviral defence mechanism and a normal host process essential for orderly development of many organisms (O'Brien, 1998). It has been shown to play a decisive role to avoid virus spreading by eliminating virus-infected cells. Khanobdee et al. (2002) reported an increasing incidence of apoptosis in the lymphoid organ of yellow head virus-injected *P. monodon* were companied by a high mortalities of host (Khanobdee et al., 2002; Wu and Muroga, 2004). The finding of caspase 3B gene which is identified as an inducer of the apoptotic pathway (Webb et al., 1997) and the increasing in its mRNA level after *V. harveyi* stimulation especially at 6 hpi implied that *V. harveyi* may induce haemocytes to undergo apoptosis in order to eliminate bacterial invasion. So apoptosis may also be involved in both antibacterial and antiviral defence mechanism.

During the course of *Vibrio* infection, lysozyme mRNA expressed in unchallenged *Litopenaeus stylirostris* is significantly higher than that of infected animals. A decrease in lysozyme transcript abundance was seen early after infection and was elevated in surviving shrimp at 96 hours (de Lorgeril et al., 2005). Unlike the result described by de Lorgeril (2005), lower level of lysozyme transcripts were observed at 0 hour *V. harveyi* infected *P. monodon*. Difference of infection methods may result in the different profile of lysozyme expression; *L. stylirostris* was infected with *V. penaeicida* by immersion resulted in increasing of lysozyme transcripts at 96 hours (de Lorgeril et al., 2005), while direct injection of *V. harveyi* to *P. monodon* resulted in increasing in lysozyme expression at 48 hpi. This infered that infection by direct injection resulted in more rapid response than immersion.

Profilin 1 has been reported to have an important role in host-pathogen interactions. Pathogens such as *Listerial monocytogenes* and *Shigella flexneri* use the host-cell actin cytoskeleton to propel themselves through the cytoplasm and to spread to neighboring cells without entering the extracellular space. These pathogens can induce polarized actin polymerization on the bacterial surface that pushes the bacteria forward and leaves behind an actin comet tail (Kocks, 1994). It is reasonable to hypothesize that this phenomenon may occur in shrimp haemocytes. Therefore, we

speculate that profilin involved in infiltrating of haemocytes through shrimp tissues especially in muscle via regulate actin polymerization. The higher epression of profilin at 6 hpi, supported the evidence that at the early phase of infection haemocytes migrated to the site of injection. Considering at 24 hpi, the expression of profilin gene was lower, implied the the lower mobility of haemocytes. At 48 hours where the total haemocyte number is going to return to the normal level (Munoz et al., 2002), the profilin mRNA again raised in this late phase of shrimp immune response corresponding to the proliferation and systemic reaction in haemocytes (Bachere et al., 2004). However, the exact relation between profilin expression and immune response should be further investigated.

For interferon-related developmental regulator 1 (IFRD1) gene, real-time PCR analysis revealed differentially expression as determined by DD-PCR. Considering IFRD1 expression profile upon microbial infection, we found that it was very similar to that of profilin. That is the gene expression is relatively high at the fisrt and late phase of *V. harveyi* infection not the intermediate phase at 24 hpi. To date, little is known about the function of this transcript. However, in human the involvement of IFRD1 in early hematopoiesis was suggested by Buanne et al. (1998). The alignment of part of shrimp IFRD1 with mammal IFRD1 revealed a very conserved of amino acid sequences of this gene. Like those of mammal IFRD1, in shrimp, this gene may contain at the C-terminus a region similar to the interferon-γ. In vertebrate, interferon-γ is a pleiotrophic cytokine that regulates hundreds of diverse genes. Many of these genes are involved in responses to viral and microbial pathogens, and their effects are manifested via the immune system (Shtrichman and Samuel, 2001), and is a molecule known for its role in cellular differentiation (Buanne et al., 1998). Action of interferon-γ against microbial pathogens are mediated through many inducible proteins such as protein kinase, oligoadenylate synthetase, GTPase, an antimicrobial peptide: human β-defensin, (Joly et al., 2004; MacMicking, 2004; Shtrichman and Samuel, 2001). We assumed that IFRD1 may be involved in the haemoctye differentiation or regulation of innate immue response in shrimp. To assign a functional role of this gene the complete sequences should be obtained.

SERPINs, normally function as proteinase inhibitors, are a group of high molecular weight (40 to 50 kDa) proteins involved in a number of biological processes such as blood coagulation cascades, complement activation, fibrinolysis, angiogenesis, inflammation, tumor suppression and hormone transport. Most serpins inhibit serine proteinases of the chymotrypsin family. However, cross-class inhibitors have been identified. Among them, SERPINB3, one member of human serpins in B clade, neutralizes the potent papain-like cysteine proteinases, cathepsins L, K and S (Silverman et al., 2001). It may function after releasing from the cells under certain conditions.

Serpins have been identified as haemolymph protein in several arthopod species, including horseshoe crabs, crayfish, insects, and recently chinese shrimp (Kanost, 1999; Shen et al., 2004). In horseshoe crabs, three serpins are synthesized in haemocytes and released in response to infection and regulate proteinases in haemolymph coagulation pathway (Iwanaga et al., 1998). In lepidopteran insect, *Manduca sexta*, three serpins have been identified and one of them, serpin-3, is a regulator of the pro-PO activation reaction in response to microbial infection by inhibiting prophenoloxidase-activating proteinases (Zhu et al., 2003). In *P. monodon*, only the low molecular weight serine proteinase inhibitors, Kazal type, were identified and characterized (Baily-Brook and Moss, 1992; Jarasrassamee et al., in press; Somprasong, 2004; Supungul, 2002). Recently, construction of cDNA library from cepharothorax of *Fenneropenaeus chinensis* could identified the serpins (Shen et al., 2004). By DD-PCR technique, the SERPINB3 was the first serpin identified in this species. Like above serpins, it was synthesized in the haemocytes. Expression analysis by real-time PCR of SERPINB3 revealed that upon microbial challenge, mRNA level gradually increased from 6 to 48 hpi. Its function and regulation should be further investigated.

An immune effector of *P. monodon*, namely anti-lipopolysaccharide factor (ALF*Pm*), was identified as a differentially expressed gene upon *V. harveyi* challenged as previously identified by EST (Supungul et al., 2002). EST analysis of cDNA libraries from haemocytes of normal and *V. harveyi*-challenged *P. monodon* resulted in identification of at least 5 types of ALF homologues to that of *Limulus*. They predominated and increased in the challenged library suggesting their

importance in shrimp immune system (Supungul et al., 2002). ALF*Pm*3 was the major isoform identified by EST. From this study, the results also indicated that ALF*Pm*3 is the major ALF*Pm* that significantly responded to bacterial challenge. Therefore, we focused on characterization of ALF*Pm*3 activity in order to understand its function on shrimp immunity. To accomplish this, production of the recombinant protein was necessary.

