ผลของโปรตีนรีคอมบิแนนต์แอนติไลโปพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 ในการป้องกันโรคเรืองแสงในกุ้งกุลาดำ *Penaeus monodon*

นางสาวศิริขวัญ พลประทีป

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF RECOMBINANT ANTI-LIPOPOLYSACCHARIDE FACTOR ISOFORM 3 PROTEIN ON VIBRIOSIS PREVENTION IN THE BLACK TIGER SHRIMP *Penaeus monodon*

Miss Sirikwan Ponprateep

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science Program in Biochemistry

Department of Biochemistry

Faculty of Science

Chulalongkorn University

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Thesis Title	EFFECTS OF RECOMBINANT ANTI LIPOPOLYSACCHARIDE FACTOR ISOFORM 3 PROTEIN ON VIBRIOSIS PREVENTION IN THE BLACK TIGER SHRIMP *Penaeus monodon*
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ศิริขวัญ พลประทีป : ผลของโปรตีนรีคอมบิแนนต์แอนติไลโปพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 ในการป้องกันโรคเรืองแสงในกุ้งกุลาดำ *Penaeus monodon.* (EFFECTS OF RECOMBINANT ANTI-LIPOPOLYSACCHARIDE FACTOR ISOFORM 3 PROTEIN ON VIBRIOSIS PREVENTION IN THE BLACK TIGER SHRIMP *Penaeus monodon)*
อ. ที่ปรึกษา : ศ.ดร.อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม : อ.ดร.กุลยา สมบูรณ์วิวัฒน์, 109 หน้า.

จากการศึกษาก่อนหน้านี้พบว่าโปรตีนรีคอมบิแนนต์แอนติไลโปพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 (rALF*Pm*3) มีฤทธิ์ยับยั้งการเจริญของจุลชีพได้หลายกลุ่ม โดยเฉพาะอย่างยิ่งฤทธิ์ของโปรตีนในการยับยั้งการเจริญของวิบริโอฮาร์วิอายที่เป็นแบคทีเรียก่อโรคเรืองแสงในกุ้ง ดังนั้นในการทดลองนี้จึงทำการผลิตโปรตีน rALF*Pm*3 ในระบบของยีสต์ *Pichia pastoris* ในปริมาณมาก จากนั้นทำโปรตีนให้บริสุทธิ์โดยเทคนิคโครมาโทรกราฟฟี แบบแลกเปลี่ยนประจุบวก นำโปรตีนที่ได้ไปศึกษาฤทธิ์ของการยับยั้งการเจริญของเชื้อวิบริโอภายใต้สภาวะรุนแรง พบว่าที่ความเข้มข้นของเกลือ (1-3%) และ ค่า pH (5-9) ที่สูงขึ้นจะมีผลทำให้ฤทธิ์ในการยับยั้งแบคทีเรียลดลง ขณะที่โปรตีนมีความคงตัว เมื่อบ่มในอุณหภูมิ 4 30 50 และ 100 องศาเซลเซียส เป็นเวลา 2 ชั่วโมง นอกจากนั้นได้ทำการเก็บโปรตีนที่อุณหภูมิห้องเป็นเวลา 3 เดือน พบว่า ฤทธิ์ในการยับยั้งเชื้อวิบริโอไม่เปลี่ยนแปลงไปจากเดิม การศึกษาผลของการทำลายเชื้อวิบริโอและการป้องกันโรคกุ้ง พบว่ากุ้งมีอัตราการรอด 100% เมื่อทำการบ่มระหว่าง เชื้อวิบริโอ (10^6 CFU)และ rALF*Pm*3 ที่ความเข้มข้นสุดท้าย 6.25 ไมโครโมลาร์ ในการศึกษาความสามารถของ rALF*Pm*3 ต่อการป้องกันโรคก่อนการติดเชื้อ พบว่ากุ้งมีอัตราการตายลดลง จาก 60% เป็น 25% เมื่อฉีดด้วย rALF*Pm*3 ก่อนทำให้ติดเชื้อ ผลของ rALF*Pm*3 ต่อระดับการแสดงออกของยีนที่เป็นตัวแทนในระบบภูมิคุ้มกันในกุ้งที่ติดเชื้อวิบริโอ ได้แก่ ยีนแอนติไลโปพอลิแซ็กคาไรด์แฟกเตอร์ไอโซฟอร์ม 3 (ALF*Pm*3) ยีนโปรฟีนอสออกซิเดส (PPA) ยีนที่เกี่ยวข้องกับการกำจัดออกซิเจนอิสระ (SOD) ยีนที่เกี่ยวข้องกับการเกิดแอพอปโทซิส (Survivin) ซึ่งมีการเปลี่ยนแปลงในทุกยีน ยกเว้นยีนที่เกี่ยวข้องกับการเกิดฟาโกไซโตซิส (PAP) จากผลการทดลองแสดงให้เห็นว่า rALF*Pm*3 มีศักยภาพที่จะนำไปใช้ในการบำบัดโรคและพัฒนาเป็นผลิตภัณฑ์ยาเพื่อประยุกต์ใช้ในการเพาะเลี้ยงกุ้งต่อไป

ภาควิชา.....ชีวเคมี..... ลายมือชื่อ นิสิต.....ศิริขวัญ พลประทีป.....
สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD :*Penaeus monodon*/ SHRIMP DISEASE/ *Vibrio harveyi*/ ANTI-LIPOPOLYSACCHARIDE FACTOR/ *Pichia pastoris* EXPRESSION SYSTEM

SIRIKWAN PONPRATEEP : EFFECTS OF RECOMBINANT ANTI-LIPOPOLYSACCHARIDE FACTOR ISOFORM 3 PROTEIN ON VIBRIOSIS PREVENTION IN THE BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR : KUNLAYA SOMBOONWIWAT, Ph.D., 109 pp.

The broad spectrum of antimicrobial activity of the recombinant anti-lipopolysaccharide factor isoform 3 protein form *Penaeus monodon* (rALF*Pm*3) was previously reported. Interestingly, the rALF*Pm*3 is highly active against *Vibrio harveyi*, a shrimp pathogenic bacterium suggesting its potential use in control or prevention of the outbreak of Vibriosis in shrimp farming. Therefore, the rALF*Pm*3 was produced in large scale in *Pichia pastoris* and purified by cation-exchange chromatography. The effects of extreme condition on anti-*Vibrio* activity of rALF*Pm*3 were examined including salt, pH and temperature. The anti-*Vibrio* activity of rALF*Pm*3 protein was reduced when the salt concentrations (1-3%) and pH (5-9) were increased. However, either incubation at various temperatures (4, 30, 50 and 100°C) for 2 hours or storage at room temperature for three months did not effect the molecule integrity and its anti-*Vibrio* activity. We further investigated its activity in vivo in terms of neutralization and protective effect on *V. harveyi* challenged shrimp. At the concentration 6.25 μM, neutralization of *V. harveyi* (10^6 CFU) with rALF*Pm*3 resulted in 100% shrimp survival. Protection of *P. monodon* from *V. harveyi* infection by pre-injected with rALF*Pm*3 revealed a reduction of cumulative mortality from 60% to 25% comparing to the control shrimps. The effects of the rALF*Pm*3 injection on expression levels of the representative immune genes, ALF*Pm*3, PAP, PPA, SOD and survivin were examined in order to observe changes in shrimp immune response. The results indicated that rALF*Pm*3 could affect several immune reactions including antimicrobial action, proPO system, antioxidant defence pathway and apoptosis but not affect the phagocytosis, Our results suggest the potential use of rALF*Pm*3 in therapeutic and pharmaceutical applications in aquaculture.

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List of Abbreviations

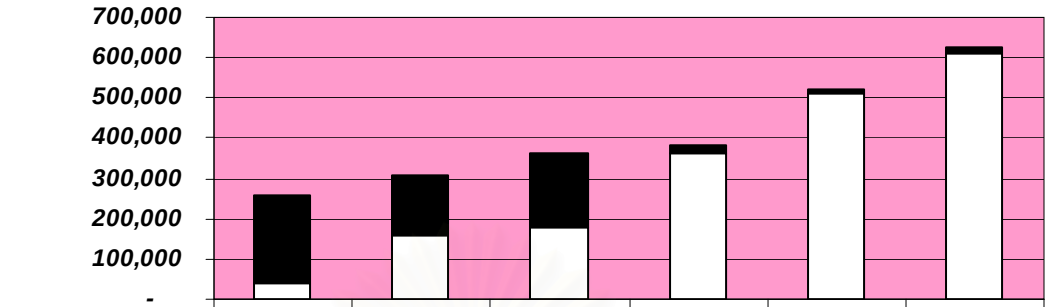
bp	base pair
^{o}C	degree celcius
SOD	cytosolic manganese superoxide dismutase precursor
DEPC	diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EtBr	ethidium bromide
hpi	hour-post injection
LPS	lipopolysaccharide
M	molar
ml	millilitre
MT	metric ton
mg	milligram
mM	millimolar
ng	nanogram
O.D.	optical density
PAP	phagocytosis activating gene
PCR	polymerase chain reaction
proPO	prophenoloxidase
PPA	prophenoloxidase activating gene
rALF*Pm*3	recombinent antilipopolysacharide factor isoform 3
RNA	ribonucleic acid
RT	reverse transcription
survivin	apoptosis inhibitor survivin
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I

INTRODUCTION

1.1 General introduction

Shrimp aquaculture started in the 1970s as an industrial activity and developed rapidly with a huge increase in the number of hatcheries and farms. The activity concerns tropical countries in South East Asia, Central and South America and the total annual production reaches about 712,000 metric tones (Rosenberry, 1997). Asia has always led world production of cultivated shrimp with a market value of billions of US dollars per year. Thailand alone has been the world's leading producer since 1992 with its export earnings reaching more than 1 billion US dollars per year (Flegel, 2006). In 1995, largely due to the peak in yellow-head virus (YHV) outbreaks, production decreased by about 5000 metric tons(equal to approximately 40 million US dollars in lost export revenue). In 1996 and 1997, peak losses by another virus called white-spot syndrome virus (WSSV) was even more disastrous, with cumulative lost export revenue estimated at approximately 1 billion US dollars. After 1997, Thai production began to recover, reaching the previous highest production of 250,000 metric tons again in 1999 and have since remained more or less at that level or higher. The rest of Asia did not fare so well. For example, WSSV outbreaks in China began in 1993, reducing export production from the 1992 high of 115,000 metric tons to 35,000 metric tons. Although, the shrimp production continuously increase but the black tiger shrimp production rapidly decrease in Thailand since 2005 to 2007 (Fig. 1.1). The examples above serve to illustrate how serious disease losses can be in the shrimp aquaculture industry (Flegel, 2006). Shrimp production is seriously affected by problems linked to environment degradation and to infectious and non-infectious diseases. The causative agents of infectious diseases in shrimp are mainly viruses and bacteria belonging to Vibrionacea (Bachere, 2000).



	2002	2003	2004	2005	2006	2007
■ *P. monodon*	221,000	150,000	180,000	19,000	10,400	12,480
□ *P. vanamei*	39,000	160,000	180,000	361,000	509,600	611,520

Figure 1.1 The shrimp production in Thailand between 2002 and 2007 (Source: http:// www.shrimpcenter.com)

1.2 Taxonomy of *Penaeus monodon*

The taxonomic definition of *Penaeus monodon*, the giant tiger shrimp, 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 (Fig. 1.2). Each body region possesses appendages specialized for different functions. The head (five somites) and thorax (eight somites) are fused into a cephalothorax, which is completely covered by the carapace. Many internal organs, such as gills, digestive system, reproductive system and heart, located in thorax, are protected by the carapace. Shrimp 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 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 abdomen has the obvious segmentation of invertebrates. The abdomen consists of six somites, the first five with paired pleopods (walking legs) (Bell and Lightner, 1988; Baily-Brook and Moss, 1992) and the sixth with uropods. A tail fan comprises of a telson, which bears the anus. The telson has deep medication groove without dorso-lateral spines. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of prawn (Anderson, 1993). The anus is on the ventral surface of the telson towards its base (Dall et al., 1990).

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 moulted. After moulting the new cuticle is soft and is stretched to accommodate the increased sized of the shrimp.

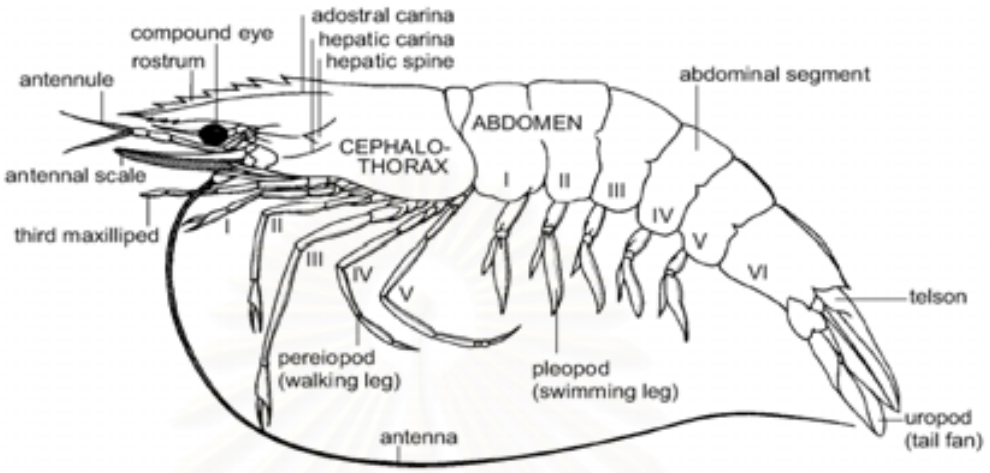


Figure 1.2 Lateral view of the external morphology of *Penaeus monodon* (Anderson, 1993)

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 (Fig. 1.3). Muscular, digestive, circulatory, respiratory, nervous, and reproductive systems are all present. The movements of the body such as walking, crawling, burrowing, swimming, feeding, and breathing are controlled by the muscles. 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.

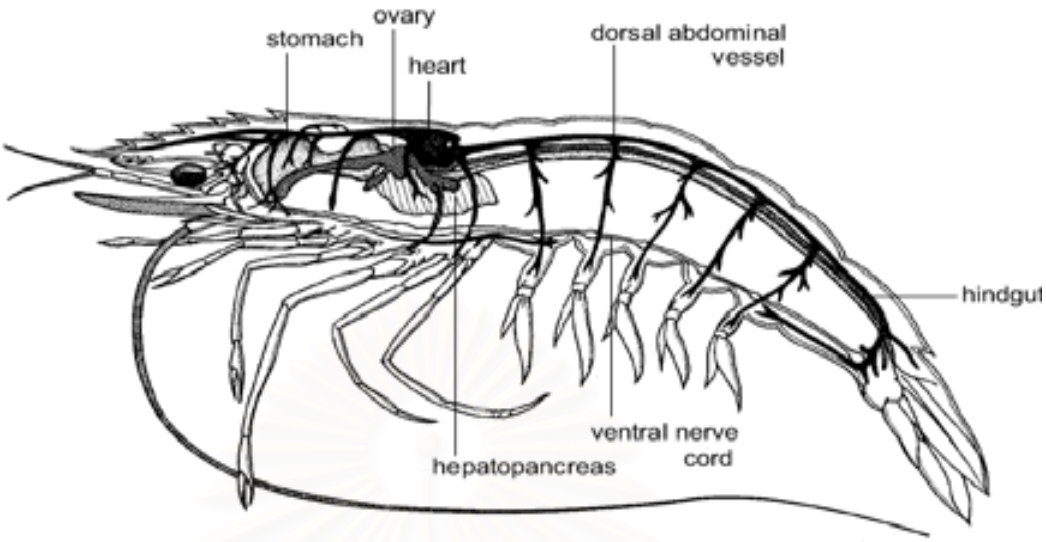


Figure 1.3 Lateral view of the internal anatomy of a female *Penaeus monodon* (Primavera, 1990)

Hepatopancreas connects to the gastrointestinal tract via the primary duct. It occupies a large portion of the cephalothorax in penaeid shrimp and functions on absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). One of the haemolymph vessels that leave the heart ends in the lymphoid organ where the haemolymph is filtered. This organ consists of two distinct lobes, each located ventro-lateral to the junction of the anterior and posterior stomach chambers. The lymphoid lobes are apposed slightly dorso-anterior to the ventral hepatopancreatic lobe. The haemocytes are produced in haematopoietic tissue. The hematopoietic tissue consists of densely packed lobules located at different parts of the shrimp anterior region. The first one is hematopoietic tissue surrounding the lateral arterial vessel, which joins the anterior recurrent artery at the base of the rostrum. The second one located within the 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.

1.4 Distribution and Life cycle

The giant black tiger shrimp is widely distributed throughout the greater part of the Indo-Pacific region, ranging northward to Japan and Taiwan, eastward to Tahiti, southward to Australia and westward to Africa. The penaeid life cycle includes several distinct stages that are found in a variety of habitats (Fig. 1.4). Juveniles prefer brackish shore areas and mangrove estuaries in their natural environment. Most of the adults migrate to deeper offshore areas at higher salinities, where mating and reproduction takes place. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). The eggs hatch into the first larval stage, which is the nauplius. The nauplii feed on their reserves for a few days and develop into the protozoeae. The protozoeae feed on algae and metamorphose into myses. The myses feed on algae and zooplankton and have many of the characteristics of adult shrimp and develop into megalopas, the stage commonly called postlarvae (PLs). Larval stages inhabit plankton-rich surface waters offshore, with a coastal migration as they develop.