The recombinant ALF of horseshoe crabs expressed in bacteria were unsuccessful, probably due to its antibacterial activity. Among baculovirus and yeast expression system, the latter system gave better yields of this protein (Paus et al., 2002; Wang et al., 2001; Wang et al., 2002). The metylotrophic yeast *P. pastoris* was chosen as an expression system because it posses many advantages which include; rapid growth characteristics of prokaryotic organism, containing the subcellular machinery for performing post-translational protein modification of a eukaryotic cell, possibility of integration of mutliple-copies of expression cassette into chromosome, small-scale fermentations, and the potential of hundreds of mg/L yields (Cereghino and Cregg, 2000). In recent works, production of an active recombinant Ch-penaeidin in *P. pastoris* showed much higher levels of secreted protein than previously reported in *Saccharomyces cerevisiae* (Destoumieux et al., 1999; Li et al., 2005). The yield of rALF*Pm*3 depended on the gene copy number which roughly correlated to level of G418 resistance. The *Pichia* transformant clones which resisted to 0.25 and 0.75 mg/ml G418 sulfate could express lower amount of protein (data not shown) whereas the ALFK9 transformant that contains multiple integrated copies of pALFPmK, implied by resistance to 1 mg/ml G418 sulfate could produce the protein at higher level. The protein of interest from ALFK9 represents the vast majority of total protein in the medium and this served as the first step in purification of the protein (Barr et al., 1992). These resulted from the fact that the yeast *Pichia* secretes only low levels of native proteins (Cereghino and Cregg, 2000). The molecular mass of crude rALF*Pm*3 determined by SDS-PAGE is about 14 kDa, which is larger than the expected size (11.3 kDa). The slow mobility was apparently caused by high ratio of positively charged amino acids of the protein.

To obtain the sufficient amount of purified rALF*Pm*3 protein, the rALF*Pm*3 from ALFK9 clone was produced by our co-workers in a fermentor. In every

purification step, the antimicrobial activity was used to monitor the rALF*Pm*3 fraction. The LALF was purified from the haemocytes of horseshoe crabs by means of cation-exchange chromatography (Morita et al., 1985; Tanaka et al., 1982). The recombinant of LALF was also purified by a weak anion-exchage chromatography followed by a strong cation exchange chromatography (Paus et al., 2002). Ion-exchange chromatography was selected for the first step of rALF*Pm*3 purification to clearify and concentrated the rALF*Pm*3 from the large volume of cell-free supernatant. At pH 7 which lower than its pI, the molecule possessed the net positive charge, the supernatant was sujected to cation-exchange catridge resulted in binding of the protein to the stationary phase. The bound protein eluted with 1 M NaCl containing the antimicrobial activity against *E. coli* 363 revealed the presence of rALF*Pm*3. The clearified elution fraction was dialysed against water to eliminate the salt. Before further purification by reversed phase HPLC, the dialysed fraction containing rALF*Pm*3 was lyophilized and re-dissolved in small volume of acidified water. The acidified rALF*Pm*3 was purified to homogeneity by C8 reverse phase HPLC. The latter purification step resulted in extremely loss of rALF*Pm*3 protein. Therefore, the more efficient procedure for rALF*Pm*3 purification should be developed.

To ensure that the recombinant protein is rALF*Pm*3, Edman degradation were performed. Mass spectrometry was used to analyze the purified rALF*Pm*3 for its exact molecular mass. Taken together, the results demonstrated that the purified rALF*Pm*3 contained two products which the expected product was the major product. The unexpected product, the rALF*Pm*3 with the extra Glu and Ala residues at the N-terminus, resulted from inefficiency of STE13 protease in processing the secreted rALF*Pm*3. This occurrence could overcome by cloning the interested genes in-frame with modified α-mating factor signal sequence devoiding of the STE13 proteolytic signal sequence (Cabral et al., 2003). The recombinant of Ch-penaeidin contained an additional tetrapeptide, Tyr-Val-Glu-Phe, aparted from the mature peptide which arose from the C-terminus of α-factor signal (Li et al., 2005). In case of rALF*Pm*3 two amino acids, Tyr and Val, were added at the N-terminus of mature peptide.

Study on antimicrobial activity revealed that rALF*Pm*3 has broad spectrum of activity against Gram-negative and Gram-positive bacteria and fungi as observed in *Limulus* ALF (LALF) (Wainwright and Norman, 1995). The MIC values of shrimp

ALF was first reported in the present study. Similar to LALF, rALF*Pm*3 activity against Gram-negative bacteria should be derived from a high affinity for the lipid A portion of endotoxin of ALF. The LPS binding domain of ALF is distinguished by an alternating series of positively charged and hydrophobic residues, and is formed in an amphipathic loop conformation stabilized by a disulfide bond at the stem of the loop and the H-bond interaction of the β-sheet structure (Hoess et al., 1993). The LPS binding domain of ALF*Pm*s, comparing to rLALF, lacks some of the positively charge residues, while some other positively charge residues were conserved and most of the bulky hydrophobic residues were also conserved, which implied their crucial role in this domain (Supungul et al., 2004). Among thousond of AMPs isolated from multicellular organisms (Bulet et al., 2004), very few anti-microbial peptides have been reported to be highly active against *Vibrio* strains apart from the mytilins, isolated also from the mussel *Mytilus galloprovincialis* (Mitta et al., 2000), and which display MIC values similar to those observed for rALF*Pm*3. The recombinant lysozyme of *M. japonicus*, has been shown to display lytic activity against several *Vibrio* species including *V. penaeicidae* which is a shrimp pathogen using a lysoplate assay (Hikima et al., 2003). Comparing with the recombinant penaidin, the anti-fungal activity against *Botrytis cinerea* and a shrimp phathogenic strain, *Fusarium oxysporum*, of the rALF*Pm*3 is higher. MIC value of recombinant penaeidin-2-1 and penaeidin-3-1 against both strains of fungi is below 10 μM (Destoumieux et al., 1999), and of recombinant Ch-penaeidin against *F. oxysporum* is 12 μM (Li et al., 2005) while at lower concentration (Table 3.5), rALF*Pm*3 caused growth inhibition to those fungi. *In vitro* bactericidal activity of rALF*Pm*3 against both *B. megaterium* and *E. coli* 363 were disclosed by rapid killing observed in this study. This support the result obtained from recombinant TALF (Wang et al., 2002). Rapid killing mechanisms on *B. megaterium* identical to that identified for penaeidin (Destoumieux et al., 1999) and pore-forming antibacterial peptides such as insect defensins (Cociancich et al., 1993). Broad antimicrobial spectrum and activity against pathogenic microorganisms revealed that rALF*Pm*3 is a potentially candidate of a clinically applicable antimicrobial agent. In addition, activity of rALF*Pm*3 implied the important role of ALF*Pm*3 in shrimp immune system.

In *Drosophila*, induction of antimicrobial peptide expression in fat body cells and surface epithelia as well as the signalling and regulatory pathways controlling peptide expression are well characterized (Imler and Hoffmann, 2000; Tzou et al., 2000). Apart from dipteran and lepidopteran, hemocytes are the site of antimicrobial peptide synthesis in different groups of invertebrate (for review; (Bachere et al., 2004). In *Limulus*, haemocytes undergo exocytosis after microbial stimulation and releasing of antimicrobial peptides will occur (Iwanaga, 2002). In shrimp, knowledge of the regulation of antimicrobial peptide encoding genes during the immune response was still not clear. The most recent progress on shrimp antimicrobial peptides is the studies on penaeidins, antimicrobial peptides form *L. vannamei*. Penaeidins are constitutively expressed in granular haemocytes of naive shrimp. Upon microbial challenge, the penaeidin expression in circulating haemocytes dropped in the first phase of immune response because the penaeidin-expressing haemocytes leave the blood circulation and most of the shrimp tissues and migrate towards site of injury (Bachere et al., 2004).

Injection of live *V. harveyi* at the second abdominal segment of *P. monodon* resulted in a dramatically drop of the total circulating haemocyte number in the early hours post-injection followed by recovery of number at about 24-48 hours as observed in *L. vannamei* injected with a cocktail of heat-killed microorganisms (Munoz et al., 2002). This indicated that the cellular component of microorganisms is an important factor for the stimulation of immune response of animals. Expression of ALF*Pm*3 gene after microbial stimulation was investigated *in vivo* by *in situ* hybridization and *in vitro* by real-time PCR analysis. Relation between expression profile and changes in circulating haemocyte number during microbial response should be considered for better understanding ALF*Pm*3 expression. ALF*Pm*3 expression may be regulated independently within each haemocyte or the observed expression profile (Figures 3.25 and 3.30) may due to variations in haemocyte population composition. According to the results of *in situ* hybridization, ALF*Pm*3 transcripts was found to be highly expressed within the very low number of the total circulating haemocytes in the first phase (observed at 6 hpi) of microbial response. We can give two possible explanations for this finding. First, at 6 hpi, if the decrease in total circulating haemocyte number caused by lysis or migration of non-ALF*Pm*3 expressed

haemocytes, it might result in the detection of higher number of ALF*Pm*3 expressing cells in the total haemocyte population. Second, the haemocytes themselves were induced by microbial challenge to produce more ALF*Pm*3.

In this study, we also reported that the haemocyte-expresssing ALF*Pm*3 distributed to most of the shrimp tissues. The infiltrating haemocytes in heart, lymphoid organ, and hematopoietic tissue not circulating haemocytes of 24 hpi, has high level of ALF*Pm*3 mRNA expression. A decrease in ALF*Pm*3 transcripts at 48 hpi in both infiltrating and circulating haemocytes was followed by a little increase in circulating haemocytes but greatly increase in tissue infitrated haemoctyes at 72 hpi. These results implied that ALF*Pm*3 genes in haemocytes were expressed higher at the early phase of response in order to function in combating with microbial invasion by migrating to shirmp organs and the peptides may release from haemocytes to kill the pathogens. The immunolocalization experiment revealed that ALF*Pm*3 peptides were produced in the haemocytes. It was shown that at the injection site, the haemocytes producing ALF*Pm*3 was not significantly found even the animals were stimulted by saline injection. Immediately after microbial injection, stimulation resulted in higher number of haemocytes containing ALF*Pm*3 that supposed to leave the circulation and migrate towards the injection site (Munoz et al., 2002) not the surrounding muscle in order to control microbial invasion to other organs. Spreading of ALF*Pm*3 expressing haemocytes in many shrimp organs could be explained by the following hypothesis. After *V. harveyi* challenging, a lot of bacteria should located at the site of injection. However some of *V. harveyi* can enter to the circulation system, this resulted in distribution of bacteria all over the shrimp body. We believed that shrimps protected themselves from microbial invasion via many immune reactions which mainly occur in haemocytes. Microbial challenge triggered the haemocytes to induce immune reactions and in the same time to reach the site of injection and elsewhere that pathogen attacked. Burgents et al. (2005), reported that the majority of *V. campbellii* (approximately 63%) was located at the site of injection and remained there for the entire 240 minutes of investigation after injection. The haemolymph contained approximately 27% of the intact *V. campbellii* while the remaining 10% were located in the examined organs such as gills, hepatopancreas, lymphoid organ and heart.

In summary, shrimp responded to microbial invasion through several immune reactions which resulted in changes of expression level of several gene transcripts. Further investigation of their roles in shrimp immunity will allow a better understanding of host defense mechanisms in this important species. In the innate immune system, antimicrobial effectors play an important role in killing or inhibiting bacterial growth. In shrimp, an anti-lipopolysaccharide factor (ALF), showed significant response to *Vibrio harveyi* challenged. The rALF*Pm*3 exhibited broad spectrum of activity against several strains of microorganisms including the shrimp pathogens. The results suggested a potential use of this molecule for disease control in shrimp aquaculture.

CHAPTER V

CONCLUSIONS

1. Identification of *Vibrio harveyi* responsive genes from *Penaeus monodon* haemocytes was performed by DD-PCR. Seventy-nine differentially expressed bands were identified from thirty-three combinations of primers. and forty-eight fragments were successfully cloned and sequenced.
2. Analysis of sequences of randomly selected transformant clones derived from each differentially expressed band showed that 44% accounted for clones containing one sequence whereas the remaining 56% contained mixture of sequences arisen from co-migration of non-homologous cDNA or a technical error.
3. Homology search against the GenBank database of the sequence clones identified 24 different genes with known function and 8 of them including balbiani ring protein3 precursor, argonaute protein, caspase 3B, serine protease inhibitor B3 (SERPINB3), Rc12 protein, glucose transporter 1 (GLUT1), interferon-related developmental regulator 1 (IFRD1), and huntingtin interacting protein E, were novel genes identified in *P. monodon*. For previously identified genes like anti-lipopolysaccharide factor type 3, (ALF*Pm*3), three additional isoforms were newly identified and a new isoform of lysozyme was also isolated
4. Some differentially expressed candidate genes including caspase 3B, GLUT 1, IRDR1, lysozyme, profilin, SERPINB3, were further confirmed for their relative expression by real-time RT-PCR. The results indicated that all selected genes were differentially expressed in *P. monodon* haemocytes upon *V. harveyi* challenge.
5. A recombinant protein of anti-lipopolysaccharide factor type 3 from the black tiger shrimp (rALF*Pm*3) was successfully produced in *Pichia pastoris* system.
6. The molecular mass of the purified rALF*Pm*3 protein was 11314.7 kDa and the N-terminal sequences corresponded to that of deduced amino acid sequence.

7. The purified rALF*Pm*3 protein has broad antimicrobial activity against Gram-negative and -positive bacteria, as well as filamentous fungi. The growth inhibition mechanism of rALF*Pm*3 is bacteriocidal effect.
8. By i*n situ* hybridization and real-time PCR analysis, it was shown that ALF*Pm*3 transcripts were produced in shrimp haemocytes and up-regulated upon *V. harveyi* infection.
9. Bacterial invasion resulted in distribution of ALF*Pm*3 expressing haemocytes throughout the shrimp cepharothorax during the first phase of infection. During this time, ALF*Pm*3 producing haemocytes migrated to the injection site. The ALF*Pm*3 transcripts reached again the high level of expression at 72 hour-post injection.

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APPENDICES

APPENDIX A

Preparation for polyacrylamide gel electrophoresis

1. Stock reagents

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

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

Adjust volume to 100 ml with distilled water.