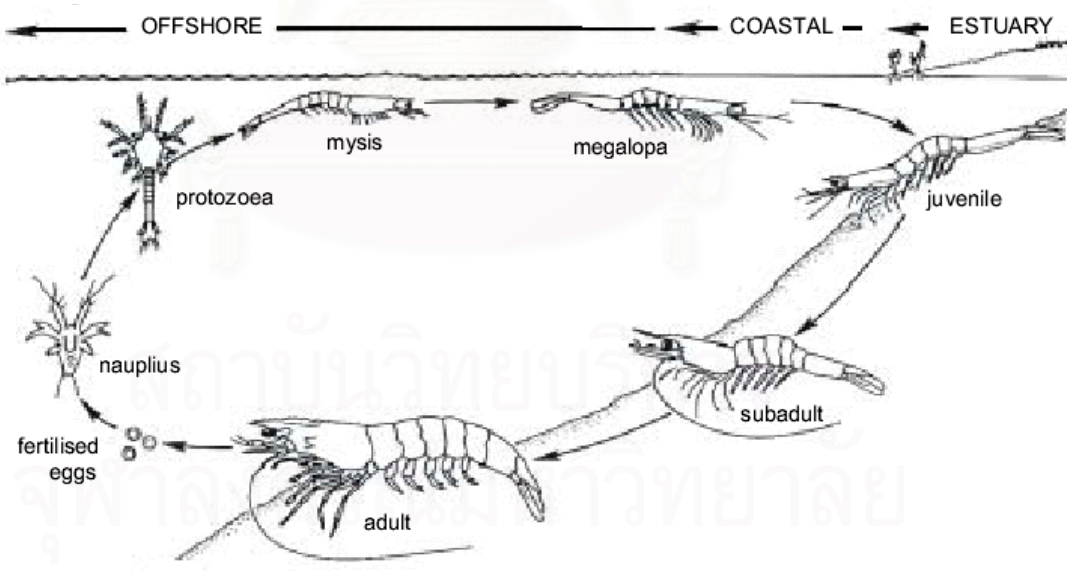


Figure 1.4. **The life history of *P. monodon* shrimp (Baily-Brook and Moss, 1992)**.

Eggs hatch within 16 h after fertilization. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The megalopa and early juvenile are called postlarvae.

Transition from juvenile to subadult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months. Pictures are not in proportion to actual size.

1.5 Shrimp diseases

The repeated outbreaks of infectious diseases occurred in the shrimp farms cause the great impact on the *P. monodon* production. . The infectious diseases in *P. monodon* are caused mainly by virus and bacteria belonging to Vibrionacea (Lightner et al., 1983). Vibriosis is the most prevalent bacterial disease causing mass mortalities both in hatcheries and in shrimp growout ponds (Saulnier et al., 2000). The major virulent strains of *Vibrio* in shrimp are *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and *V. anguillaram*. The presences of *V. harveyi* have been reported in *P. monodon* and *P. vannamai* in Indonesia (Sunaryanto and Mariam, 1986), Thailand (Jiravanichpaisal et al., 1994) and India (Karunasagar et al., 1994). The major viruses of concern (in estimated order of past economic impact for Thailand) are white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV) and monodon baculovirus (MBV). However, with the introduction of shrimp, Taura syndrome virus (TSV) and infectious hypodermal and hematopoeitic virus (IHHNV) have now become important (Flegel, 2006). Moreover, shrimp aquaculture is presently based on wild animals that are adopted to natural conditions and not to the artificial conditions of shrimp hatcheries and farms, where water quality, microbiological flora and nutrition are vastly different from those in the sea. Intensive rearing conditions are stressful for shrimp and lead to physiological disturbances or immunodeficiencies that increase sensitivity to pathogens.

1.5.1 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 identified 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 spread cellular degeneration and severe nuclear hypertrophy in most tissue derived from ectodermal and mesodermal origin, especially from subcuticular shell epithilum, gill epitelium, sublcuticular stomach epithelium, lymphoid organ, connective tissue, hematopoietic tissue and nervous tissues (The ASEAN Fisheries Sub-Working group, 1998). The other clinical sign of this disease is a rapid reduction in food consumption of shrimp. Mortalities were low during the initial two to three days. Mass mortalities 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.

1.5.2 Vibriosis

Vibrio species are a normal part of the bacterial flora in aquatic environment and formerly considered to be mostly opportunistic pathogens (Lightner and Redman, 1998). 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). However, recently occurring disease syndromes of penaeid shrimp have been caused by *Vibrio* species which behave more like true pathogens than opportunistic invader (Lightner, 1992). Vibriosis is a major disease problem in shrimp aquaculture, causing high

mortality and severe economic loss in all producing countries (Mohney et al., 1994). Disease outbreaks attributed to *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus* and *V. penaeicida* have been observed in nursery or grow-out ponds of *P. monodon*, *L. vannamei*, *P. japonicus* and *P. stylirostris* (Saulnier et al., 2000).

V. harveyi is a Gram-negative bacterium. It is a rod shape, 0.5-0.8 μm in width and 1.4-2.6 μm in length. It is able to emit light of a blue-green color. This bacterium is claimed to be the most causative agent associated with shrimp mortality. The disease is widely known as luminous disease or Kung-rungsang in Thai. The bacterial pathogen results in mortality up to 100% for nauplius to Zoea stages of *P. merguiensis*. Living and dead shrimp larvae and even the seawater in the disease outbreak areas are luminescent in dim light (Fig. 1.5).

Extracellular products (ECP) have been considered to be important determinants of virulence in *V. harveyi* such as cysteine protease, phospholipase, haemolysin. Thus, Saeed (1995) studied the association of *V. harveyi* with mortalities, and noted that ECP was toxic to brown spotted grouper but not tosil very black porgy. Furthermore, Liu et al. (1996) studied the pathogenicity of strains, which were recovered from diseased *P. monodon* and determined that virulence occurred with both live bacteria and ECP (Liu et al., 1996). Both the live virulent bacteria and their ECP exhibited stronger proteolytic (caseinase), phospholipase and haemolytic activities than those of non-virulent reference strains (Austin and Zhang, 2006).

1.6 Shrimp disease control

The control of disease became a priority at the world level if shrimp production is to be ecologically and economically sustainable. To a greater extent, the durability of the production is dependent on the equilibrium such as the environment quality, the prevention of diseases by diagnosis and epidemiological surveys of the pathogens and the health status of the shrimp. The shrimp aquaculture is also dependent on the selection of animals resistant to diseases. Therefore, the prevention and the control of shrimp diseases need an integrated approach in which knowledge of shrimp immunity must be improved, consideration given to other research areas

related to pathology and shrimp physiology, and in close connection to research in genetics (Bachere, 2000).

The main technique for disease prevention in vertebrates is vaccination. Lacking adaptive immunity, invertebrates cannot be protected by a previous contact with the antigen. Treatments of formalin-killed *Vibrio* vaccine significantly enhance the resistance of post-larvae shrimp to Vibriosis (Teunissen et al., 1998). Immunostimulants are the substances, which enhance the non-specific defence mechanism and provide resistance against the invading pathogenic microorganisms. In order to increase the immunity of shrimp against the WSSV, the methanolic extracts of five different herbal medicinal plants like *Cyanodon dactylon*, *Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa* and *Eclipta alba* were selected and mixed thoroughly in equal proportion. Among the different concentrations of herbal immunostimulant supplemented diets, the shrimp fed on had more survival and reduction in the viral load. Also the better performance of haematological, biochemical and immunological parameters was found in the immunostimulant incorporated diets fed shrimp. (Citarasu et al., 2006). Moreover, the efficacy of immunostimulants composed of inactivated WSSV, with or without β-1, 3-glucan or killed *Vibrio penaeicida* and of recombinant proteins of WSSV (rVP26, rVP28), were tested by intramuscular injection into kuruma shrimp followed by an intramuscular challenge with WSSV. The stimulated shrimp showed a resistance to WSSV (Namikoshi et al., 2004; Witteveldt et al., 2004). In shrimp ponds, some treatment processes can be applied to the water. For instance, tons of so-called immuno-stimulants such as β-glucans or laminarin, soluble/insoluble extracts of fungi or alga cell walls, are added to shrimp ponds although the reduction of diseases-related mortalities has not been proven. Moriarty (1998) who found that the value of adding selected strains of *Bacillus* as probiotics resulted in control of the Vibrios in shrimp farms in Indonesia (Moriarty, 1998). The farms that did not use probiotics suffered high mortality of the shrimps on or before the 80th day of culture due to luminescent *Vibrio* disease. Moreover, Selvin and Lipton (2004) showed that Dendrilla feed elicited complete protection (100% survival) against the most common shrimp pathogens such as luminescent *V. harveyi* and *V. alginolyticus.* Ciprofloxacin was found to be the most effective in controlling the isolates from hatcheries and ponds

compared with the other antibiotics. However, it revealed that antibiotic-resistant bacteria are wide spread in the shrimp culture hatcheries and ponds in India (Vaseeharan et al., 2005). Antibiotics are extensively used but the pathogens resistance and international regulations are aimed at limiting their use. While the knowledge of shrimp immune system is developing, current knowledge of cellular and molecular effectors can be employed to reduce the impact of diseases. For instance, hemocytic parameters such as phagocytosis, associated oxidative radical production, and anti-microbial peptide levels, might be reflective of immune status. Selection of the most resistant animals could be based on a screening of these parameters. In the same way, gene characterization and antibody-based immunoassays of molecular effectors will may facilitate genetic selection (Roch, 1999).

1.7 The shrimp immune system

1.7.1 Heamocyte

Crustacean has an open circulatory system. The circulating haemocytes of crustacean are essential in immunity, performing functions such as phagocytosis, encapsulation, and lysis of foreign cells (Johansson and Soderhall, 1989). 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 present in penaeid shrimp and freshwater crayfish (Rodriguez et al., 1995).

Hyaline cells, which lack the cytoplasmic granules, are the smallest group. It was found only 1% of the total haemocytes (Iwanaga et al., 1998). The hyaline haemocyte is reportedly involved in phagocytosis (Soderhall et al., 1986). Granular and semigranular haemocytes were oval, plate-shaped structure with 15-20 μm in their longest dimension. The semigranular cell is the most abundant type of haemocyte. This haemocyte responses by phagocytosis and encapsulation (Persson et al., 1987). The granular cells are involved in phagocytosis, encapsulation and storage

of the prophenoloxidase (proPO) system (Hose et al., 1990; Gargioni and Barracco, 1998; Burgents et al., 2005).

1.7.2 Phagocytosis

Cellular defense actions include such processes as phagocytosis, encapsulation and nodule formation (Millar and Ratcliffe, 1994). Phagocytosis is a phenomenon that appears to occur in all organisms, and includes attachment to the foreign body, ingestion and destruction. Encapsulation, a process wherein layers of cells surround the foreign material, occurs when a parasite is too large to be ingested by phagocytosis. Nodule formation, which appears similar to capsule formation, occurs when the number of invading bacteria is high. These structures, capsules and nodules, are always melanized in arthropods.

1.7.3 The prophenoloxidase (proPO) system

Shrimp proPO system have been elucidated in *Penaeus californiensis* (Vargas-Albores et al., 1996; Gollas-Galvan et al., 1999), *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 the crayfish proPO than to the insect proPO according to the amino acid comparison.

The proPO activating system comprises several proteins involving in the immune defense in invertebrates leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Sritunyalucksana and Söderhäll, 2000) (Fig. 1.5). It is activated by extremely low quantities of lipopolysaccharides or peptideoglycans from bacteria and β–1, 3-glucans from fungi, through pattern-recognition proteins (Ariki et al., 2004). The proPO system is a proteinase cascade involving several zymogenic proteinases, and proPO (Cerenius and Soderhall, 2004). Upon stimulation, proprophenoloxidase-activating enzyme (proppA) is converted to an active ppA, a serine proteinase, which then converts the proPO to the active phenoloxidase (PO) by proteolysis. PO catalyses the two successive reactions: the hydroxylation of a monophenol to *o*-diphenol (monophenoloxidase activity) and the oxidation of the *o*-diphenol to *o*-quinone (diphenoloxidase activity) (Decker et al., 2000; Decker and

Tuczek, 2000; Cerenius and Soderhall, 2004). Production of the *o*-quinones by PO leads to melanization. The production of melanin pigment can often be seen as dark spots in the cuticle of arthropods 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 (Cerenius and Soderhall, 2004).

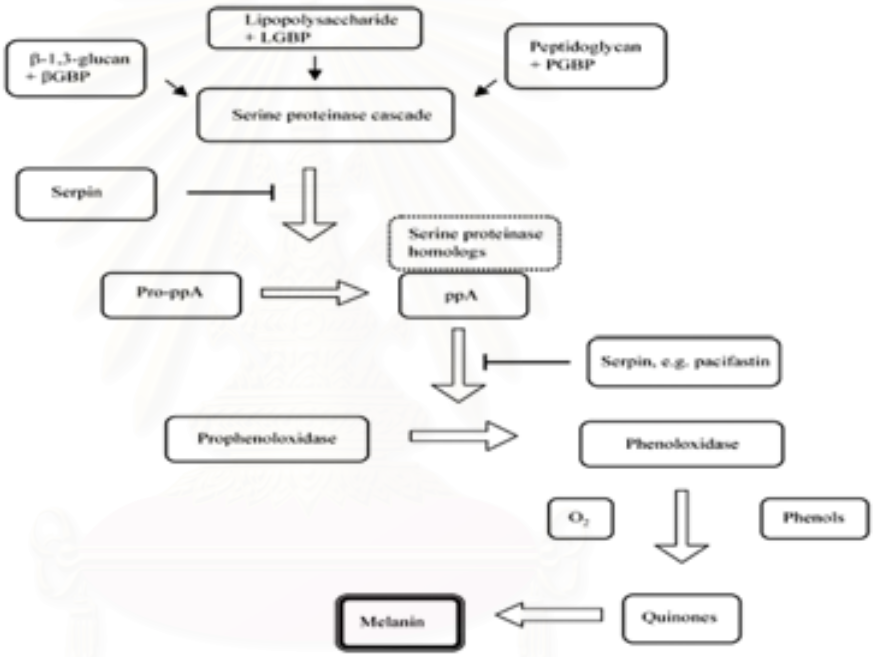


Figure 1.5 Overview of the arthropod prophenoloxidase (proPO)- activating system (Cerenius and Soderhall, 2004).

1.7.4 Reactive oxygen

In animal, the synthesis of reactive oxygen species (ROS) has important roles in activated phagocytes are potentially deleterious to microorganism. ROS include oxygen free radicals and non-radical oxygen derivatives such as superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2), singlet oxygen ($O_2^{\cdot}$) and hydroxyl radical ($OH^{-\cdot}$), which are highly microbiocidal (Munoz et al., 2000). The process starts when stimulation leads to increased consumption of oxygen, and the reduction of oxygen, catalysed by a membrane-bound NADPH oxidase, gives rise to superoxide anion (Eq.

(1)). Although these microbiocidal agents are generated in the phagocytic vacuoles, an important quantity crosses into the extravacuolar and extracellular environment and may cause damage to cells (Warner, 1994). ROS can damage all kinds of biochemical substances present in the cell, i.e. DNA, lipids, proteins and carbohydrates (Gutteridge, 1977). To prevent oxidative damage, cell contains widely distributed enzyme: superoxide dismutase (SOD), glutathione peroxidase and reductase (GPx and GR) and catalase (Cat) that detoxify the ROS. The superoxide anion can easily form in reactions where molecular oxygen is present, and is accelerated by the cytoplasmic enzyme superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2) and O_2 (Eq. (2))

$$2O_2 + NADPH \xrightarrow{\text{NADPH oxidase}} 2\,O_2^{-} + NADP^{+} + H^{+} \quad (1)$$

$$O_2^{-} + O_2^{-} + 2H^{+} \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (2)$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad (3)$$

$$H_2O_2 + 2GSH \xrightarrow{\text{GPx}} GSSG + 2H_2O \quad (4)$$

Hydrogen peroxide is scavenged by catalase to form water and oxygen (Eq. (3)) and by peroxidase (such as Glutathione peroxidase (GPx)) in the presence of a reducing agent (Glutathione (GSH)) (Eq. (4)). SOD, a cytosolic enzyme that is specific for scavenging superoxide radicals, is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. However, only few studies of SOD in crustaceans are related to oxidative status (Bell and Smith, 1995), immunity (Munoz et al., 2000), and disease indication (Neves et al., 2000). In shrimp, SOD has been measured in *Palaemontes argentinus* (Neves et al., 2000) and *L. vannamei* (Campa-Cordova et al., 2002). Glutathione peroxidase (GPX) is a selenium-containing enzyme that detoxifies hydrogen peroxide and various hydrogen

peroxides in cell using glutathione as a reducing agent (Rice-Evans and Burdon, 1993). The antioxidant superoxide dismutase (SOD) converts this microbiocidal metabolite into hydrogen peroxide that passes freely through membranes. The antioxidants catalase and glutathione peroxidase remove the hydrogen peroxide from cells. These are mechanism to help protect host cell from free-radical damage.

1.7.5 Apoptosis

Apoptosis, or programmed cell death, plays a major role in differentiation, development, tissue homeostasis and cell-mediated immunity, defense against environmental insults including pathogen attack (Thompson et al., 1995). Recent studies have shown that many pathogens exert control on the processes that regulate apoptosis in the host. The induction of apoptosis upon infection results from a complex interaction of parasite proteins with cellular host proteins. Induction of apoptosis in the infected cells significantly imparts protection to the host from the pathogen (Hasnain et al., 2003). However, if over apoptosis, it will be implicated in shrimp death so apoptosis inhibitor is necessary. For example, both survivin and P109 protein involved in apoptosis inhibitor (Liston et al., 1996). Besides, survivin is involved in regulation of cell division during HIV-1 infection (Zhu et al., 2003).

1.7.6 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are effector molecules that play an important role in innate immune system. In all kingdoms, from bacteria to human, a variety of AMPs have been identified and characterized (Dathe and Wieprecht, 1999; Douglas et al., 2001; Hiemstra, 2001; Buhimschi et al., 2004; Xu et al., 2005; Tew et al., 2006; Zhou et al., 2006). All AMPs share common biochemical features such as small size, generally less than 150-200 amino acid residues, amphipathic structure and cationic property though the anionic peptides also exist. AMPs have a 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 (Murakami et al., 1991; Hancock and Diamond, 2000; Tang et al., 2005) and may also exhibit an anti-tumor property (Cruciani et al., 1991). In artropoda, a broad activity of AMPs against

bacteria and filamentous fungi has been reported in insects, horseshoe crab, and shrimp (Bulet et al., 1999; Munoz et al., 2002; 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 the secretory cells, others are induced upon microbial stimulation (Hancock and Diamond, 2000). In 2004, Bulet et al. assessed that over 1000 AMPs, mostly from insectd, have been isolated and characterized from multicellular organisms at the level of their primary structure.