1.5 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

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

2.0 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane	24.2	g

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

0.5 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	6.06	g

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

1.0 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

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

Solution B (SDS PAGE)

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

Solution C (SDS PAGE)

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

2. SDS-PAGE

15 % Seperating gel

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

5.0 % Stacking gel

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

5X Sample buffer

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

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

Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

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

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

APPENDIX B

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Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*

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Abstract

Anti-lipopolysaccharide factors (ALFs), originally characterized from horseshoe crabs, have been recently identified from hemocytes of the black tiger shrimp, *Penaeus monodon*, by a genomic approach. In order to characterize the properties and biological activities of this immune effector in shrimp, ALF*Pm3*, the most abundant isoform found in *P. monodon*, was expressed in the yeast *Pichia pastoris*. Large-scale production in fermentor provided 262 mg/l of recombinant ALF*Pm3* which was purified to homogeneity by single chromatography step on expanded-bed Streamline SP6XL. The rALF*Pm3* was further characterized in terms of N-terminal sequencing and mass spectrometry. Anti-microbial assays demonstrated that rALF*Pm3* has a broad spectrum of anti-fungal properties against filamentous fungi, and anti-bacterial activities against both Gram-positive and Gram-negative bacteria, associated with a bactericidal effect. Interestingly, rALF*Pm3* is highly efficient against various *Vibrio* species including strains pathogenic for shrimp. Finally, a synthetic peptide corresponding to a part of the putative LPS-binding site of ALF*Pm3* was shown to display activities mainly directed against Gram-positive bacteria indicating the involvement of the full molecule to the anti-microbial activity for Gram-negative bacteria.

Keywords: Shrimp; Crustacean; Anti-lipopolysaccharide factor; LPS-binding; Anti-microbial activities; *Vibrio*; Hemocytes

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1. Introduction

Anti-lipopolysaccharide factors (ALFs) have been first isolated from large granule hemocytes of horseshoe crab and they consist of basic proteins of around 100 amino acids [1,2]. It was shown that exposure of limulus hemocytes to bacterial endotoxins resulted in rapid degranulation and activation of an intracellular coagulation cascade [3]. *Limulus* ALF is able to inhibit this cascade by binding and neutralizing LPS and was shown to have an anti-bacterial effect on the growth of the Gram-negative bacteria *Salmonella minnesota* but not on the Gram-positive strain *Staphylococcus aureus* [1]. Since, the discovery of ALFs, various lipopolysaccharide-binding molecules and derivatives have attracted great interest in mammalian health as candidate therapeutic and/or prophylactic agents for the management of septic shocks [4]. Indeed, in humans, sepsis caused by Gram-positive and Gram-negative bacteria may cause shock that result in inflammatory responses with tissue injury, multi-organ failure and often in death [5,6]. It is known that lipid A, the conserved hydrophobic region of lipopolysaccharides (LPS) constitutes the bioactive center and toxic component of LPS. Among the molecules evaluated for properties of lipid A neutralization, *Limulus* anti-lipopolysaccharide factor (LALF) and derived peptides are intensively studied [7–9]. Comparatively, few studies have been devoted to the anti-microbial properties and the activity spectrum of this effector. Indeed, increased resistance of bacteria towards antibiotic drugs has stimulated intensive effort for discovery and characterization of anti-microbial peptides as sources or templates for the design of new therapeutic antibiotics.

Recently, cDNA clones homologous to the horseshoe crab ALFs were identified from the hemocytes of the black tiger shrimp, *Penaeus monodon*, and the atlantic white shrimp, *Litopenaeus setiferus*, by expressed sequence tag (EST) approach [10,11]. At least five different ALF sequences (ALF*Pm1*–ALF*Pm5*) have been identified in *P. monodon* hemocytes and ALF*Pm3* was shown to be the most abundant isoform found in both normal and *Vibrio harveyi*-infected shrimp hemocyte cDNA libraries, whereas the others were only evidenced in the infected ones [12]. The deduced amino acid sequence of the ALF*Pm3* (98 amino acids) showed 57–65% homology with those of *Tachypleus* ALF and *Limulus* ALF (Fig. 1). All ALF sequences including ALF*Pm3* have two conserved cysteine residues and the clustering of positive charges mainly within the disulfide loop (30–51 residues) [2,13]. This positive cluster is defined as the putative LPS-binding site. Until now in shrimp, only penaeidins, a family of anti-microbial peptides, have been intensively studied in terms of biological properties, anti-microbial activities, gene expression and localization in response to infection [14]. The other anti-microbial effectors recently identified such as crustins or ALFs remain to be characterized for their biological properties and immune functions. As done for a lysozyme sequence identified in *Marsupenaeus japonicus* [15], the production of recombinant molecule is a good alternative to chromatographic purification approaches, particularly when the effector is weakly expressed and produced in shrimp tissues.