In general, the net positive charge together with the amphipathic structure accounts for the preferential binding to the negatively charged membrane interface of microorganisms, which is different from the predominantly zwitterionic surface of normal mammalian cells. Thus, they are toxic to the microbes and not to the mammals. The features required for AMPs are the selective toxicity on microbial cells, short bacterial killing time, broad antimicrobial spectra, and no bacterial resistance development (Matsuzaki, 2001). AMPs can interact and insert into the lipid bilayer, causing the collapse of electrochemical gradients across the cell membrane. Consequently, the microorganisms lose their cellular components and source of energy for surviving.

Several antimicrobial peptides are isolated and characterized in arthropods, mainly in insects, especially *Drosophila* and chelicerates (horseshoe crabs) (Iwanaga et al., 1998). In horseshoe crabs, these proteins are mainly synthesized in the 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 the granules after stimulation by LPS. This antimicrobial system in insect differs from those of the crustaceans in that the fat body of the insects is the main site for the antimicrobial peptide synthesis (Hoffmann and Reichart, 1997; Engström, 1999). Upon injury, the transcription of the antimicrobial peptide gene is induced, resulting in their immediate synthesis and subsequent secretion into the blood.

There are only a few reports on antimicrobial peptides in crustaceans. achyplesin family and anti-LPS factors which act against Gram-negative bacteria

were observed in horseshoe crab (Aketagawa et al., 1986; Nakamura et al., 1988; Muta et al., 1990; Yamakawa et al., 2001). 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). Recently, a new family of antimicrobial peptide which acting against Gram-positive bacteria and fungi, penaeidins, was reported in penaeid shrimp, *L. vannamei* (Destoumieux et al., 1997). These peptides contain both a proline rich domain at the N-terminus and a C-terminal domain containing 6 cysteines forming three disulfide linkages. The cDNA clones of penaeidin isoforms were also isolated from the haemocytes of *L. vannamei* and *L. setiferus* (Gross et al., 2001) and *P. monodon* (Supungul et al., 2004). A cysteine-rich 11.5 kDa antibacterial protein was purified and characterized from the haemolymph of shore crab, *Carcinus maenas* (Relf et al., 1999). Crustins, an antimicrobial peptide homologues of the 11.5 kDa antibacterial peptide, were identified from 2 species of *Penaeid* shrimp, *L. vannamei* and *L. setiferus* (Vargas-Albores et al., 2004). Peptides with antiviral activity, derived from the hemocyanin of *L. vannamei and P. stylirostris* and hemocyanin of *P. monodon*, have been identified (Altschul et al., 1997; Destoumieux-Garzon et al., 2001; Patat et al., 2004). Recently, the histones and histone derived peptides of *L. vannamei* have been reported as an innate immune effectors because they can inhibit the growth of Gram-positive bacteria (Patat et al., 2004)

1.8 Anti-Lipopolysaccharide 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 (Tanaka et al., 1982; Morita et al., 1985). 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., 2002; Supungul et al., 2004). 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 semi-quantitative 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 anti-lipopolysaccharide factor type 3 from the black tiger shrimp (rALF*Pm*3) was successfully produced in *Pichia pastoris* system. 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 (Somboonwiwat et al., 2005).

1.9 Objective of the thesis

We aim to investigate the effects of extreme environmental conditions on the anti-*Vibrio* activity of rALF*Pm*3. The rALF*Pm*3 was produced in small and large scale in *Pichia pastoris* and purified by cation-exchange chromatography. We further studied its activity *in vivo* in terms of neutralization and protective effect on *V. harveyi* challenged shrimp. Additionally, the expression analysis of selected immune-related genes in hemocyte of *V. harveyi* challenge shrimp that was pre-injected with the rALF*Pm*3 was observed using semi-quantitative RT-PCR.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

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

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

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

Biophotometer (eppendorf)

FlUOstar Optima (BMG Labtech)

-20 °C Freezer (Whirlpool)

-80 °C Freezer (Revco)

Gel dryer (Bio-RAD)

Gel document (Syngene)

Incubator 30 °C (Heraeus)

Incubator 37 °C (Memmert)

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

MFCS/win 2.1 fermentor (B.Braun Biotech International)

Microcentrifuge tube 0.5 and 1.5 ml (Axygen)

Nipro disposable syringe (Nissho)

Orbital Shaker (Gallenkamp)

Oven series 8000 (Contherm)

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)

Peristaltic pump P-1 (Amersham Biosciences)

pH meter Model # SA720 (Orion)

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

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

Refrigerated centrifuge Model # J2-21 (Beckman)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Stirring hot plate (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Vacuum pump (Bio-RAD Laboratories)

XK 26/20 column (Amersham Biosciences)

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

2.1.2 Chemicals and reagents

Absolute ethanol, C_2H_5OH (BDH)

Acetic acid glacial, CH_3COOH (BDH)

Acrylamide, C_3H_5NO (Merck)

Agarose (Sekem)

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

Ammonium solution (Carlo Erba)

Bacto agar (Difco)

Bacto tryptone (Merck)

Bacto yeast extract (Scharlau)

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

Biotin (Merck)

Boric acid, BH_3O_3 (Merck)

Bromophenol blue (BDH)

Calcium sulfate (Ajax)

Chloroform, $CHCl_3$ (Merck)

Cobalt chloride (Ajax)

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

Cupric sulfate.$5H_2O$ (Ajax)

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

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

Ethidium bromide (Sigma)

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

Ferrous sulfate.7H_2O (Ajax)

Formaldehyde, CH_2O (BDH)

GeneRuler™ 100bp DNA Ladder (Fermentus)

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

Glycine NH_2CH_2COOH (Scharlau)

Hydrochloric acid, HCl (Merck)

Isopropanol, C_3H_7OH (Merck)

Maxnesium sulfate (Ajax)

Manganese sulfate (Ajax)

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)

Nitroblue tetrazolium (NBT) (Roche, Germany)

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

Phosphoric acid (Lab scan)

Potassium hydroxide (Carlo Erba)

Potassium sulfate (Ajax)

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, $NaHCO_3$ (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)

Sodium iodide (Ajax)

Sodium molybdate.$2H_2O$ (Ajax)

SP Sepharose High Performance (Amersham Biosciences)

Sulfuric acid (Carlo erba)

RNA markers (Promega)

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

TRIreagent (Molecular biology)

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

Tryptic soy broth (Difco)

Tween^{TM}-20 (Fluka)

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

Zinc chloride (Ajax)

2.1.3 Enzymes

RQ1 RNase-free DNase (Promega)

Taq DNA polymerase (Fermentus)

2.1.4 Microorganisms

Escherichia coli strain 363

Vibrio harveyi 639

2.1.5 Kits

ImProm-II^{TM} Reverse Transcription system kit (Promega)

2.1.6 Animals

P. monodon Juveniles(approximately 3 month-old, 15-20g of body weight) were obtained from shrimp farm (Chuntaburi Province), Thailand. The animals were acclimated for 3 days in indoor tanks, water temperature ranged between 26 and 32 °C, salinity at 15 parts per thousand (ppt) before experimentation.

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

A full-length cDNA clone of the anti-lipolysaccharide factor isoform 3 (ALF*Pm*3) was isolated from haemocytes of non-stimulated shrimps by Supungul (2002). Its recombinant protein was successfully expressed in a *Pichia pastoris* methylotrophic yeast system (Invitrogen) by Somboonwiwat et al. (2005). To obtain an adequate amount of the rALF*Pm*3 for further study, it was produced with modification from previous study, both in shake flask and a fermentor.

2.2.1 Small scale expression system

The rALF*Pm*3 was produced in a shake flask (Invitrogen). The stock culture of the yeast recombinant clone containing ALF*Pm*3 gene was transferred into a YPD agar (1% yeast extract, 2% peptone, 2% dextrose and 1.5% agar) plate and incubated at 30^{o}C for 36-39 hours. A single colony of the recombinant clone was transferred into Erlenmeyer flask containing 20 ml of sterilized YPD broth (1% yeast extract, 2% peptone and 2% dextrose) (the YPD volume was 20% of total flask volume). The flasks were then incubated overnight at 30^{o}C, 300 rpm. The overnight culture was inoculate (1/100) to 200 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM poteassium phosphate, pH 6.0 , 1.34% YNB, 4x10^{-5}% biotin and 1% glycerol) in a liter flask and was grown at 30^{o}C, 300 rpm until the culture reach an OD_{600} of 4-6. The cells were harvested by centrifugation at 5,000 rpm for 10 min at room temperature. To induce the recombinant expression, the cell pellets were resuspended in 40 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM poteassium phosphate, pH 6.0, 1.34% YNB, 4x10^{-5}% biotin and 0.5% methanol). The pure methanol was added to culture to a final volume concentration of 0.5% every 24 hours in order to maintain the induction. One milliliter of the culture supernatant was collected at expression culture and then centrifuged at 5,000 rpm for 10 min at room temperature at each time point (0, 1, 2, 3 days). The supernatant was kept and stored at -80^{o}C until use. The rALF*Pm*3 expression was checked by silver stained SDS-PAGE and antimicrobial activity assay against *Escherichia coli* strain 363 *(E. coli* 363).

2.2.2 Large scale expression system

2.2.2.1 Inoculum preparation

A single colony of the recombinant clone was transferred into Erlenmeyer flask containing 60 ml of sterilized YPG medium (containing of 2% Bacto-peptone, 1% yeast extract, 2% Glycerol). The culture was then incubated at 30^{o}C, 300 rpm for 24 h. The culture was inoculated (1/10) to 200 ml of YPG medium in a liter flask and was grown at 30^{o}C at 300 rpm for 24 hours.

2.2.2.2 Fermentor experimental set up

The rALF*Pm*3 was produced in a fermentor using a method modified from lssaly et al. (2001). The inoculum culture was transferred into a 20 liters fermentor vessel (MFCS/win 2.1 fermentor, B.Braun Biotech International) containing 7 liters working volume of basal salt medium containing 4% (w/v) glycerol. After medium sterilization, 4.35 ml.L^{-1} of PTM trace salts (Appendix A) was added aseptically into the medium. The vessel temperature was maintained at 30^{o}C. The pH of the culture was monitored and controlled within 5.0$\pm$0.5 using pH probe via automatic addition of 15% (v/v) NH_4OH, which was also used as a nitrogen source and 2 N H_2SO_4. The initial dissolved oxygen (DO) was maintained above 30% air saturation by varying agitation speed from 500-1,000 rpm. Aeration was set at 2 vvm (volume of oxygen (liters) per volume of fermentation culture (liters) per minutes). Whenever, the cell density is high, the induction phase was started by an addition of 100% methanol containing 12 ml PTM trace salt per liter of methanol. The methanol concentration was controlled by fixing the feeding rate at 0.7 ml.h^{-1}L^{-1}. However, during methanol addition the level of the dissolved oxygen was monitor. The methanol fed would be stopped for a short period of time until the dissolved oxygen level immediate increased which means the methanol depletion. The spike in dissolved oxygen level could indicate the lack of methanol accumulation. Total induction period was 144 hours. Samples were collected in every 8-24 hours for expression analysis.

2.2.2.3 Biomass determination

The cell suspension was collected at different time point and monitored the cell density by measuring the absorbance at 600 nm. The cell dry weight or biomass was subsequently determined. The cell pellet was collected by centrifugation at 5,000xg for 10 minutes and then dried by incubation in the hot-air incubator at 80^{o}C for 24 hours. The dried cell was weighted and the biomass production was plotted.

2.2.2.4 Total protein determination

The total protein content was determined using Bradford assay (Bradford, 1976). The recombinant protein in the maximum volume of 100 µl was mixed with 1 ml of Bradford working buffer. The experimental buffer was added to make a total volume of 100 µl. The reaction mixture was incubated for 2 minutes and the absorbance of 595 nm was measured. The total protein was determined by comparing with a standard curve generating from known amount of BSA standard.

2.2.3 Detection of the recombinant protein by SDS-PAGE

The SDS-PAGE system was performed according to the method of Bollag et al. (1996). The slab gel (10x10x0.75 cm) system consisted of 0.1% (w/v) SDS in 12% (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 B. 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 silver staining. The silver staining solution was described in protein method. The gel was fixed in 50 ml of the fixing solution for 60 minutes. The fixing solution was decanted and the gel was rinsed 3 times with the deionised water for 10 minutes each. The gel was then incubated in the staining solution for 15 minutes. The gel was rinsed twice with the deionised water as above and soaks for 2 minutes. The gel was developed for 3-10 minutes in the developing solution. The stop solution was added directly to the gel when the desired staining intensity was reached. The stop solution was decanted and the gel was washed three times in the deionised water.

2.2.4 Detection of the recombinant protein by Western blot analysis

The recombinant protein was separated on 12 % SDS-PAGE gels. Gel was removed from slab and incubated for 10 minutes in the blotting buffer (48 mM Tris pH 9.2, 39 mM glycine and 20% methanol). The nitrocellulose membrane was soaked in the blotting buffer for 15 minutes. The two sheets of the blotting paper were wet in the blotting buffer. A Sheet of blotting paper, the membrane; the gel and the other sheet of blotting paper were laid on the Trans-Blot® SD (Bio-Rad) machine in a sandwich model as shown in figure 2.1. Avoid air bubbles between layers.

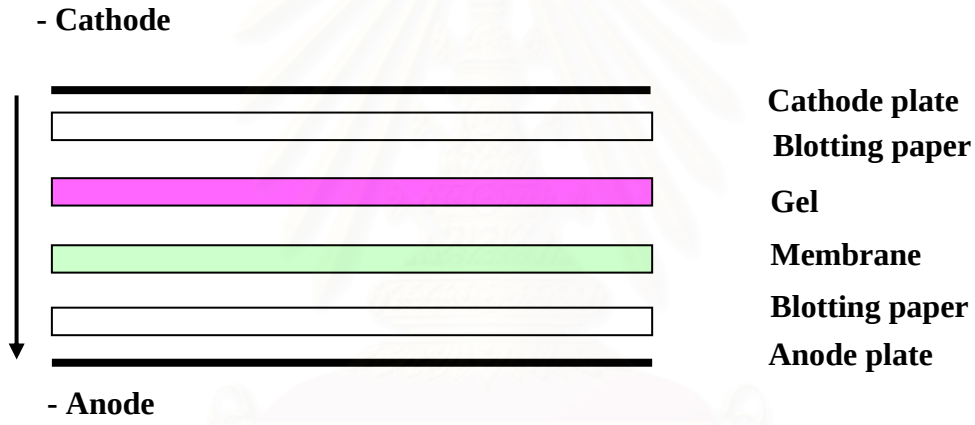


Figure 2.1 The layers of gel was transferred into the membrane

Protein transfer was performed at constant electricity of 80 mA for 1 hour. After transfered, the membrane was soaked overnight in blocking buffer (5% (w/v) skim milk, 0.05% (v/v) Tween 20 in PBS buffer) at room temperature. The membrane was washed 3 times for 10 minutes each in PBS-Tween buffer (0.05% (v/v) Tween 20 in PBS buffer) and incubated with rabbit anti-rALF*Pm*3 antibody diluted 1/20,000 in 1% (w/v) skim milk, 0.05% (v/v) Tween 20 in PBS buffer at 37ºC for 3 hours. The membrane was washed as above and incubated with secondary antibody, anti-rabbit IgG conjugated with alkaline phosphatase, diluted 1/20,000 in the same buffer as primary antibody for 1 hour at room temperature. After washing, the membrane was dipped into the detection solution

(100 mM Tris-HCl pH 9.5, 100mM NaCl, 50 mM $MgCl_2$). After the band was observed, the chromogenic reaction was stopped by rinsing the membrane twice with water.

2.2.5 Antimicrobial activity assay

The bacterial strains used in this study included Gram-negative bacteria, *E. coli* 363 and marine pathogenic organisms for shrimps, *Vibrio harveyi* 639.

Antimicrobial activities and minimum inhibitory concentration (MIC) values were determined by liquid growth inhibition assay as previously described (Somboonwiwat et al., 2005). Ten microliters of each diluted protein, or sterile deionised water as a control, were incubated in a sterile microliter plates with 100 µl of a suspension of mid-logarithmic growth phase culture of bacteria diluted in culture medium to OD_{600} = 0.001. Poor-broth nutrient medium (1% bactotryptone, 0.5% NaCl, pH 7.5) was used for *E. coli* 363 cultures. For *V. harveyi*, it was grown in saline peptone water (1.5% peptone, 1.5% NaCl, pH 7.2). Bacteria were grown overnight under vigorous shaking at 30^{o}C. Microbial growth was controlled by measurement of the optical density at OD_{600} after incubation for 24 hours 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.

2.2.6 Purification of the rALF*Pm*3

The culture supernatant containing the rALF*Pm*3 was first diluted 1:1 with the start buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.0) and then purified by a strong cation exchange chromatography, SP Sepharose High Performance column, (GE healthcare). The Sepharose High Performance media was packed in 26nmx20nm column equilibrated with the start buffer (20 mM Tris-HCl, 200 mM NaCl, pH 7.0). After sample loading, the unbound protein was washed with the start buffer until the A_{280} was decrease to zero. Elution was performed with elution buffer (20 mM Tris-HCl, 1 M NaCl, pH 7.0). The flow rate was controlled at 2 ml/minutes throughout the purified process. The fractions were analyzed by measurement of the optical density at 280 nm and running on 12% silver staining SDS-PAGE stained with silver solution. The antimicrobial activity was performed against *E. coli* 363. The fractions with antimicrobial activity were then pooled. The eluates were dialyzed overnight against

sterile deionised water at 4^{o}C to eliminate salt. The purified rALF*Pm*3 were then evaporated to concentrate and then measured the protein concentration at optical density at A_{280}. The purified protein was kept at -80^{o}C until use.

2.3 Antibacterial properties of the rALF*Pm*3 under extreme incubation conditions

2.3.1 Effect of salt

The purified rALF*Pm*3 stock solution was diluted in sterile water to the final concentration of 25-0.195 μM in 96 well costar 3599 and incubated with 0-6 %salt variations by adding NaCl to EPS medium (2% peptone, 1% yeast extract and various salt (w/v)) and *V. harveyi* 639 (dilution in EPS medium to OD_{600} = 0.001). Bacteria were grown overnight under vigorous shaking at 28^{o}C. Microbial growth was controlled by measurement of the optical density at OD_{600} after incubation for 24 hours by Fluor meter.