In order to further characterize the penaeid shrimp ALF in terms of biological activity, three-dimensional structure and immune functions, a large-scale production system of the molecule was considered. We report the recombinant expression of ALF*Pm3* in the methylotrophic yeast *Pichia pastoris*, its purification to homogeneity and characterization. We have studied the anti-microbial activity of rAFL*Pm3* and compared to that of a synthetic peptide corresponding to

```
LALF     1 DG-IWTQLIFTLVKNLATLWQSGDFQFLDHECHYRIKDTFRRLKWKYKGKFWCPSWTSITGRATKSSRSGAVEHSVRNFVGQAKSSGLITQRQAEQFISQYN 101
TALF     1 EGGIWTQLALALVKNLATLWQSGDFQFLGHECHYRVNPTVKRLKWKYKGKFWCPSWTSITGRATKSSRSGAVEHSVRDFVSQAKSSGLITEKEAQTFISQYE 102
ALFPm3   1 QG--WEAVAAAVASKIVGLWRNEKTELLGHECKFTVKPYLKRFQVYYKGRWWCPGWTAIRGEASTRSQSGVAGKTAKDFVRKAFQRGLISQQEANQWLSS--  98
             *  *  :.  .:: . ::.*.    : : ***:: :.*..:*:.: ***:.***.**.*   *   *.**:: :::::**. *   ***:..:*. ::*
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The sequence of the synthetic peptide ALF*Pm3*#35-51 is in bold face.
LALF, anti-LPS factor sequence from *Limulus polyphemus*, P07086; TALF from *Tachypleus tridentatus*, P07087; ALF*Pm3* anti-LPS factor from *Penaeus monodon*.

Fig. 1. Amino acid sequence alignment of ALF*Pm3* with anti-LPS factor from *Tachypleus tridentatus* (TALF; PO7087) [2] and *Limulus polyphemus* (LALF; PO7086)[38]. The sequence of the synthetic peptide, ALF*Pm3*#35–51, deduced from the proposed LPS-binding region of ALF*Pm3* is in bold face.

the putative LPS-binding domain of the molecule. Finally, the availability of large amount of protein led us to the preparation of a specific antibody for further detection and localization of this immune effector in shrimp hemocytes.

2. Materials and methods

2.1. Construction of ALFPm3 expression cassette

To obtain the mature ALF*Pm3* peptide, ALF*Pm3* cDNA was amplified using Isis DNA polymerase™ (Qbiogene) with specific primers. For the convenience of cloning, a *Sna*BI site was added to the 5′-end of the forward PCR primer and a *Not*I site was added to the 3′-end of the reverse primer after the stop codon (Fig. 2). Primer sequences were ALFFw*Sna*BI: 5′-ATTACATACGTACAAGGGT GGGAGGCTGTG-3′ and ALFRv*Not*I: 5′-AAT-TATTGCGGCCGCCTATGAGCTGAGCCACTGG-3′, where restriction sites are underlined. The purified PCR product was digested with the restriction enzymes and cloned in-frame into *Sna*BI/*Not*I sites of the *Pichia* expression vector pPIC9K (Invitrogen), downstream of the sequence of the *Saccharomyces cerevisiae* α-mating factor signal peptide and the Glu-Ala-Glu-Ala repeat sequence. *Escherichia coli* Top10F' (Invitrogen) was used in the construction of ALF*Pm3* expression vector. The sequence of recombinant plasmid, pALF*Pm*K, was confirmed by nucleotide sequencing.

2.2. Pichia pastoris transformation and selection of recombinant clones

P. pastoris KM71 was grown overnight at 30 °C, 250 rpm in YPD medium. The yeast cells were harvested, washed twice with ice-cold sterile water, and resuspended in 1 M sorbitol. The purified pALF*Pm*K plasmid was linearized by *Sac*I and 5 μg of it was transformed into *P. pastoris* by electroporation as recommended by manufacturer instructions (Invitrogen). The cells were then spread on MD plates containing 1.34% yeast nitrogen base with ammonium sulphate without amino acid, 4×10^{-5}% biotin, 2% dextrose, 1.5% agar. Transformants were resuspended in sterile water and pooled. Positive *P. pastoris* transformants were selected by spreading 10^5 cells on YPD plates supplemented with G418-sulphate at

Fig. 2. Partial nucleotide sequence of ALF*Pm3* cDNA fused in-frame downstream the α-factor secretion signal and two Glu-Ala repeats of pPIC9K expression plasmid. *Sna*BI and *Not*I above the nucleotide sequence indicate the restriction sites added in the primers. Part of the nucleotide and amino acid sequence corresponding to α-factor secretion signal is represented by a box. Arrows above the nucleotide sequence indicate the Kex2 and Ste13 cleavage sites necessary for proteolytic processing of the fusion protein formed by the α-factor secretion signal and ALF*Pm3*. The mature amino acid sequence of ALF*Pm3* is shown in bold.

a final concentration of 0, 0.25, 0.75, 1, 1.5, and 2 mg/ml. Plates were incubated at 30 °C and checked daily until colonies growth. In order to purify putative G418-sulphate resistant clones, single colonies were streaked on YPD plates and then, on YPD-G418-sulphate plates to confirm G418-sulphate resistance.

2.3. Production of recombinant ALF3Pm3 (rALFPm3)

Single colonies of each clone were grown overnight in YPD broth medium at 30 °C and used to inoculate 100 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB (yeast nitrogen base with ammonium sulphate without amino acid, 4×10^{-5}% biotin, and 1% glycerol) in an 1 l flask. The cells were grown at 30 °C until an OD_{600} of 4–6. Then, they were harvested by centrifugation at 1500*g* for 5 min at room temperature and resuspended in 20 ml BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, and 0.5% methanol). To induce the expression, methanol (100%) was added every 24 h to a final volume concentration of 0.5%. To monitor the cell growth, culture medium (1 ml) was collected and centrifuged at 9000 rpm for 2 min at room temperature at each time point (0, 1, 2, 3, 4, 5, and 6 days). The supernatant and the cell pellets were stored at −80 °C. ALF*Pm3* expression was analyzed by SDS-PAGE. The clone which expressed the highest amount of recombinant protein was selected for large-scale production.

2.4. Large-scale production and purification of rALFPm3 in bioreactor

Cultures were carried out as described previously [16–18]. Cellular concentrations were followed by optical density (OD) using a spectrophotometer at 600 nm. One OD unit corresponds to 0.472 ± 0.001 g/l dry cell weights on glycerol and 0.442 ± 0.085 g/l dry cell weight on methanol.

The recombinant protein was purified using a single step ion-exchange chromatography performed with a Pharmacia Streamline 50 column (id 5 cm × 1 m). The matrix (Streamline SP XL) was equilibrated in 20 mM Tris, 200 mM NaCl, pH 7.0. The gel was expanded using upside flow at 300 cm/h until the top of the bed was stable. The crude culture was applied after dilution (1/2) in 20 mM Tris, 200 mM NaCl, pH 7.0 to the column (upside flow). The flow was subsequently inverted (downside flow) and the plunger was lowered to 1 cm from the top of the bed. The proteins were eluted with 307 ml of buffer containing 1 M NaCl at 100 cm/h flow rate. The elution of rALF*Pm3* was monitored by SDS-PAGE analyses. The excluded fractions were again purified and the rALF*Pm3* was eluted with 315 ml of 20 mM Tris–HCl, pH 7.0 and then 220 ml of 1 M NaCl buffer. The rALF*Pm3*-containing fractions were pooled and dialyzed in a spectro/por 3.5 kDa dialyzing tubing against 10 l of water and then lyophilized. SDS-PAGE was carried out under reducing conditions with 15% running gels and the protein bands were colored by Coomassie blue staining. The recombinant rALF*Pm3* was quantified by densitometry using cytochrome *c* (Sigma) as reference, with ImageMaster TotalLab software (Amersham Biosciences).

N-terminal sequence analysis of purified rALF*Pm3* was performed by automated Edman degradation and detection of the phenylthiohydantoin (PTH) derivates on an automated Procise Applied Biosystems Sequencer. Molecular mass of purified rALF*Pm3* was determined using matrix-assisted laser desorption ionization mode (MALDI-TOF) mass spectrometry.

2.5. Peptide synthesis

Part of putative LPS-binding region of ALF*Pm3* protein (KPYLKRFQVYYKGRMWC), ALF*Pm3* #35–51, was synthesized on an Abimed AMS 422 synthesizer by Fmoc chemistry [19]. The N-terminal residue of peptide was blocked by acetylation, and the C-terminal residue was amidated. The peptides were deprotected and released from the Rink amide resin (Novabiochem) by trifluoroacetic acid treatment in the presence of appropriate scavengers, and lyophilized. The peptides were purified onto a preparative C18 reverse-phase HPLC column (Waters) by elution with acetonitrile in 0.1% (v/v) trifluoroacetic acid acidified water. Then, the homogeneity of the purified peptide was analyzed on analytical C18 HPLC column and peptide integrity was checked by MALDI-TOF mass spectrometry. Lyophilized

synthetic peptide was reconstituted in sterilized water in order to minimize the risk of LPS contamination.

2.6. Anti-microbial activity assay

The bacterial strains used in this study included Gram-negative bacteria (*Escherichia coli* 363, *Salmonella thyphimurium*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Erwinia carotovora*) and Gram-positive bacteria (*Aerococcus viridans*, *Micrococcus luteus*, *Bacillus megaterium*, *Staphylococcus aureus*). In addition, the filamentous fungi *Botrytis cinerea* and *Penicillium crustosum* were used. Moreover, marine pathogenic organisms for shrimps were also considered including Gram-negative bacteria of the *Vibrio* genus (*V. harveyi*, *V. alginolyticus*, *V. penaeicida* and *V. anguillarum*) and the filamentous fungus *Fusarium oxysporum*.

Anti-microbial activities and determination of minimum inhibitory concentration (MIC) values were determined by liquid growth inhibition assays as previously described [20]. Briefly, 10 μl of each diluted molecules, or sterile deionized water as a control, were incubated in a sterile microtitration plates with 100 μl of a suspension of mid-logarithmic growth phase culture of bacteria diluted in culture medium to $OD_{600} = 0.001$, or with 80 μl of fungal spores (final concentration 10^4 spores/ml) and 10 μl of water. Poor-broth nutrient medium (1% bactotryptone, 0.5% NaCl, pH 7.5) was used for standard bacterial strain cultures. For marine bacteria strains, they were grown in saline peptone water (1.5% peptone, 1.5% NaCl, pH 7.2). Anti-fungal assays were performed in potatoes dextrose broth (Difco) at half-strength supplemented with 10 μg/ml tetracycline. Bacteria were grown overnight under vigorous shaking at 25 or 30 °C according to the strains. Fungi were grown at 25 °C in the dark without shaking for 48 h in a moist chamber. With the exception of fungal strains, microbial growth was controlled by measurement of the optical density at OD_{600} after incubation for 24 h. Inhibition of filamentous fungi growth was observed at microscopic level after 24 h and measured at 600 nm after 48 h. The MIC value was recorded as the range between the highest concentration of the peptide where bacterial growth was observed and the lowest concentration that caused 100% of inhibition bacterial growth [21].

2.7. Bacteriostatic/bactericidal activity assay

Mid-logarithmic-phase cultures of *E. coli* 363 and *B. megaterium* in poor-broth nutrient medium were incubated at 30 °C in the presence of rALF*Pm3* or water (control). The final concentration of the molecule tested was 10 times over the MIC value. Twenty microliter aliquots were removed at different time interval and plated on nutrient agar. The number of colony forming units (cfu) was determined after incubation for 24 h at 30 °C.

2.8. Hemolytic activities

The hemolytic activity of rALF*Pm3* and the derived ALF*Pm3* peptide were determined using human red blood cells (RBCs) isolated from heparinized blood as described by Dathe et al. [22]. Briefly, the RBCs were prepared from freshly collected human blood (4 ml) by centrifugation at 1500 rpm for 10 min at 4 °C. The cells were washed three times with phosphate buffer saline (pH 7.2–7.4) and diluted to 10% hematocrite. The RBCs (1%) were incubated with the peptide dissolved in phosphate buffered saline for 1 h at 37 °C. The concentration series used were as follows: 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, and 1.95 μM. The samples were centrifuged at 3500 rpm for 5 min and the absorbance of the supernatant was measured at 405 nm. Zero hemolysis (blank) and 100% hemolysis (control) were determined in phosphate buffered saline and Triton X-100, respectively.

2.9. Anti-ALF antibody and immunodetection of ALF in shrimp hemocytes

A rabbit was immunized by injection of the recombinant ALF (200 μg) diluted in PBS. Immunoglobulin G (IgG) was purified from immune serum on a Hitrap protein G sepharose column (Pharmacia). Native ALF was detected in hemocytes by immunocytochemistry according to the method already described [23]. Briefly, hemocytes cytocentrifuged on slides were successively incubated overnight with anti-ALF polyclonal antibody purified IgG (4 μg/ml) and for 1 h with alkaline phosphatase (PAL)-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories, Baltimore Pike, PA, USA). Control consisted in the omission of the first antibody specific for ALF.

3. Results and discussion

3.1. Large-scale production and purification of rALFPm3

EST analysis of cDNA libraries from hemocytes of normal and *V. harveyi*-challenged *P. monodon* resulted in the identification of at least five types of ALF homologues to that of *Limulus*. The representativeness of these transcripts appeared to be increased in the challenged library suggesting their potential involvement in the shrimp immune response to infection [10]. Investigations on the immune function and properties of this immune effector in shrimp require a large amount of ALF*Pm3* and its large-scale production in a heterologous system is an alternative to the purification from shrimp tissues which is often a tedious approach when the effectors are weakly expressed in the animals [24–26]. In this study, we have chosen to produce ALF*Pm3* in the methylotrophic yeast *P. pastoris*, an expression system which possesses many advantages including the potential to get hundreds mg per liter of recombinant protein [27].