2.3.2 Effect of acid-alkali

The stock rALF*Pm*3 solution was diluted in sterile water for 25-0.195 μM final concentration into 96 well costar 3599 and incubated with pH variations between 3-9 by adding HCl or NaOH to EPS medium (2% peptone, 1% yeast extract and 2% salt) and *V. harveyi* 639 (dilution in culture medium to OD_{600} = 0.001). Bacteria were grown overnight under vigorous shaking at 28^{o}C. Microbial growth was controlled by measurement of the optical density at OD_{600} after incubation for 24 hours by Fluor meter.

2.3.3 Effect of temperature

The stock rALF*Pm*3 solution were incubated at 4 (by using refrigerator), 30, 50 and 100 ^{o}C by oven for 40, 80, 120 minutes. The incubated rALF*Pm*3 solution was diluted in sterile water for 25-0.195 μM final concentration into 96 well costar 3599 and then were incubated with EPS medium (2% peptone, 1% yeast extract and 2% salt) and *V. harveyi* 639 (dilution in culture medium to OD_{600} = 0.001). Bacteria were grown overnight under vigorous shaking at 28^{o}C. Microbial growth was controlled by

measurement of the optical density at OD_{600} after incubation for 24 hours by Fluor meter.

2.3.4 Effect of stored condition

The stock rALF*Pm*3 solution was stored at -20, 4 and room temperature for 1, 2 and 3 month. After that, it was diluted in sterile water for 25-0.195 μM final concentration into 96 well costar 3599 and then were incubated with EPS medium (2% peptone, 1% yeast extract and 2% salt) and *V. harveyi* 639 (dilution in culture medium to OD_{600} = 0.001). Bacteria were grown overnight under vigorous shaking at 28^{o}C. Microbial growth was controlled by measurement of the optical density at OD_{600} after incubation for 24 hours by Optima Fluor meter. A quantity and quality of the stored protein was determined by SDS-PAGE and Mass Spectrophotometer, respectively.

2.4 Shrimp

Sub-adult *P. monodon* (approximately 3 month-old, 15-20 g of body weight) were purchased from local farms and acclimatized in aquaria at the ambient temperature (28±4°C) and at the salinity of 15 ppt for at least 3 days before used in the experiments. Shrimps were fed twice daily with compound shrimp diet based on 5% of body weight

2.5 Bacterial challenge for LD_{50} determination

A *V. harveyi* 639 for injection was prepared as followed. Stock were streaked on to a trypic soy agar (TSA) plate supplemented with 2% NaCl and grown overnight at 30 ^{o}C. A single colony was inoculated in tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl at 30 ^{o}C for 12-16 hours. The overnight culture was inoculated to TSB (1:100). After that, culture were grown at 250 rpm, 30 ^{o}C until the A_{600} was 0.6 where the cell density was 10^{8} CFU/ml. The titer of this dilution was monitored by a plate count method in tryptic soy agar (TSA) supplemented with 2% (w/v) NaCl (modified from Austin, 1988).

P. monodon were assigned into 5 groups, 15 individual each in duplicate. The first group, control group, was intramuscularly injected with 100 μl of sterile 0.85% (w/v) NaCl. The second to fifth groups were intramuscularly injected with 100 μl of 10^5, 10^6, 10^7 and 10^8 CFU/ml *V. harveyi* 639. The mortality rates were observed for 24 hours.

2.6 Preparation of *V. harveyi* infected shrimp

A single colony of *V. harveyi* 639 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 250 rpm, 30 ^{o}C until the A_{600} was 0.6 where the cell density was 10^8 CFU/ml and then diluted 1:10 with a sterile 0.85% (w/v) NaCl (10^7 CFU/ml). The 100 μl of 10^7 CFU/ml diluted culture was intramuscularly injected into the second abdominal segment, whereas the control group was injected with 100 μl of 0.85% (w/v) NaCl solution.

2.7 Mortality rate determination of *in vivo* neutralization between the rALF*Pm*3 and *V. harveyi* 639 in *P. monodon*

P. monodon were assigned to 7 groups of 10 shrimps each in triplicates. The first group was *P. monodon* experimentally intramuscularly injected with 100 μl of 10^7 CFU/ml *V. harveyi* 639. The second to seven groups were *P. monodon* intramuscularly injected with 100 μl of 10^7 CFU/ml *V. harveyi* 639 incubated with 1.56, 3.125, 6.25, 12.5, 25 and 50 μM purified rALF*Pm*3 (final concentration) for 30 minutes, respectively. The mortality rates of each experimental group were observed for 7 days.

2.8 Mortality rate determination of protective effect of the rALF*Pm*3 on *V. harveyi* 639 injected *P. monodon*

P. monodon were assigned into 3 groups of 10 shrimps each in duplicate. The first group was the control shrimps, which were injected with 100 μl of 0.85% NaCl. A second group of shrimps were injected with 100 μl of 0.85% NaCl before 100 μl of *V. harveyi* injection at 10^7 CFU/ml for 24 hours. A third group, shrimps were injected with 100 μl of 100 μM of purified rALF*Pm*3 protein before 100 μl of *V. harveyi* injection at 10^7 CFU/ml for 24 hours. The mortality rates of each experimental group were observed for 7 day.

2.9 Gene expression analysis of a protective effect of the rALF*Pm*3 on *V. harveyi* 639 injected *P. monodon*

2.9.1 Challenge experiments

For the experimental infection, shrimps were divided into two groups, first group was the control shrimp, which shrimps were injected with 100 μl of 0.85% NaCl before 100 μl of *V. harveyi* injection at 10^6 CFU/ml for 24 hours. A second group, shrimps were injected with 100 μl of 100 μM of purified rALF*Pm*3 protein before 100 μl of *V. harveyi* injection at 10^6 CFU/ml for 24 hours.

Hemolymph was collected from an individual shrimp after the rALF*Pm*3 injection (0 and 24 hours) and *V. harveyi* injection (0, 6, 24 and 48 hours). Each time point, hemolymph was collected from a 5 individual shrimps.

2.9.2 Diagnosis of *Vibrio harveyi*

Shrimps were checked for *V. harveyi* infection using the PCR technique (modified from Thaithongnum et al., 2006). This procedure detects the presence and the severity of *V. harveyi* infection. A gill of shrimp *P. monodon* was crushed in 200 μl lysis buffer (0.05 N NaOH and 0.025% sodium dodecyl sulphate), followed by incubation in boiling water for 5 minutes before immediate incubation on ice. After a brief centrifugation at 7,500 rpm for 5 minutes, 1/100 dilution of the 2 μl supernatant

was used as the template for PCR amplification. The 25 μl PCR reaction contained 2 μl of DNA template, 1.25 mM of each dNTPs, 2.5 mM MgCl2, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM $(NH_4)_2SO_4$ and 0.1% TritonX-100), 1 U of Taq DNA polymerase, 2 μM of the forward primer (5'TCTAACTATCCACCGCGG3') and reverse primer (5'AGCAATGCCATCTTCACGTTC3'), amplifying a 363-bp fragment of *V. harveyi* DNA. The PCR reaction was conducted by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, the initial denaturation was 94°C for 5 s and the final extension was 72 °C 5 minutes. Following PCR, 10 μl of the reaction solution was examined following electrophoresis through a 1.5% w/v agarose gel and ethidium bromide staining.

Furthermore, the suspensions of hepatopancreas were streak on TCBS medium and incubated at 30 °C overnight. Colonies of *V. harveyi* 639 from infected shrimps showed strong luminescence in the dark.

2.9.3 Haemocytes 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)

2.9.4 Formaldehyde-agarose gel eletrophoresis

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 (0.66 M 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.).

2.9.5 DNase treatment of total RNA samples

Chromosomal DNA contamination in total RNA samples was removed by treating 10 μg of total RNA with 2 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 2.8.4

2.9.6 First-stranded cDNA synthesis

The first stranded cDNA was synthesized from 0.5 µg of total RNA using an ImProm-II^{TM} Reverse Transcription system kit (Promega). Total RNA was combined with 0.5 µg of oligo (dT_{15}) primer and appropriate DECP-treated H_2O in final volume of 5 µl. The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. After that, 2 µl of 5x reaction buffer, 1.3 µl of 25 mM $MgCl_2$, 0.5 µl of dNTP Mix (10 mM each), 10 units of Recombination RNasin and 0.5 µl of ImProm-II reverse transcriptase were added and gently mixed. The reaction mixture 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.

2.9.7 PCR

Amplification reaction was performed in 15 µl mixture containing 5 µl of a 1:10 dilution of cDNA template, 1.25 mM of each dNTP, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM $(NH_4)_2SO_4$ and 0.1% TritonX-100), 1 U of *Taq* DNA polymerase, the primers (0.4-.08µm), $MgCl_2$ concentration (1.5-2.5 mM) and the number of cycle (24-26 cycles). The amplification was consisted of predenaturation at 95 °C for 2 minutes followed by 24-26 cycle of denaturation at 94 °C for 30 sec, annealing at 50-58 °C for 30 sec and extension at 72 °C for 30 sec and a final

extension at 72 °C for 5 minutes. The final concentrations of the reaction are shown in Table 2.1.

Table 2.1 Primer pairs and conditions for the RT-PCR

Gene	Primer	Primer Conc. (μm)	$MgCl_2$ Conc. (mM)	Cycles	Annealing Temp(°C)
ALF*Pm*3	5'CCCACAGTGCCAGGCTCAA 3' (F) 5'TGCTGGCTTCTCCTCTGATG3' (R)	0.2	2.5	25	58
PPA	5'CTGGGGCACCATCTACTACGGC 3' (F) 5'CTGTCACCCTGGCACGAATCCT3' (R)	0.2	2.5	26	55
PAP	5'ATTGCATCATCCACCATG 3' (F) 5'GGGACTTTGTCATCTTCA3' (R)	0.4	2.5	25	50
SOD	5' AATGTTGGCTCTGGTGTAGG 3' (F) 5' TGGATTTAACCGAAGAGACTG 3' (R)	0.20	2.5	24	55
Suvivin	5' GGGAGGAGCACAAAAACCAT 3' (F) 5' ACAAGAACAGAGGAGTGAA 3' (R)	0.08	1.5	27	55
β-actin	5'GCTTGCTGATCCACATCTGCT 3' (F) 5'ATCACCATCGGCAACGAGA3' (R)	0.04	2.5	26	58

2.9.8 Gel electrophoresis and quantitative analysis

PCR products were determined by electrophoresis on 1.5% agarose gels. The agarose gel (2% (w/v)) was prepared in 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). The slurry of agarose in TBE buffer was melted in a microwave oven until completely dissolved. The solution was allowed to cool at 55-60 °C before pouring into a casting tray with a well comb. After hardening, the gel was submerged in a chamber containing an enough amount of 1xTBE buffer covering the gel for approximately 0.5 cm.

The PCR products were mixed with 3 μl of the 10x loading dye (0.25% bromophenol blue and 25% Ficoll in water) before loading of 5 μl PCR products into the well. A DNA ladder (100 bp marker) was used as standard DNA markers. Electrophoresis was carried out in 1x TBE buffer at 100 volts until the bromophenol blue dye marker migrated about 2/4 of the gel length. After electrophoresis, the gel was stained in a 2.5 μg/ml ethidium bromide (EtBr) solution for 30 sec and destained

to remove unbound EtBr by submerged in distilled water for 20 minutes. Fractionated PCR product was visualized under a UV transilluminator and photographed.

The intensity of target band was detected using Gel Documentation System (GeneCam FLEX1, SynGene) and further quantified using the Genetools analysis software.

2.9.9 Data analysis

Significantly different expression levels were treated using One Way Analysis of Variance (ANOVA) following by a post hoc test (Duncan's new multiple range test). Significant differences were indicated at $p < 0.05$.

CHAPTER III

RESULTS

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

3.1.1 Small scale expression system

The stock culture of the yeast recombinant clone containing ALF*Pm*3 gene (ALFK9) was streaked on a YPD agar plate and incubated at 30^{o}C for 36 hours. A single colony of the recombinant clone was transferred into BMGY medium (1% yeast extract, 2% peptone, 100 mM poteassium phosphate, pH 6.0, 1.34% YNB, 4x10^{-5}% biotin and 1% glycerol) to increase biomass then cells were harvested and transferred into BMMY medium (1% yeast extract, 2% peptone, 100 mM poteassium phosphate, pH 6.0 , 1.34% YNB, 4x10^{-5}% biotin and 0.5% methanol). The pure methanol was added to a final volume concentration of 0.5% every 24 hours (0, 1, 2 and 3 days) to maintain the induction. During the methanol induction, the rALF*Pm*3 protein containing α-factor mating signal sequence was secreted into the culture medium. The signal sequence was proteolytically removed by KEX2 and STE13 gene products resulting in the mature rALF*Pm*3 protein (Cregg et al., 2000). The expression culture was collected and the supernatant was further analyzed. The rALF*Pm*3 expression was checked by 12% silver stained SDS-PAGE. Fig. 3.1 showed the major band of approximately 14 kDa at 1, 2 and 3 days after induction, which is larger than the expected size (11.3 kDa) for rALF*Pm*3 as reported previously (Somboonwiwat et al., 2005). Western blot analysis using antibody specific to rALF*Pm*3 showed that the location of the positive bands corresponded to that of the major bands observed in the silver stained gel (Fig. 3.1 B). This result revealed the successful expression of the rALF*Pm*3 protein. Determination of the total protein concentration was performed by Bradford assay. The total protein concentration in the expression culture at 0, 1, 2 and 3 days after induction was 0.052, 0.094, 0.105 and 0.11 mg/ml, respectively. To determine whether the crude supernatant containing rALF*Pm*3 could exhibit its activity, the antimicrobial activity test against *Escherichia coli* 363 was performed. The result showed growth inhibitory activity of the crude

supernatant on *E. coli* 363. The crude supernatant was subsequently purified to homogeneity using cation exchange chromatography (section 3.2.3).

3.1.2 Large scale expression system

The rALF*Pm*3 protein was produced in a bioreactor using a defined medium known as basal salt medium (Appendix A). It was chosen for this study because it was the most cited medium reported in many literatures (Inan et al., 1999; Issaly et al., 2001). The cells were grown to high density in the medium containing 4% (w/v) glycerol as a carbon source during the growth phase. After 48 hours of cultivation, the dry weight of 20.1 g/l and the specific growth rate close to 0.047 h^{-1} were reached. The fed-batch phase was consequently initiated by continuous feeding the production feed medium consisting of 100% methanol and 4.35 ml/l PTM trace element solution. The feeding rate was constant at 0.7 ml/l.h. This production phase was performed continuously from 48 to 192 hours of culture. Fig. 3.2 showed the evolution of biomass and the quantities of methanol added during the production phase.

The culture supernatant was collected and the total protein content was determined. It was found that the total protein content was gradually increased and reaching 0.22 mg/ml at the end of the experiment. The rALF*Pm*3 expression was checked by 12% silver stained SDS-PAGE. The result showed the major band of approximately 14 kDa at 96 hours to the final hour (Fig. 3.3 A). Western blot analysis of duplicate gel confirmed the expression of the recombinant protein (Fig. 3.3 B). The antimicrobial activities test against *E. coli* 363, using liquid broth assay revealed the activity of the crude protein.

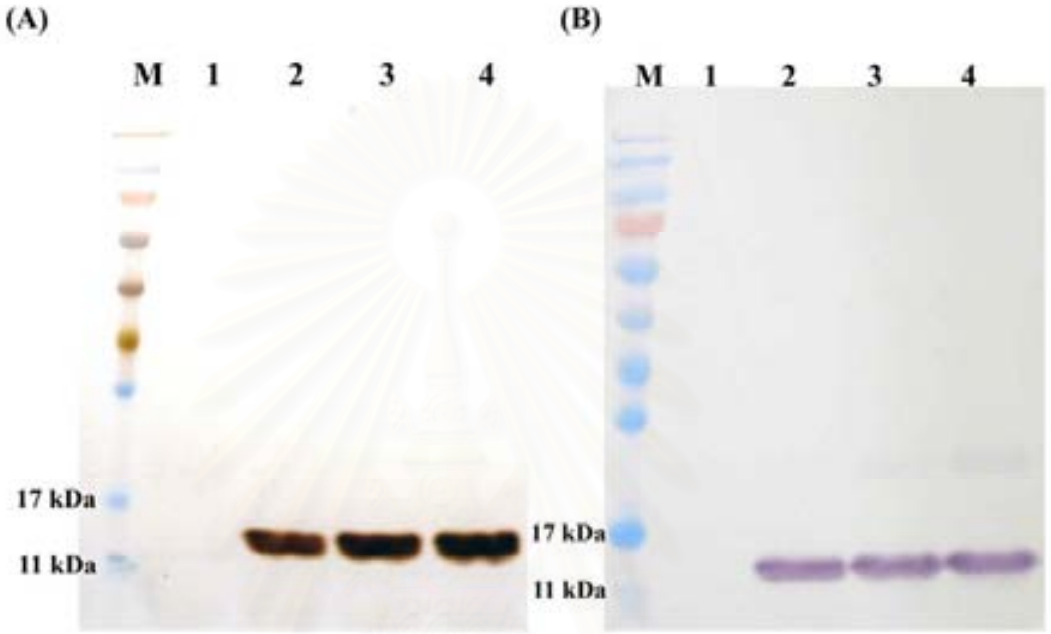


Figure 3.1 Expression analysis of the rALF*Pm*3 protein in the yeast *Pichia pastoris.* The expression of the rALF*Pm*3 was investigated at various time after methanol induction by the silver stained 12% SDS-PAGE analysis (A) and Western blot analysis using a polyclonal antibody specific to rALF*Pm*3 (B); Lane M: Pre-stained protein ladder, Lanes 1 to 4: crude supernatant at 0, 1, 2 and 3 days after methanol induction, respectively.