P. pastoris KM71 transformants were screened on YPD containing various concentration of G418-sulphate as described in Section 2. Three rALF*Pm3* clones were randomly picked from plates at each concentration of G418-sulphate, grown in medium culture for small-scale expression trials and culture supernatants were analyzed by SDS-PAGE (data not shown). We selected for large-scale production in a 5 l bioreactor (Fig. 3) the clone, named ALFK9, which showed the highest apparent level of expression of ALF*Pm3*. The culture supernatants were analyzed by SDS-PAGE and one major protein with an apparent molecular weight around 10 kDa was detected (data not shown). The ratio of the rALF*Pm3* content estimated by densitometry to the total protein content indicates that rALF*Pm3* represented about 78% of protein secreted by *P. pastoris*. The recombinant rALF*Pm3* was produced during the induction phase and secreted into the culture medium. The recombinant molecule rALF*Pm3* was further purified from cell-free supernatant (2.8 l) by ion-exchange chromatography on a Streamline SP column. This single step purification on expanded-bed chromatography

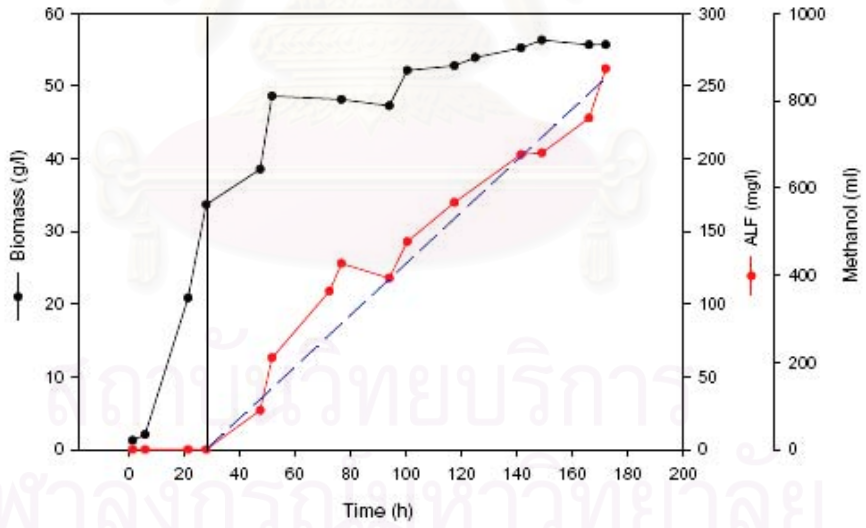

Fig. 3. Evolution of biomass (g/l) and of rALF*Pm3* expression during fed-batch culture of ALFK9 clone in bioreactor. In the first phase, two successive batches with 40 g/l of glycerol were performed to increase biomass. In these conditions, 33.7 g/l of dry cell weight were obtained and the fed-batch phase was consequently initiated with specific growth rate close to 0.005 g/l/h. After 130 h of induction, the final biomass was 55.7 g/l, the biomass yield on methanol was 0.27 g/g, and the concentration of rALF*Pm3* was 262 mg/l. The specific production was 4.7 mg rALF*Pm3*/g biomass. Methanol induction began at 24 h (dashed blue line) and the culture was harvested at 170 h. (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.)

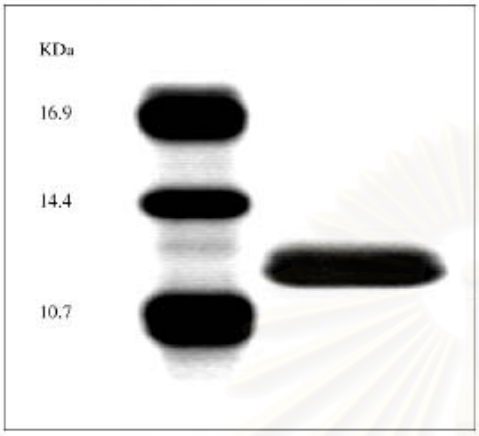

Fig. 4. SDS-PAGE of rALF*Pm3* produced in culture medium by *P. pastoris* after Streamline SP XL purification.

appeared to be a fast and efficient procedure since 118.4 mg of pure rALF*Pm3* were obtained and used for further characterization (Fig. 4).

3.2. Structural characterization and immunolocalization of ALFPm3

Purified rALF*Pm3* was analyzed by Edman degradation and MALDI-TOF mass spectrometry. Two molecules were detected: a major product with a molecular mass of 11,314.17±0.43 Da which corresponds to the expected calculated average mass (11314.96) of rALF*Pm3* including additional Tyrosine and Valine residues, and an additional molecule at 11,514.83±0.54 Da. Edman degradation on the 11,314.17 Da-major product revealed that the first 12 N-terminal residues were identical to the expected mature rALF*Pm3* including the additional Tyrosine and Valine residues brought by the cloning procedure in *Pichia*. With respect to the minor product of 11,514.83 Da, which represented about 15% of the amount of rALF*Pm3* produced, Edman degradation showed in the N-terminal amino acid sequence additional Glutamic and Alanine residues contributing to the mass excess of 200.66 Da. These extra amino acid residues could result from an inefficient STE13 protease cleavage at the N-terminal part of the secreted rALF*Pm3* which have already been reported in the *Pichia* production system [28].

The availability of great amount of rALF*Pm3* led us to raise a specific polyclonal antibody in rabbits and to detect and verify that native ALF is expressed in shrimp. Immunodetection assays showed that the different parts of the cytocentrifuged hemocyte population reacted to the specific anti-ALF*Pm3* antibody with various intensity (Fig. 5) revealing the presence of native ALF.

3.3. Study of rALFPm3 anti-microbial activity spectrum and properties

The anti-microbial activity spectrum of the purified rALF*Pm3* was investigated against a panel of Gram-positive and Gram-negative bacteria and filamentous fungi strains, including pathogenic strains for shrimps or molluscs. rALF*Pm3* displayed a large

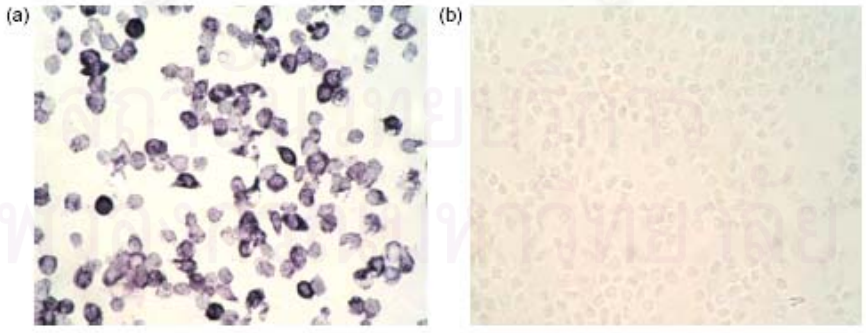

Fig. 5. Immunoreactivity of ALF*Pm3*-specific antibody on circulating hemocytes from *Penaeus monodon* challenged by *V. harveyi*. Hemocytes were cytocentrifuged on slides and stained with anti-ALF*Pm3* IgG followed by alkaline phosphatase-conjugated anti-rabbit IgG (a). Labeling without specific anti-ALF*Pm3* IgG was performed as negative control (b).

Table 1
Range of activity of rALF*Pm3* and synthetic peptide

Microorganisms	MIC value (μM)	
	rALF*Pm3*	ALF*Pm3*#35–51
Gram (+) bacteria		
Aerococcus viridans	1.56–3.12	3.12–6.25
Bacillus megaterium	0.19–0.39	1.56–3.12
Micrococcus luteus	1.56–3.12	3.12–6.25
Staphylococcus aureus	50–100	25–50
Gram (−) bacteria		
Enterobacter cloacae	3.12–6.25	12.5–25
Erwinia carotovora	1.56–3.12	25–50
Escherichia coli 363	0.095–0.19	12.5–25
Klebsiella pneumoniae	3.12–6.25	12.5–25
Salmonella thyphimurium	6.25–12.5	12.5–25
Vibrio alginolyticus	0.39–0.78	12.5–25
Vibrio anguillarum	0.78–1.56	50–100
Vibrio harveyi	0.78–1.56	25–50
Vibrio penaeicida	25–50	50–100
Filamentous fungi		
Fusarium oxysporum	1.56–3.12	6.25–12.5
Botrytis cinerea	3.12–6.25	6.25–12.5
Penicillium crustosum	12.5–25	12.5–25

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

range of anti-microbial activities and was highly effective against most of the microorganisms tested (Table 1). It showed an anti-microbial activity against all the Gram-positive bacteria, with the exception of *S. aureus* which remained unaffected in the concentration range of 50–100 μM. Comparatively, rALF*Pm3* was highly effective against *B. megaterium* (MIC = 0.19–0.39 μM). rALF*Pm3* showed high activities against Gram-negative bacteria. It strongly inhibited the growth of *E. coli* 363 (MIC = 0.095–0.19 μM) and of most of the *Vibrio* strains tested with low MIC values, such as 0.39–0.78 μM against *V. alginolyticus* or 0.78–1.56 μM against *V. harveyi*, pathogen for shrimps. However, weak activity was observed against the highly pathogenic *V. penaeicida* (MIC = 25–50 μM) which is highly pathogenic for shrimp [29]. Anti-microbial activity was also observed against filamentous fungi as shown against the shrimp pathogen *F. oxysporum* (MIC = 1.56–3.12 μM) or *P. crustosum* (MIC = 12.5–25 μM). The mechanism of action against filamentous fungi of rALF*Pm3*, either inhibition of spore germination or delayed growth of hyphae, should be further investigated. To date, very few anti-microbial peptides have been reported to be highly active against *Vibrio* strains apart from the mytilins, isolated also from a marine invertebrate, the mussel *Mytilus galloprovincialis* [30], and which display MIC values similar to those observed for rALF*Pm3*. Identified from the shrimp *M. japonicus*, recombinant lysozyme have been shown to display lytic activity against several *Vibrio* species including *V. penaeicidae* using a lysoplate assay [15]. Regarding the anti-microbial immune response in shrimp, anti-LPS factor would have complementary activity and function to those displayed by the penaeidins, a family of anti-microbial peptides also expressed in shrimp hemocytes [14]. Indeed, penaeidins have been shown to be strictly active in vitro against Gram-positive bacteria and fungi [20], but they also contribute, in shrimp tissues, to the elimination of *Vibrio* due to opsonic activity [23,31]. It will be of a prime importance to further analyze the respective kinetics of expression and localization of both penaeidins and ALF effector in response to infection, considering also lysozyme activity.