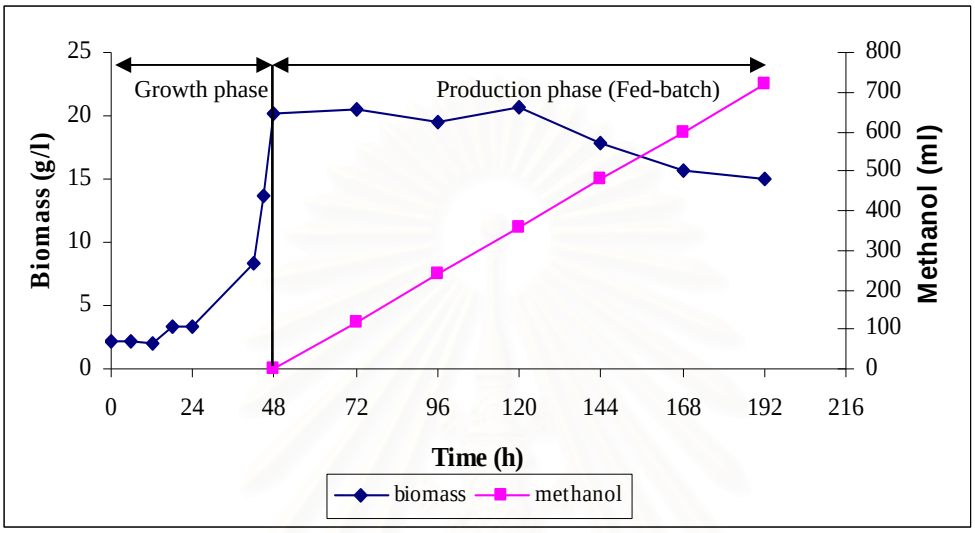


Figure 3.2 Evolution of the biomass (g/l) in bioreactor. In the first phase, 40 g/l of glycerol were performed to increase biomass (g/l) (dark blue line) in 20 l bioreactor containing 7 l of basal salt medium. In these conditions, 20 g/l of dry cell weight were obtained and the fed-batch phase was consequently initiated with specific growth rate close to 0.047 h^{-1}. After 144 h of induction, the final biomass was 15 g/l. Methanol induction began at 48 h (pink line) and the culture was harvested at 192 h.

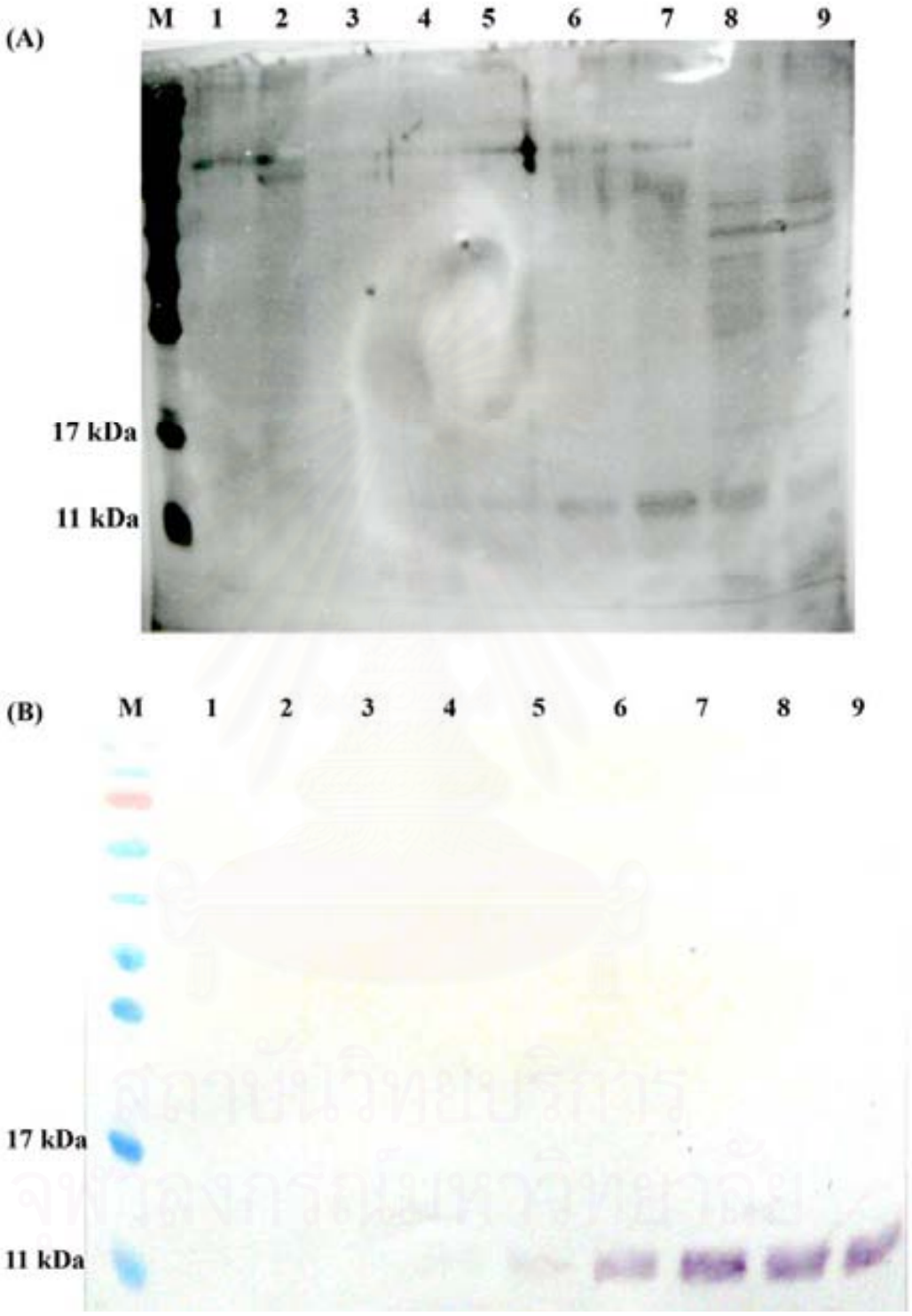


Figure 3.3 Expression analysis of the rALF*Pm*3 protein produced in a bioreactor. The 12% silver stained SDS-PAGE (A) and Western blot analysis (B) of the culture supernatant sampling at 0, 24, 48, 72, 96, 120, 144, 168 and 192 hours from a bioreactor are shown in lanes 1 to 9, respectively. Lane M: Pre-stained protein ladder. The rALF*Pm*3 was produced since 48 h to 144 h after methanol induction

3.2 Purification of rALF*Pm*3 protein

The culture supernatant containing rALF*Pm*3 protein was purified by strong cation exchange chromatography (SP Sepharose High Performance) packed in the HiLoad column as described in section 2.2.6. Each fraction was measured for the presence of protein by spectrophotometry (A_{280}). A chromatogram of the purification is shown in Fig. 3.4. The representative fractions of the flow-through, wash fractions and all elution fractions were run on a 12% silver stained SDS-PAGE (Fig. 3.5) and tested for its antimicrobial activity against *E. coli* 363 to identify the fraction containing the rALF*Pm*3 protein. The purified fractions were then pooled and dialyzed overnight against sterile deionised water at 4^{o}C to eliminate salt. The purified rALF*Pm*3 were then concentrated by rotary evaporation and the protein concentration was determined by measuring the absorbance at 280 nm (Appendix B). The recombinant protein stock solution was stored at -20^{o}C. In the experiment, the total crude supernatant of approximately 500 mg yielded 100 mg of purified rALF*Pm*3 protein.

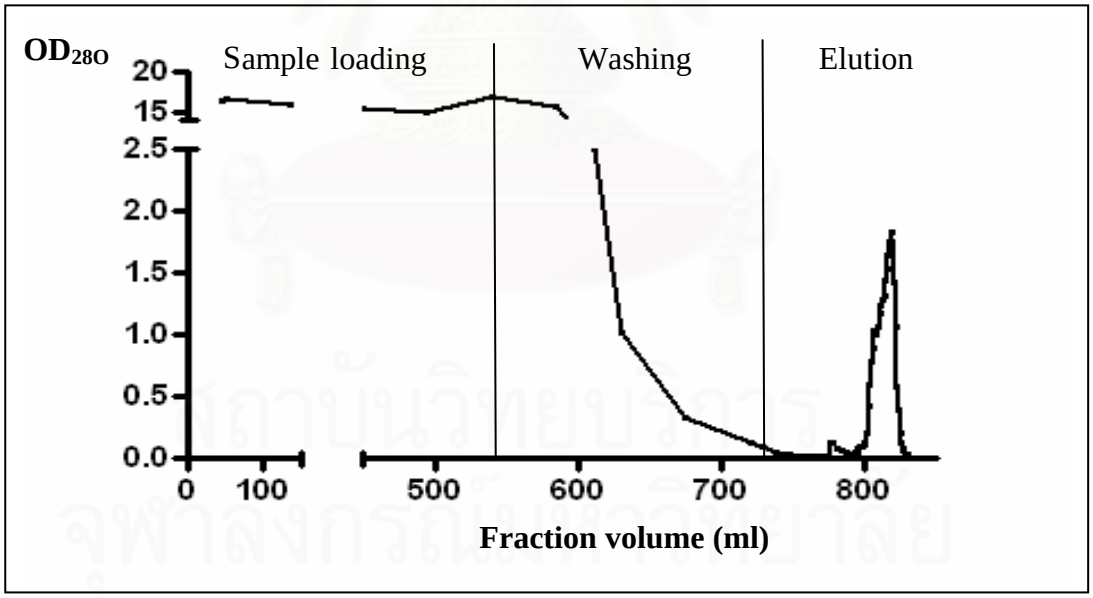


Figure 3.4 A chromatogram of rALF*Pm*3 protein purification by cation-exchange chromatography. The purification was separated into 3 phases; sample application, washing and elution. The elution phase revealed the one peak protein at optical density of 280 nm.

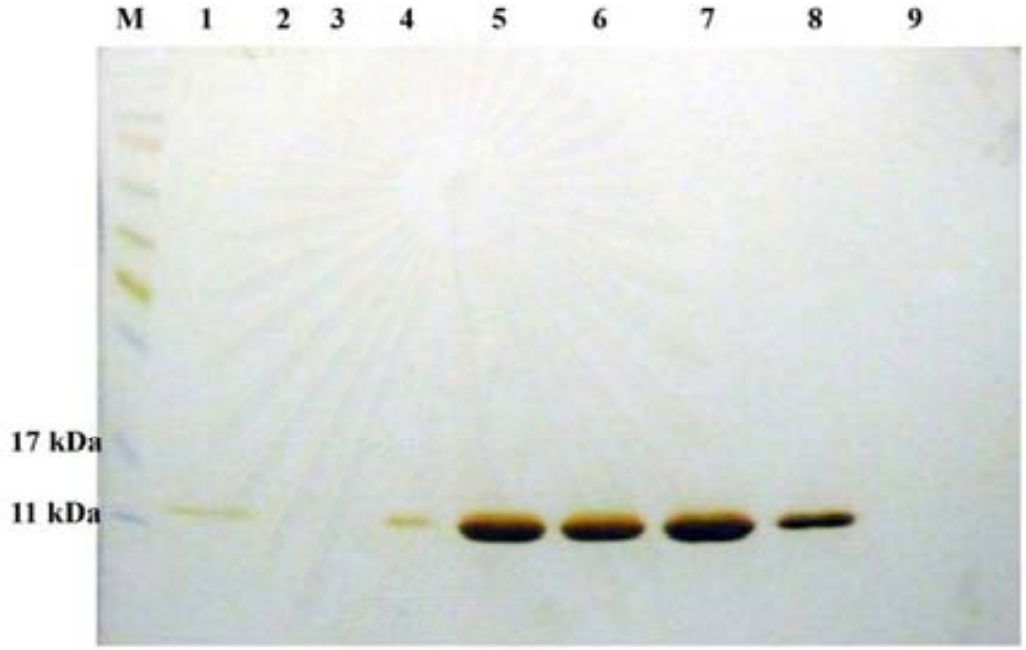


Figure 3.5 A 12% silver stained SDS-PAGE analysis of rALF*Pm*3 protein purified by cation-exchange chromatography

Lane M: Protein Marker

Lane 1: crude rALF*Pm*3 protein

Lane 2: flowthrough fraction

Lane 3: washing fraction

Lanes 4 to 9: elution fraction no.1-6

3.3 Antibacterial properties of the purified rALF*Pm*3 protein under extreme incubation conditions

The purified rALF*Pm*3 protein was examined for the stability on its antibacterial activity under extreme incubation conditions such as salt concentration, temperature and under acidic or basic conditions. The data obtained from this study will reveal the possibility to develop the shrimp feed supplemented with the rALF*Pm*3.

3.3.1 Effect of salt on antibacterial activity of rALF*Pm*3

The purified rALF*Pm*3 stock solution was diluted in sterile water to the tested concentration and incubated with mid-logarithmic phase culture of *V. harveyi* 639 in the medium containing 0-6% (w/v) NaCl. The antibacterial activity against *V. harveyi* 639 was determined. The growth of *V. harveyi* 639 was observed by measuring OD_{600} and converted into log CFU and plotted against NaCl concentration to showed effect of salt on rALF*Pm*3 antimicrobial activity (Fig. 3.6). As shown from the graph, no growth of *V. harveyi* was observed at 0% NaCl. Moreover, high salt concentration (4-6% NaCl) caused the retardation of bacterial growth comparing to that at 1-3% NaCl. Therefore, effect of salt on antibacterial activity of rALF*Pm*3 was determined at 1-3% NaCl. The rALF*Pm*3 at the concentration as low as 0.78 µM could cause 100% growth inhibition (MIC Value) of *V. harveyi* in a medium containing 1% NaCl, When the salt concentration was increased to 2% ad 3%, the decrease in the antimicrobial activity was observed. The MIC value was increased to 12.5 µM. The results indicated that a high salt concentration could result in a low anti-bacterial activity of rALF*Pm*3 (high MIC values).

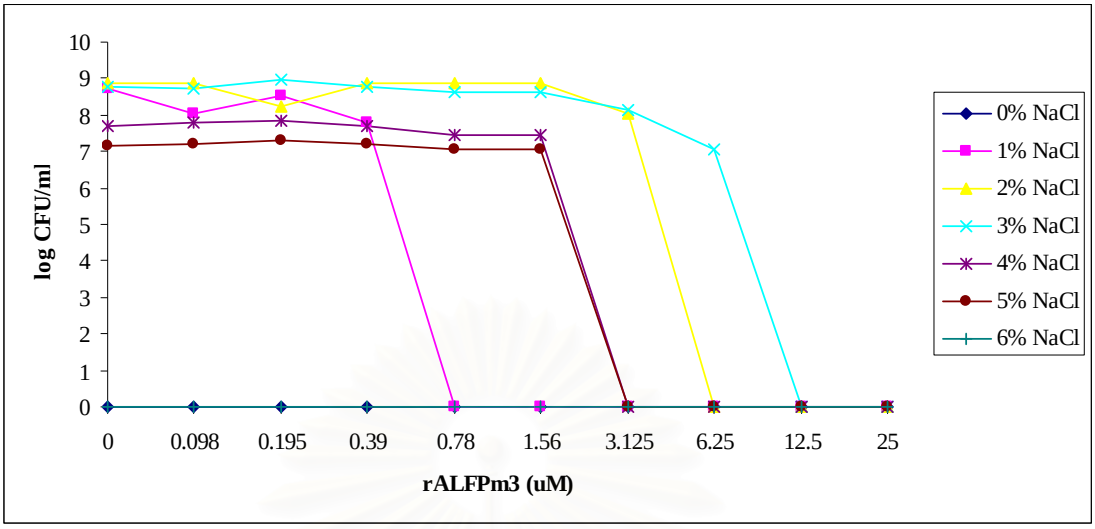


Figure 3.6 Effect of salt concentration on antimicrobial activity of rALF*Pm*3 against *V. harveyi* 639. Various concentration of the rALF*Pm*3 were incubated with the culture of *V. harveyi* 639 (OD=0.001) in a EPS broth containing 1, 2, 3, 4, 5 and 6% (w/v) NaCl, pH 7.0. After 24 h of incubation at 28^{o}C, the bacterial growth was measured by determining the absorbance at 600 nm and the OD_{600} was converted to log CFU/ml by calculation. The data represent average values from two independent experiments.

3.3.2 Effect of pH on antibacterial activity of rALF*Pm*3

The purified rALF*Pm*3 stock was diluted in sterile water and incubated with mid-logarithmic phase culture of *V. harveyi* 639 in the culture medium whose pH varied from 3 to 9. The growth of *V. harveyi* 639 observed by measuring OD_{600} and converted into log CFU and plotted against various pH to show effect of pH on the rALF*Pm*3 antimicrobial activity (Fig. 3.7). It was found that the pH values of 3, 4 and 5 affected the growth of *V. harveyi* under the tested conditions, thus, they were excluded. At pH 6, the MIC value against *V. harveyi* was 3.125 μM and increased to 12.5 μM, 25 μM and 25 μM at pH 7, 8 and 9, respectively. The result indicated that the high alkaline condition could result in the low anti-bacterial activity of rALF*Pm*3 (high MIC values).