To study the nature of inhibition mechanism displayed by rALF*Pm3* in terms of bacteriostatic or bactericidal effect, we performed kinetics experiments using the bacteria the most sensitive to rALF*Pm3*: *E. coli* 363 as Gram-negative and *B. megaterium* as Gram-positive bacteria. To overcome a possible dose dependent effect, rALF*Pm3* was incubated with the bacteria at a concentration 10-fold higher than their MIC values. For the two bacteria strains, incubation of rALF*Pm3* resulted in the immediate killing of the bacteria as indicated by the absence of surviving cells as soon as 1–5 min incubation and revealing a strong bactericidal mechanism for rALF*Pm3*.

3.4. Characterization of an ALFPm3-derived synthetic peptide and comparison of its toxicity with ALFPm3

The anti-microbial activity of a synthetic peptide, ALF*Pm3*#35–51, corresponding to a part of the LPS-binding region proposed for *Limulus* ALF [32], was compared to that of the full rALF*Pm3*. This peptide was initially prepared for the production of a polyclonal antibody specific for ALF*Pm3*, but

Table 2
Hemolytic activity of rALF*Pm3* and synthetic peptide

Molecules	Percentage of hemolysis							
	Concentration (μM)							
	250	125	62.5	31.3	15.6	7.8	3.9	1.95
RALF*Pm3*	2	3	2	2	2	2	0	0
Synthetic ALF*Pm3*#35–51	22	20	9	8	7	6	4	0

The activity is measured as the percentage of lyzed red blood cells according to the peptide concentration.

recognition of the full molecule have been unsuccessful probably due to a lack of antigenicity of the peptide or its rapid neutralization during rabbit immunization (data not shown). The anti-microbial activity of ALF*Pm3*#35–51 appeared to be quite similar to that of the full ALF regarding the Gram-positive bacteria with MIC ranging from 1.56 to 3.12 μM for *B. megaterium* and *A. viridans*, respectively, or 25 μM for *S. aureus*. However, significant weaker anti-microbial activity than that displayed by the full rALF*Pm3* was observed for this peptide against all the Gram-negative bacteria tested, with MIC values about 12.5 or 50 μM. This result suggests that whereas the ALF*Pm3*#35–51 sequence would be involved in the recognition of the LPS molecules of the Gram-negative bacteria cell wall, it contributes to a minor anti-microbial activity against these micro-organisms. By contrast, in the case of the interaction with Gram-positive bacteria, when this peptide sequence would not be involved in the binding to LPS, it appears to display strong anti-bacterial activities as observed for the full rALF*Pm3* molecule. However, anti-microbial activity differences observed between the full-length ALF and the peptide would also be likely explained by differences in affinities for bacterial membranes. Further studies on rALF*Pm3* structural characteristics and interactions with bacteria will address this issue. Additionally, the synthetic ALF*Pm3*#35–51 revealed also an anti-fungal activity slightly weaker but close to that observed for the full ALF sequence. In an attempt of therapeutic strategies, this peptide may have the advantages of the dual property of LPS-binding and LPS-neutralizing, together with strong anti-microbial activity against Gram-positive bacteria, which merits to be further investigated.

Finally, an attention was accorded to the toxicity of rALF*Pm3* and its derived peptide, ALF*Pm3*#35–51 considering their effect on human red blood cells. For that, serial dilutions of both molecules ranging from 1.95 to 200 μM were incubated with the cells. After 1 h of incubation, no hemolysis activity has been observed with rALF*Pm3* whatever the concentration of the molecule (Table 2). This is in agreement with early work showing that ALF from *Tachypleus tridentatus* was able to lyze red blood cell sensitized with Gram-negative bacterial LPS but not unsensitized cells [33]. We showed that the synthetic peptide displayed a weak hemolysis (around 22%) at a concentration of 100 μM, which may suggest that an amphipathic motif would be unmasked in the peptide than in the full protein. To complete these preliminary data, studies on the toxicity of these molecules on various eukaryotic cells have been undertaken in an attempt to investigate their potential use as therapeutic anti-microbial agents in aquaculture.

4. Concluding remarks

The goal of this study was to investigate and characterize the biological properties of anti-LPS factor molecule identified in the shrimp *P. monodon*. For that, we have chosen to produce this immune effector in the recombinant system of *P. pastoris* which have been shown to be appropriate for the production of large amount of active molecules. Among the different ALF isoforms identified in the EST program, ALF*Pm3* was chosen because it predominated and first gene expression studies revealed an increase in transcripts abundance in *P. monodon* hemocytes after

a bacterial challenge [12], suggesting its involvement in the immune response of shrimp. Gram-negative bacteria and particularly those belonging to *Vibrionaceae* genera are remarkably the most present in marine environment. They are reported as commensal or probiotic but also they can be opportunistic even indeed pathogenic, often associated with mortalities of shrimp larvae or juveniles [34–36]. Consequently, it is of prime importance to understand the function of anti-LPS factor and its effect against *Vibrio* strains. We showed here that ALF*Pm3* has a large spectrum of activity against all microorganisms tested, from Gram-positive, Gram-negative bacteria as well as filamentous fungi. They are highly effective against most of the *Vibrio* strains assayed apart against *V. penaeicidae* which is pathogenic for juvenile shrimp. Interestingly, ALF*Pm3* could have a synergic effect with the shrimp lysozyme molecule which was found active against these bacteria [15] and which could also display LPS-binding properties as shown for chicken egg lysozyme [37]. Apart for a direct anti-microbial activity against potentially harmful bacteria, ALF*Pm3* would be a major effector of the shrimp immune system, which could be involved due to its LPS-binding property to various reactions such as inhibition of inflammatory reaction or anticoagulant activity. The availability of a specific antibody will now permit to investigate first the localization of shrimp ALF in hemocyte population but also to study the production and localization of this effector during the response of the shrimp to an infection. Finally, due to its anti-microbial properties, the shrimp ALF appears to be a good candidate for further investigation about its potential use in larviculture as therapeutic agent (http://www.immunaqua.com) and as alternative to conventional antibiotics.

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Biography

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