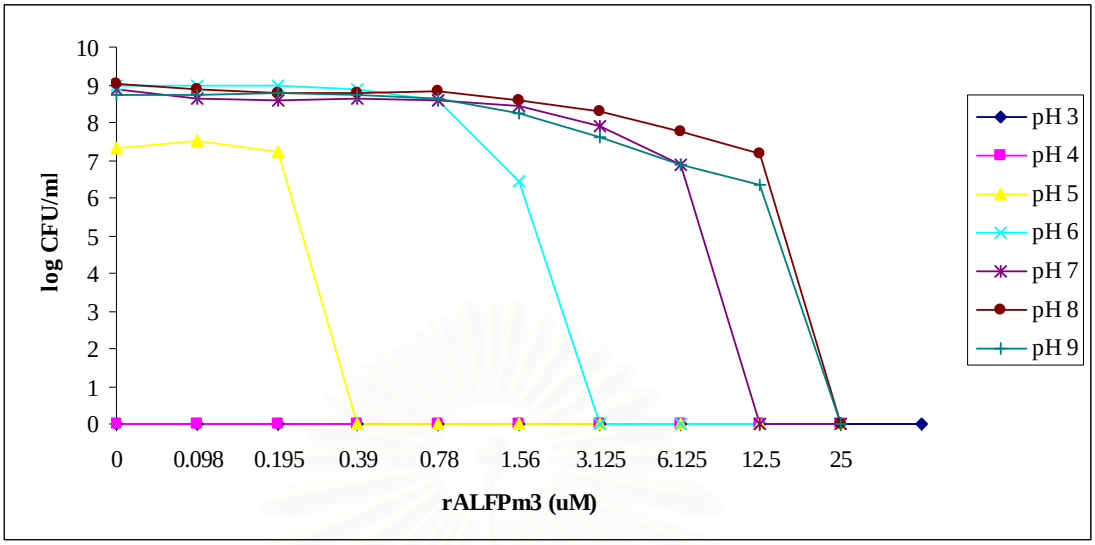


Figure 3.7 Effect of pH on antimicrobial activity of rALF*Pm*3 against *V. harveyi* 639. Various concentration of rALF*Pm*3 were incubated with the culture of *V. harveyi* 639 (OD=0.001) in a medium having pH 6-9 by adding 1 M NaOH or 1M HCl of EPS broth, 2% NaCl. After 24 h of incubation at 28^{o}C, the bacterial growth was measured by determining the absorbance at 600 nm and the OD_{600} was converted to log CFU/ml by calculation. The data represent average values from two independent experiments.

3.3.3 Effect of temperature on antibacterial activity of rALF*Pm*3

The purified rALF*Pm*3 stock solution was diluted in sterile water to the tested concentration and incubated at 4, 30, 50 and 100^{o}C for 40, 80 and 120 minutes. Upon incubation, the antibacterial activity against *V. harveyi* 639 was determined. The growth of *V. harveyi* 639 observed by measuring OD_{600} was converted into log CFU and plotted against various temperature to show the effect of extreme temperature on rALF*Pm*3 antimicrobial activity (Fig. 3.8) The results showed that there was no difference of anti-microbial activity in every incubation temperature and time. These revealed the thermal stable property of the purified rALF*Pm*3.

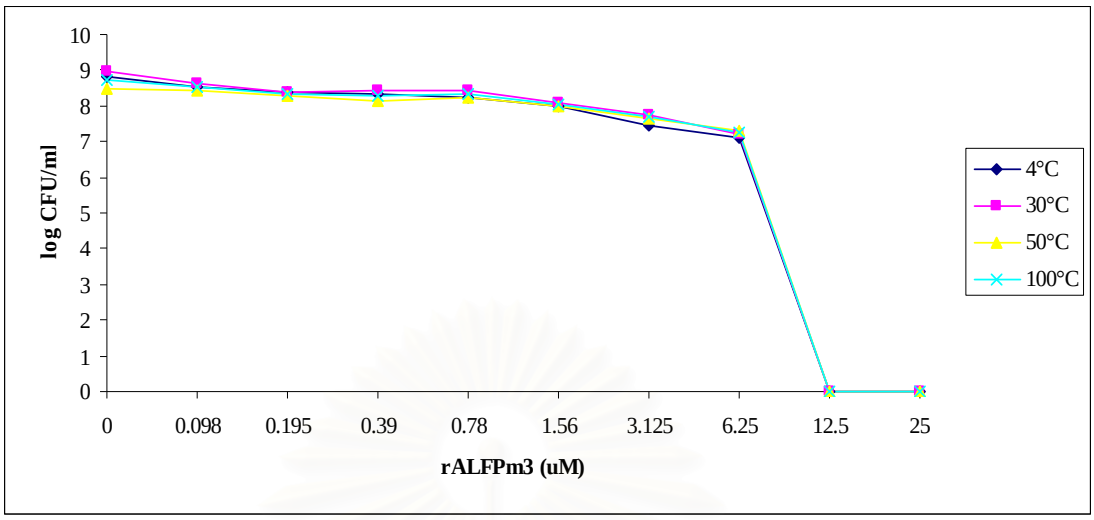


Figure 3.8 Effect of temperature on antibacterial activity of rALF*Pm*3 against *V. harveyi* 639. Various concentration of the rALF*Pm*3 were incubated at 4, 30, 50 and 100^{o}C for 2 hours and tested against the culture of *V. harveyi* 639 (OD=0.001) in EPS broth (2% NaCl, pH 7). After 24 h of incubation at 28^{o}C, the bacterial growth was measured by determining the absorbance at 600 nm and the OD_{600} was converted to log CFU/ml by calculation. The data represent average values of two independent experiments.

3.3.4 Stability of the purified rALF*Pm*3 protein under different storage conditions

The purified rALF*Pm*3 stock solution was stored at -20^{o}C, 4^{o}C and room temperature for 1, 2 and 3 months. The protein was then tested for the MIC values. It was found that the MIC values against *V. harveyi* 639 were the same at all storage conditions (6.125-12.5 µM) (Table 3.1). The rALF*Pm*3 stored for 3 months at different temperatures were analyzed by 12% SDS-PAGE (Fig. 3.9). The result showed that there was no difference of the major band at the storage temperatures. Moreover, the rALF*Pm*3 stored at -20^{o}C and at room temperature for 3 months were checked for their integrity by mass determination using Mass spectrometry (Fig. 3.10). The MS spectrum of the rALF*Pm*3 stored at room temperature for 3 month revealed a mixture of three proteins with molecular mass of 11296.24 Da (for the major one), 11498.75 and 11702.27 Da (for the minor ones). The two peaks at

11296.24 and 11498.75 Da corresponded to those found in the previous study (Somboonwiwat et al., 2005). The major product of the rALF*Pm*3 included additional Tyr and Val residues. The minor rALF*Pm*3 product is the molecule with higher molecular mass due to the presence of extra Glu and Ala residues at the N-terminus (Somboonwiwat et al., 2005). The other minor rALF*Pm*3 product (11702.27 Da) has not been reported in the previous study. The MS spectrum of rALF*Pm*3 stored at -20^{o}C for 3 months showed a broad peak with molecular mass of 11333.75 Da (Fig. 3.10 B).

Table 3.1 Minimum inhibitory concentration (MIC) of rALF*Pm*3 against *V. harveyi* 639 at storage conditions.

Temperature (^{o}C)	Time (months)	MIC value (μM)*
-20	1	6.125-12.5
	2	6.125-12.5
	3	6.125-12.5
4	1	6.125-12.5
	2	6.125-12.5
	3	6.125-12.5
room temperature	1	6.125-12.5
	2	6.125-12.5
	3	6.125-12.5

* MIC are is the average from two independent experiments and expressed as the interval a-b, where a is the highest concentration tested at which *V. harveyi* is growing and b is the lowest concentration that cause 100% growth inhibition.

Figure 3.9 The silver stained 12% SDS-PAGE analysis of the rALF*Pm*3 stored at different temperatures after 3 months.

Lane M: Protein markers

Lane 1: rALF*Pm*3 stored at -20^{o}C

Lane 2: rALF*Pm*3 stored at 4^{o}C

Lane 3: rALF*Pm*3 stored at room temperature

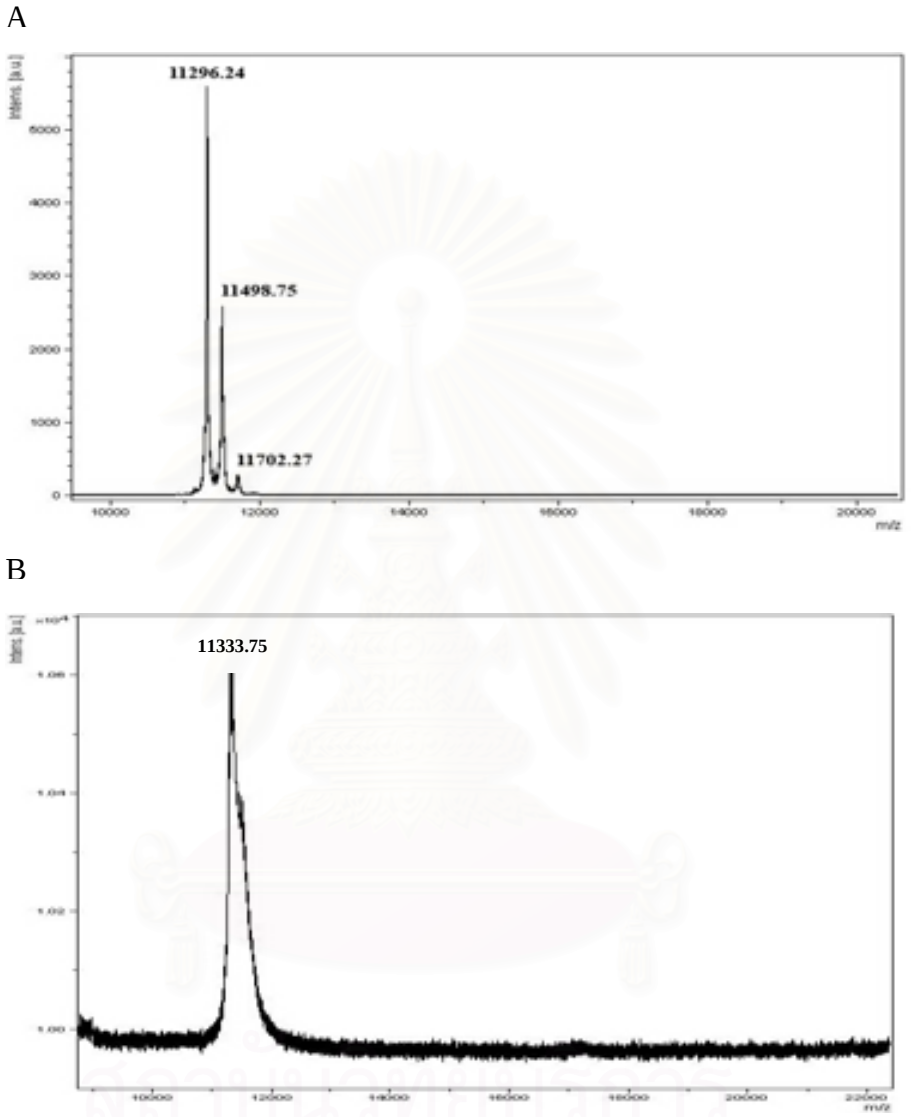


Figure 3.10 Spectrum of the rALF*Pm*3 stored at room temperature (A) and -20^{o}C (B) for 3 months. The rALF*Pm*3 was expressed in *P. pastoris* and purified by cation-exchange chromatography. The purified rALF*Pm*3 stored at room temperature at -20^{o}C for 3 months were checked for their integrity by mass determination using Mass spectrometry.

3.4 Determination of LD_{50}

P. monodon, with average body weight of *V. harveyi* 15 g, was used in the challenge experiments. In each experiment, shrimps were divided into 5 groups. The first group was the control shrimp injected with sterile 0.85% (w/v) NaCl. The second to the fifth groups were the shrimps injected with 10^4, 10^5, 10^6 and 10^7 CFU of live *V. harveyi* 639 per individual. The mortality was observed at 24 hours post-injection. The result showed that the LD_{50} value was log 5.8 of CFU/ml in average of duplicate experiments which corresponded to $4.3x10^4$ CFU of *V. harveyi* per gram of shrimp body weight (Fig 3.11).

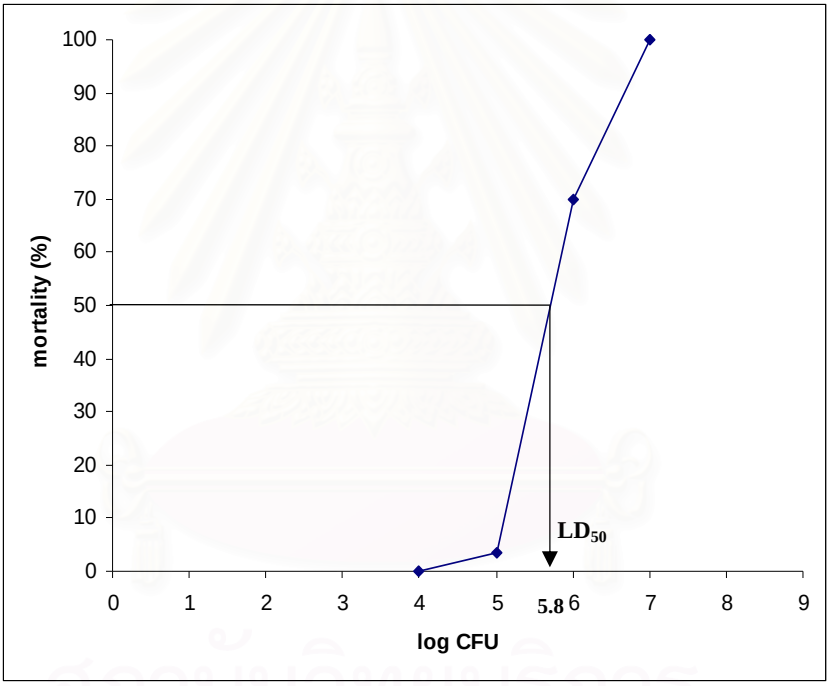


Figure 3.11 Mortality of sub-adult *P. monodon* injected with various concentration of *V. harveyi*. Shrimps were injected with 10^4, 10^5, 10^6 and 10^7 CFU of *V. harveyi* 639 (*n*=10 per group). The cumulative mortality was recorded for 24 hours post injection. The data represent the mean value from two replications.

3.5 *In vivo* neutralization activity of the rALF*Pm*3 protein against *V. harveyi* 639

Shrimps (15 g of body weight) were divided into three experimental groups. Each experiment was performed in triplicate. Two control groups were individually injected with 0.85% (w/v) NaCl and 10^6 CFU (about 1.5 LD_{50}) of *V. harveyi* 639. The last group was injected with 10^6 CFU of *V. harveyi* pre-incubated at 30^oC for 30 minutes with the purified rALF*Pm*3 at the final concentration of 50 μM. The cumulative mortality was recorded and showed in Table 3.2 and Fig. 3.12. The result showed that no shrimp died in the group that pre-incubated with *Vibrio* whereas the group that was injected with *Vibrio* alone had 88.6% mortality.

Dose-effect of the rALF*Pm*3 inhibition on *V. harveyi*-induced mortality was further investigated. Shrimps were divided into one control and six experimental groups. The experiments were performed in triplicate. The control group was individually injected with 10^6 CFU of *V. harveyi* 639 and the percentage of cumulative mortality was 86.6% in 7 days of observation. In the experimental groups, shrimp were injected with 10^6 CFU of *V. harveyi* 639 pre-incubated at 30^oC for 30 minutes with the purified rALF*Pm*3 at the final concentration of 1.56, 3.125, 6.25, 12.5 and 25 μM. The number of shrimp died within 7 days post injection was obviously decreased in these experimental groups. Neutralization of *V. harveyi* 639 with the rALF*Pm*3 protein at 1.56 and 3.125 μM decreased the percentage of cumulative mortality from 86.6% to 80% and 6.66%, respectively, comparing to the control group, respectively (Table 3.3 and Fig. 3.13.). The mortality rate decreased as the quantities of rALF*Pm*3 added to neutralize *V. harveyi* increased. At the concentration of 6.25 μM, neutralization of *V. harveyi* with rALF*Pm*3 resulted in 100% shrimp survival.

Table 3.2 Cumulative mortality of *P. monodon* in the *in vivo* neutralization experiment.

Group	Replicate	No. of dead shrimp						
		D1	D2	D3	D4	D5	D6	D7
0.85% (w/v) NaCl	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	% cumulative mortality	0	0	0	0	0	0	0
V. harveyi (10^6 CFU)	1	14	0	0	0	0	0	0
	2	12	1	0	0	0	0	0
	3	11	2	0	0	0	0	0
	% cumulative mortality	82 ± 10.2	88.6 ± 4.2	88.6 ± 4.2	88.6 ± 4.2	88.6 ± 4.2	88.6 ± 4.2	88.6 ± 4.2
rALF*Pm*3 (50 μM final conc.) + *V. harveyi* (10^6 CFU)	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	% cumulative mortality	0	0	0	0	0	0	0

D: days post challenge

Table 3.3 Cumulative mortality of *P. monodon* injected with *V. harveyi* (10^6 CFU) pre-incubated with rALF*Pm*3 before injection.

rALF*Pm*3 concentration* (μM)	Replicate	No. of dead shrimp							% cumulative mortality
		D1	D2	D3	D4	D5	D6	D7	
0	1	9	0	0	0	0	0	0	86.6±5.7
	2	8	0	0	0	0	0	0	
	3	9	0	0	0	0	0	0	
1.56	1	9	0	0	0	0	0	0	80±10.0
	2	6	2	0	0	0	0	0	
	3	7	0	0	0	0	0	0	
3.125	1	0	0	0	0	0	0	0	6.66±11.5
	2	2	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	
6.25	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	
12.5	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	
25	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	

D: days post challenge; *n*=10

* *V. harveyi* was pre-incubated with various concentration of rALF*Pm*3 at 30°C for 30 min before injection.

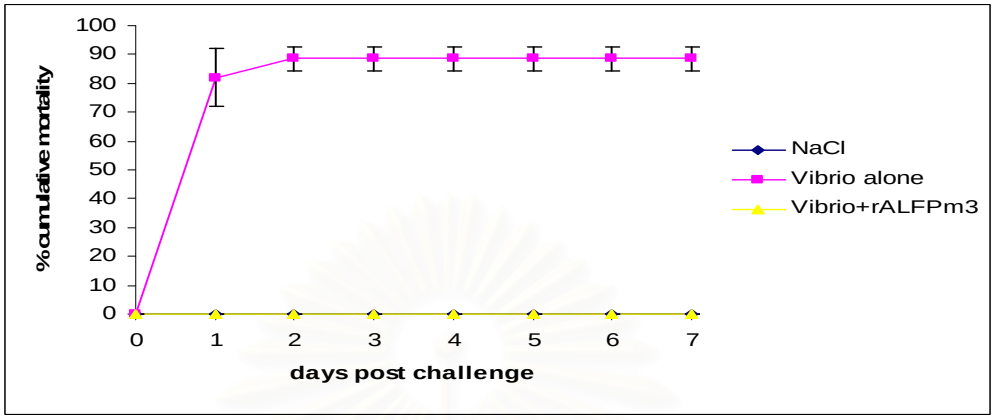


Figure 3.12 *In vivo* neutralization of rALF*Pm*3 on *V. harveyi*. Cumulative mortality rates of shrimps form the experimental groups injected with NaCl (—◆—), *Vibrio harveyi* (—■—) or *Vibrio* + rALF*Pm*3 (—▲—) were plotted at various time post injection. On day 0, shrimps were injected as follows: group 1, NaCl; group 2, *V. harveyi* (10^6 CFU/shrimp); group 3, *V. harveyi* plus rALF*Pm*3 (50 µM final concentration). Cumulative mortality data represent the mean value from three replications (*n*=15 for each group). Error bars indicate standard deviations.

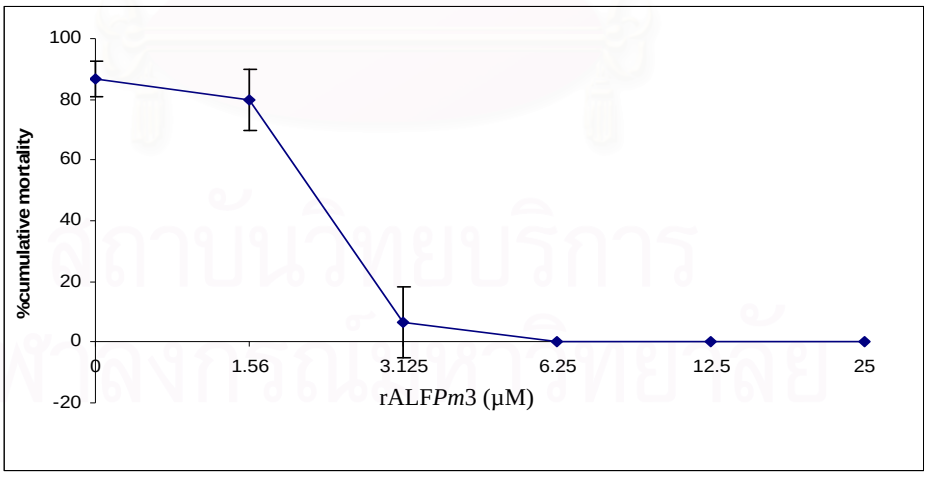


Figure 3.13 Dose-effect of the rALF*Pm*3 inhibition on *V. harveyi*-induced mortalily in *P. monodon*. Data are form 7 days post-injection of 10^6 CFU of *V. harveyi* 639 incubated with 1.56, 3.125, 6.25, 12.5 or 25 µM rALF*Pm*3 at 30°C for 30 minutes before injection. Cumulative mortality data represent the mean value from three replications (*n*=10 for each group). Error bars indicate standard deviations.

3.6 Protective effect of the rALF*Pm*3 on *V. harveyi* 639 injected *P. monodon*

Effect of the rALF*Pm*3 protein on protection of shrimp against *V. harveyi* infection was investigated. Firstly, the duration of protection efficacy of the purified rALF*Pm*3 was optimized by vary the time of injection of the 100 µM rALF*Pm*3 (100 µl) prior to the injection of 10^6 CFU of *V. harveyi* 639. Injection of the purified rALF*Pm*3 for 12, 24, 48 and 72 hours before *V. harveyi* injection resulted in 40%, 40%, 80% and 100% cumulative mortality, respectively (Table 3.4). The results indicated that the effective protection of rALF*Pm*3 was obtained at least for 24 h after infection. Therefore, the duration of 24 hours of the injection of purified rALF*Pm*3 was chosen for the test. After 24 hours of injection of 100 µM rALF*Pm*3 (100 µl), the shrimp were then injected with 10^6 CFU of *V. harveyi* and the number of dead shrimp was observed for 7 days post injection. The experiment was done in duplicate. The data showed that the injection of the purified rALF*Pm*3 to shrimp prior to *V. harveyi* infection could decrease the cumulative mortality from 60% to 25% (Table 3.5, Fig. 3.14). These revealed the ability of rALF*Pm*3 in protection of shrimp from *V. harveyi* infection.

Table 3.4 The number of dead shrimp observed at 0, 12, 24, 48 and 72 hours post injection with 100 µM rALF*Pm*3 (100 µl) followed by injection with 10^6 CFU of *V. harveyi 639* (*n*=5 per group).

Time post injection of rALF*Pm*3 (hours)	No. of dead shrimp	% cumulative mortality
12	2	40
24	2	40
48	4	80
72	5	100

Table 3.5 Cumulative mortality rate of *P. monodon* showing protective effect of rALF*Pm*3 against *V. harveyi* 639 (*n* = 10 per group).

Group	Replicate	No. of dead shrimp						
		D1	D2	D3	D4	D5	D6	D7
0.85% NaCl + 0.85% NaCl	1	1	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	% cumulative mortality	5	5	5	5	5	5	5
0.85% NaCl + *V. harveyi* (10^6 CFU)	1	7	0	0	0	0	0	0
	2	4	1	0	0	0	0	0
	% cumulative mortality	55	60	60	60	60	60	60
100 µM rALF*Pm*3 (100 µl) + *V. harveyi* (10^6 CFU)	1	1	0	0	0	0	0	0
	2	2	2	0	0	0	0	0
	% cumulative mortality	15	25	25	25	25	25	25

D: days post challenge

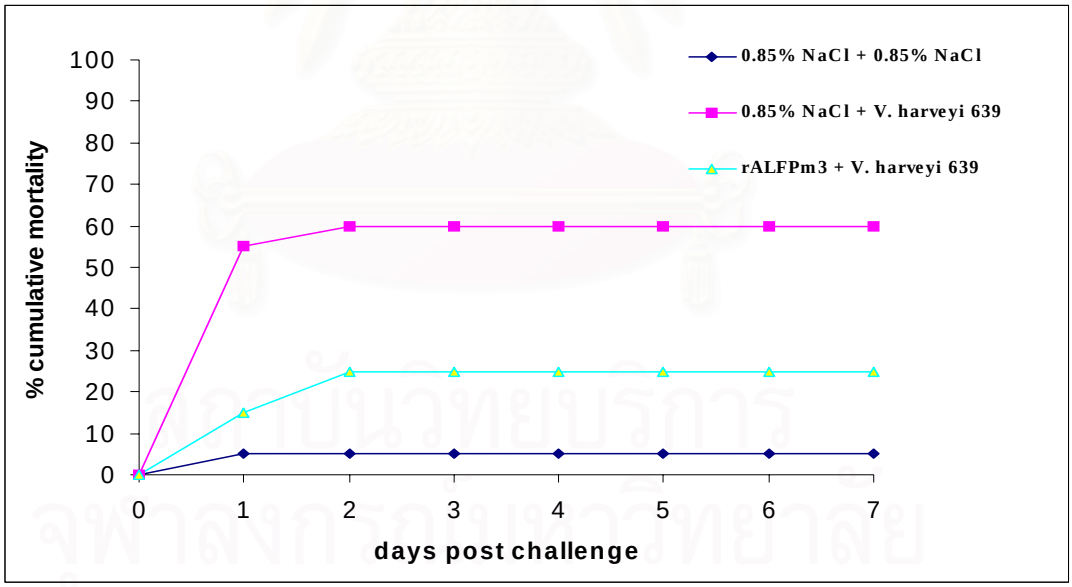


Figure 3.14 Protective effect of the rALF*Pm*3 on *V. harveyi* 639 injected *P. monodon*. Shrimps were injected with 100 µM of rALF*Pm*3 (100 µl) or with saline (100 µl) alone. Twenty four hours later, shrimps were challenged with a lethal dose (10^6 CFU) of *V. harveyi* 639. Cumulative mortality was recorded everyday for 7 days. The data represent the mean value from two replications (*n*=10 for each group).

3.7 Selection of immune-related genes and optimization of PCR conditions

In this experiment, the representative genes of *P. monodon* belonging to the major immune reactions such as those involved in the antimicrobial action, phagocytosis, apoptosis, pro-phenoloxidase system and oxidative response, were selected for their expression analysis in order to investigate the effect on the immune system of *V. harveyi* infected shrimp that received rALF*Pm*3 before infection. These genes included anti-lipopolysaccharide factor isoform 3 (ALF*Pm*3), phagocytosis activating protein (PAP), an apoptosis inhibitor; survivin, prophenol oxidase activating enzyme (PPA) and cytosolic manganese superoxide dismutase precursor (SOD), respectively.

Expression of the interesting genes was analyzed by a semi-quantitative RT-PCR technique. The expected size of PCR product were 203 bp, 360 bp, 168 bp, 166 bp and 320 bp for ALF*Pm*3 (Fig. 3.15 A), PAP (Fig. 3.15 B), PPA (Fig. 3.15 C), SOD (Fig. 3.15 D) and survivin (Fig. 3.15 E), respectively. Amplification cycle was optimized for each gene and shown in the Fig. 3.15. The appropriate number of amplification cycles was chosen for detection of the amplified product at the exponential phase not the plateau amplification phase. The cycle numbers for amplification of ALF*Pm*3, PAP, PPA, SOD, survivin and β-actin genes were 25, 25, 26, 24, 36 and 26 cycles, respectively.

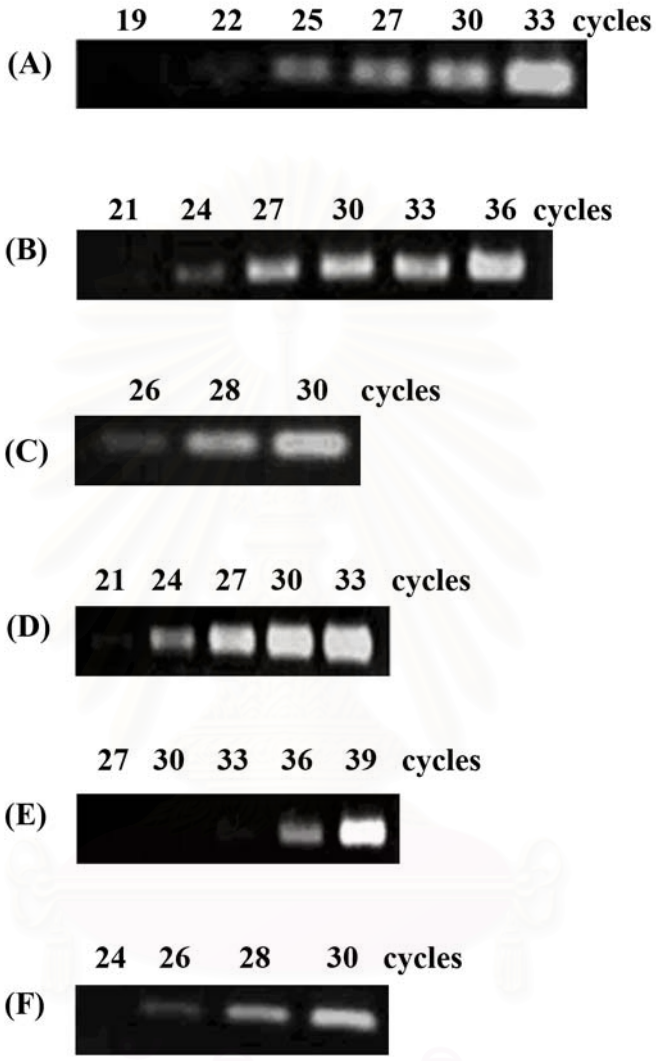


Figure 3.15 Optimization of PCR cycles for RT-PCR amplification of selected immune genes: ALF*Pm*3 (A), PAP (B), PPA (C), SOD (D), Survivin (E) and β-actin (F). Amplification cycle was optimized for each gene and the PCR products were analyzed on 1.5% agarose gel.

3.8 Expression analysis of immune-related genes after *V. harveyi* challenge by semi-quantitative RT-PCR

3.8.1 RNA preparation

For the experimental infection, shrimp were divided into two groups. After 24 h of injected with normal saline or 100 μM of purified rALF*Pm*3 (100 μl), shrimps were injected with 10^5 CFU *V. harveyi.* Hemolymph was separately collected from 5 individuals at different time points. The total RNA extracted from the hemocytes was analyzed for the quality and quantity by measuring A_{260} and A_{280}. The A_{260}/A_{280} ratio of total RNA was 1.4-1.8 indicating acceptable quality of total RNA. The quality of total RNA was monitored by running on 1% agarose-formaldehyde gel. The total RNA from hemocytes of challenged and control shrimps revealed a predominant band of 18S rRNA (1.9 kb) (Fig. 3.16).

3.8.2 Detection of *V. harveyi*

Gills were collected form shrimps injected with *V. harveyi* and used for DNA extraction in the infected *P. monodon*. Shrimps were checked for the *V. harveyi* infection by the PCR technique (modified from Thaithongnum et al., 2006). The primers that specific to gyrB gene of *V. harveyi* were used. The expected size of PCR product was 363 bp. *V. harveyi* infection was detected in shrimp injected with rALF*Pm*3 or normal saline followed by *V. harveyi* infection at 0, 6, 24 and 48 hours. A major band of expected size was observed in all individuals tested (Fig 3.17). However, slightly higher intensity was observed in samples injected with normal saline prior to *V. harveyi* injection (lanes 1-4) than those injected with rALF*Pm*3 (lanes 5-8)

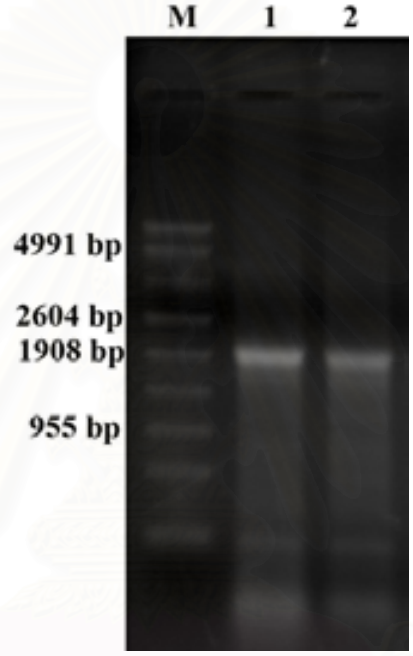


Figure 3.16 Total RNA from hemocyte of shrimps analyzed by electrophoresis on a 1% formaldehyde agorose gel.

Lane M: RNA marker

Lane 1: shrimps injected with normal saline followed by *V. harveyi* 639 injection after 24 hours.

Lane 2: shrimps injected with rALF*Pm*3 followed by *V. harveyi* 639 injection after 24 hours.

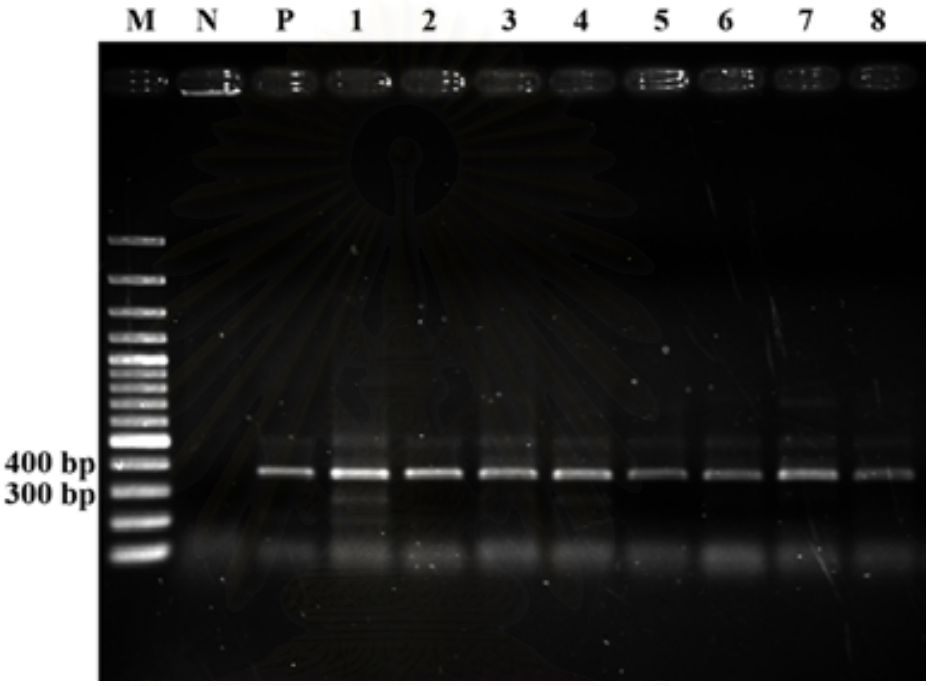


Figure 3.17 Detection of *V. harveyi* by PCR in the injection sample. Note the amplified band at 363 bp corresponded to the presence of the *Vibrio* genome.

Lane M : 100 bp ladder

Lane N : negative control

Lane P : positive control

Lanes 1-4 : shrimps injected with normal saline followed by *V. harveyi* injection at 0, 6, 24 and 48 hours.

Lanes 5-8 : shrimps injected with rALF*Pm*3 followed by *V. harveyi* injection at 0, 6, 24 and 48 hours.

3.8.3 Time course analysis of immune-related gene expression in the *V. harveyi*-challenged shrimp

The expression level of immune-related gene transcripts in haemocytes of shrimp injected with the rALF*Pm*3 followed by *V. harveyi* injection was investigated by a semi-quantitative RT-PCR assay. Shrimps injected with saline followed by *V. harveyi* injection were used as control. The amplification products of the target gene and the control gene (β-actin) were run on the same 1.5% of agarose gel and a ratio of band intensity of the target gene and the control gene was analyzed using Genetools analysis software (Syngene). Determination of the signal ratio of interested gene was normalized with β-actin by comparing the intensity of β-actin band to 1. The results of RT-PCR and data analysis were shown in appendices C and D, respectively.

Effects of the rALF*Pm*3 on the expression of various immune genes were first observed at 0 and 24 hours after injection of rALF*Pm*3 (-24 h) and subsequently after *V. harveyi* injection at 6, 24, 48 h (Fig. 3.18). The results showed no difference of mRNA expression level of ALF*Pm*3 gene compared with the control group injected with normal saline at 0 and 24 hours after the rALF*Pm*3 injection. The shrimps were then injected with *V. harveyi*. The expression levels of ALF*Pm*3 gene of shrimp pre-injected with rALF*Pm*3 significantly increased at 24 hours ($p<0.05$) post *V. harveyi* injection and subsequently decreased at 48 hours. Whereas, the expression level of ALF*Pm*3 gene of the control shrimps pre-injected with normal saline rapidly increased at 6 hours and decreased at 24 hours.

The mRNA expression level of PAP gene of shrimps injected with rALF*Pm*3 at 0 and 24 hours before challenge showed no difference compared with the control shrimp (Fig 3.17 B). After injection with *V. harveyi*, the expression level of PAP decreased at 6 hours and then increase at 24 hours in the control shrimps and the shrimps pre-injected with rALF*Pm*3. No significant difference ($p<0.05$) in the expression level of PAP between the two experimental groups was observed.

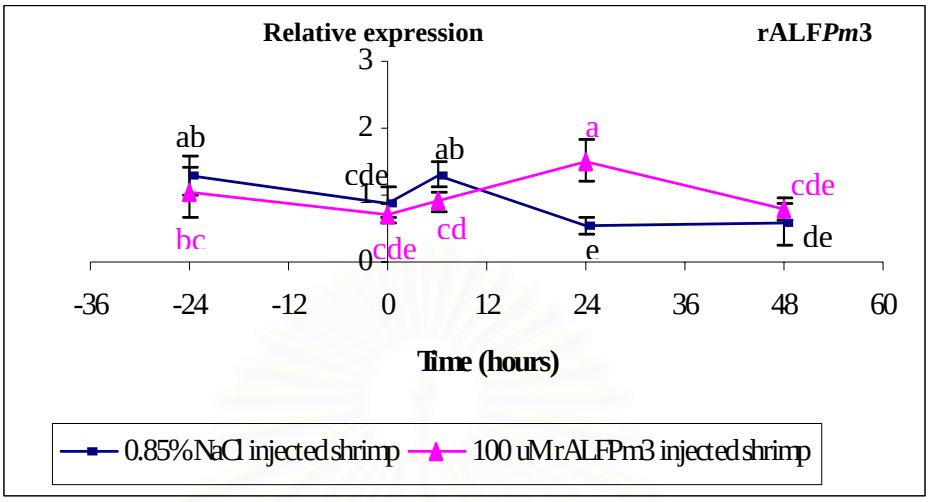
The expression level of PPA of the shrimp injected with the rALF*Pm*3 was lower than the control shrimp at 0 hour and increased to the same level as that of the shrimps injected rALF*Pm*3 at 24 hours (Fig 3.17 C). Subsequent injection of *V. harveyi* resulted in a significant decreased of PPA mRNA level at 6 hours ($p<0.05$) in

both groups of samples. However, the expression level of PPA of the control shrimp increased significantly at 48 hours ($p<0.05$). Whereas, the shrimp pre-injected with the rALF*Pm*3 did not significant change in the expression level of PPA during 6 to 48 hours. Comparing with the shrimp pre-injected with rALF*Pm*3, the expression level of PPA gene of the control shrimp was 2.8 and 2.7 folds higher at 24 and 48 hours post *V. harveyi* injection, respectively.

The expression level of SOD of the shrimp injected with rALF*Pm*3 decreased significantly about 3 folds at 0 hour ($p<0.05$) and increased significantly at 24 hours ($p<0.05$) comparing with the control shrimp (Fig. 3.17 D). The expression levels of SOD after *V. harveyi* injection showed that the control shrimp significantly decreased at 6 hours and 48 hours. Whereas, the shrimp pre-injected with rALF*Pm*3 significantly decreased at 6 hours ($p<0.05$) and increased at 48 hours. Comparing with the control shrimp, the expression level of SOD gene of the shrimp pre-injected with rALF*Pm*3 was 2.2 folds higher at 48 hours post *V. harveyi* injection.

No difference in the expression level of survivin was observed at 0 hour in both of the control and the shrimps injected with the rALF*Pm*3 (Fig. 3.17 E). The expression level of survivin significantly decreased about 2 folds in the shrimp injected with the rALF*Pm*3 at 24 hours. After *V. harveyi* injection, survivin mRNA expression of the shrimp pre-injected with rALF*Pm*3 significantly increased at 24 hours ($p<0.05$) and decreased at 48 hours. Comparing with the control shrimp, survivin mRNA expression of shrimps pre-injected with rALF*Pm*3 showed higher inducting at 24 h after *V. harveyi* injection.

A.



B.

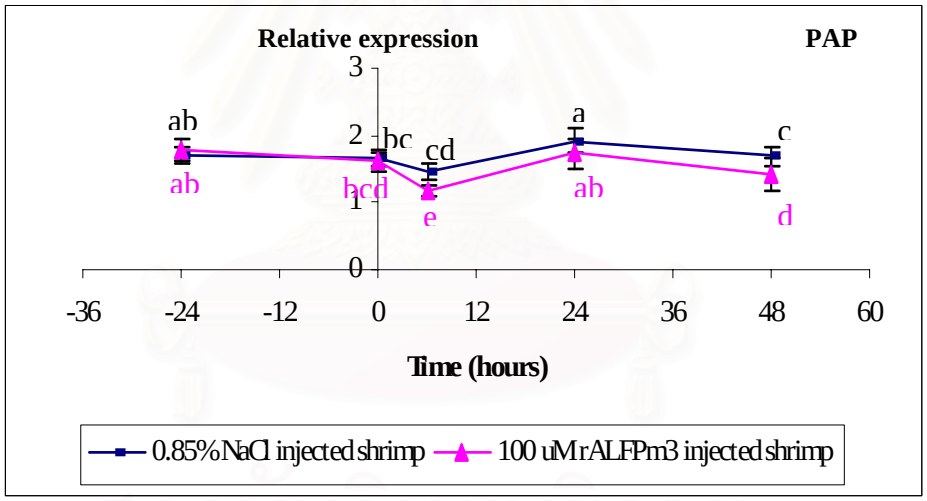
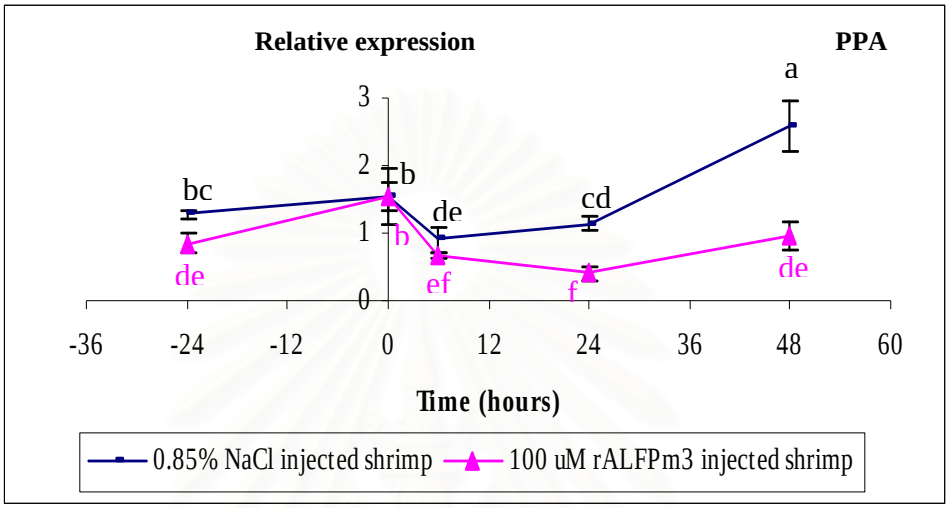


Figure 3.18 Expression analysis of immune-related genes, ALF*Pm*3 (A), PAP (B), PPA (C), SOD (D) and Survivin (E). The expression analysis of the representative genes were observed at 0 and 24 h after 0.85% NaCl or rALF*Pm*3 (-24 h and 0 h) injection and at 6, 24 and 48 h post injection with *V. harveyi* (*n*=5). Relative expression levels were normalized with β-actin (relative expression = 1). Different letters indicate significant ($p<0.05$) difference in the mean expression level of the immune-related genes.

C.



D.

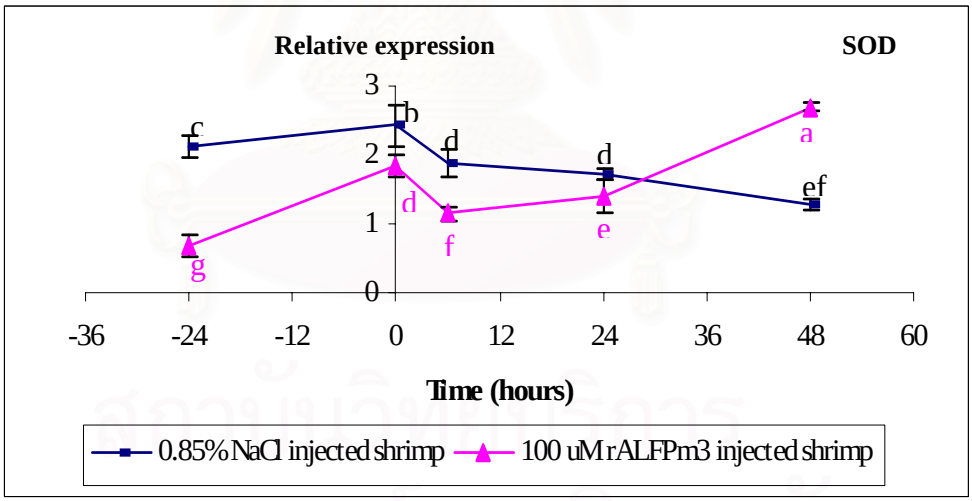


Figure 3.18 (continued) Expression analysis of immune-related genes, ALF*Pm*3 (A), PAP (B), PPA (C), SOD (D) and Survivin (E). The expression analysis of the representative genes were observed at 0 and 24 h after 0.85% NaCl or rALF*Pm*3 (-24 h and 0 h) injection and at 6, 24 and 48 h post injection with *V. harveyi* (*n*=5). Relative expression levels were normalized with β-actin (relative expression = 1). Different letters indicate significant ($p<0.05$) difference in the mean expression level of the immune-related genes.

E.

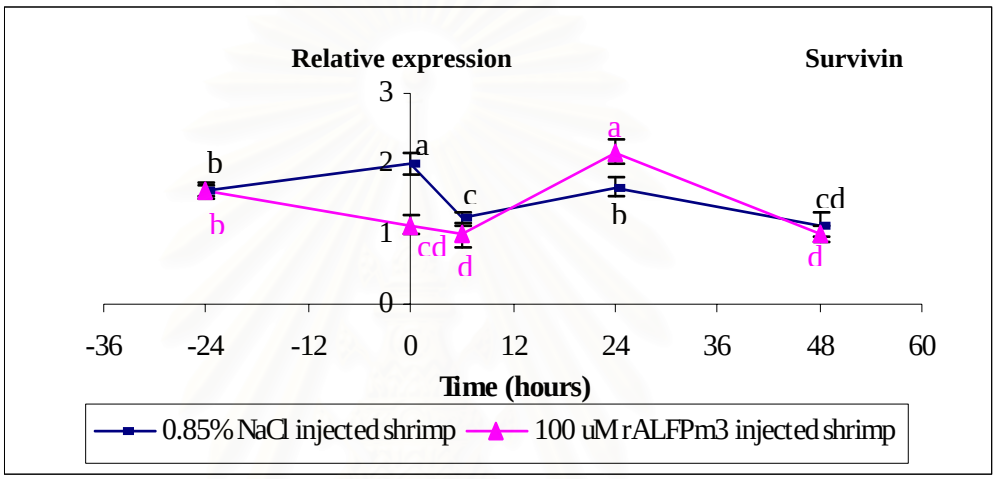


Figure 3.18 (continued) Expression analysis of immune-related genes, ALF*Pm*3 (A), PAP (B), PPA (C), SOD (D) and Survivin (E). The expression analysis of the representative genes were observed at 0 and 24 h after 0.85% NaCl or rALF*Pm*3 (-24 h and 0 h) injection and at 6, 24 and 48 h post injection with *V. harveyi* (*n*=5). Relative expression levels were normalized with β-actin (relative expression = 1). Different letters indicate significant ($p<0.05$) difference in the mean expression level of the immune-related genes.

Table 3.6 Expression levels of the five immune-related genes by using a semi-quantitative RT-PCR.

Genes	Relative Expression*				
	-24 hours	0 hours **	6 hours	24 hours	48 hours
NaCl injected shrimp					
ALF*Pm*3	1.29^{ab} (±0.28)	0.88^{cde} (±0.23)	1.30^{ab} (±0.18)	0.55^{e} (±0.12)	0.57^{de} (±0.32)
PAP	1.27^{ab} (±0.12)	1.66^{bc} (±0.12)	1.464^{cd} (±0.13)	1.92^{a} (±0.17)	1.69^{c} (±0.14)
PPA	1.28^{bc} (±0.07)	1.54^{b} (±0.42)	0.90^{de} (±0.20)	1.136^{cd} (±0.12)	2.58^{a} (±0.37)
SOD	2.10^{c} (±0.16)	2.42^{b} (±0.31)	1.88^{d} (±0.20)	1.73^{d} (±0.07)	1.28^{ef} (±0.07)
Survivin	1.6^{b} (±0.07)	2.00^{a} (±0.16)	1.21^{c} (±0.08)	1.66^{b} (±0.13)	1.12^{cd} (±0.17)
rALF*Pm*3 injected shrimp					
ALF*Pm*3	1.05^{bc} (±0.37)	0.71^{cde} (±0.15)	0.90^{cd} (±0.15)	1.50^{a} (±0.32)	0.80^{cde} (±0.16)
PAP	1.78^{ab} (±0.15)	1.61^{bcd} (±0.14)	1.18^{e} (±0.07)	1.73^{ab} (±0.21)	1.43^{d} (±0.24)
PPA	0.84^{de} (±0.15)	1.54^{b} (±0.20)	0.65^{ef} (±0.04)	0.4^{f} (±0.12)	0.96^{de} (±0.19)
SOD	0.69^{g} (±0.16)	1.84^{d} (±0.17)	1.14^{f} (±0.11)	1.40^{e} (±0.24)	2.70^{a} (±0.06)
Survivin	1.62^{b} (±0.10)	1.13^{cd} (±0.14)	0.99^{d} (±0.18)	2.17^{a} (±0.18)	1.02^{d} (±0.11)

* Relative expression level were normalized with β-actin (relative expression = 1). Data represent the average from five individual animals. Different letters indicate significant (p<0.05) difference in the mean expression level of the immune-related genes.

**0 hour: the shrimps were injected with *V. harveyi*

CHAPTER IV

DISCUSSIONS

The repeated outbreaks of infectious diseases occurred in the shrimp farms cause the great impact on the *Penaeus monodon* production. One of the major bacterial diseases in shrimp aquaculture is Vibriosis causing mass mortalities both in hatcheries and in shrimp growout ponds (Saulnier et al., 2000). The major virulent strains of *Vibrio* in shrimp are *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and *V. anguillaram*. The presences of *V. harveyi* have been reported in *P. monodon* and *P. vannamai* in Indonesia (Sunaryanto and Mariam, 1986), Thailand (Jiravanichpaisal et al., 1994) and India (Karunasagar et al., 1994). Many strategies have been used to control these bacterial diseases, one of which is the use of antibiotics such as oxytetracycline, chloramphenicol, amplicillin, ciprofloxacin and kanamycin. Ciprofloxacin was the most effective for shrimp disease control in India (Vaseeharan et al., 2005). Using high dose of antibiotics causes the development of antibiotic resistance bacterial strain (Vaseeharan et al., 2005; Nakayama et al., 2006) From this reason, the antibiotic replaceable substances including feeding mixture of yeast extracts, probiotics and oral vaccine are used instead to protect shrimp from pathogenic microorganisms (Itami et al., 1998; Teunissen et al., 1998; Witteveldt et al., 2004). The cationic amphipathic peptides or antimicrobial peptides are also the interesting molecules because they have broad spectrum of anti-fungal properties and antimicrobial activities against both Gram-positive and Gram-negative bacteria.

From the *Penaeus monodon* EST database, the antimicrobial peptides (AMPs) such as antilipopolysaccharide factors (ALFs), penaeidins and crustins were identified in the *V. harveyi* challenged shrimp (Supungul et al., 2004). The recombinant ALF*Pm*3 and the recombinant lysozyme could effectively inhibit the growth of various *Vibrio* species including *V. harveyi* (Somboonwiwat et al., 2005; Tyagi et al., 2007) Therefore, the antimicrobial peptides have potential to apply for therapeutic and pharmaceutical applications in aquaculture.

The recombinant ALF*Pm*3 protein (rALF*Pm*3) was successfully expressed in the yeast *Pichia pastoris* and had high antimicrobial activity against various *Vibrio* species (Somboonwiwat et al., 2005). In this study, we produced the rALF*Pm*3 protein in a fermentor to obtain an adequate amount of protein. Identification of the rALF*Pm*3 protein using silver-stained SDS-PAGE showed the major band of approximately 14 kDa which is higher than the expected size (11.3 kDa) because the rALF*Pm*3 protein was cationic peptides with the highly positive charge. This result is similar to that of the previous study (Somboonwiwat, 2004). To identify the rALF*Pm*3, Western blot analysis using anti-rALF*Pm*3 antibody and antimicrobial test were performed. The results showed that the major band of approximately 14 kDa was the rALF*Pm*3 and the crude protein contained the antimicrobial activity as the previous report (Somboonwiwat et al., 2005). Production of the recombinant protein in a fermentor gave about two folds higher protein concentration than that in a shake flask. However, much more protein species were found in the crude protein produced in a fermentor. This may be due to the condition of rALF*Pm*3 production phase such as feed rate of methanol, dissolved oxygen percentage and biomass concentration was not appropriate (Jahic et al., 2006). The protein purification was done as described previously (Somboonwiwat, 2004). The purification of the rALF*Pm*3 yield approximately 20% of the crude protein. Precipitations after thawing the crude supernatant and during dialysis the purified fraction were the causes of losing the purified protein.

To characterize the stability of the rALF*Pm*3 under extreme conditions, the antimicrobial activity of the recombinant protein against *V. harveyi* 639 was investigated. The result showed that the ionic strength and pH were important to the antibacterial activity of rALF*Pm*3 as found in other AMPs such as lactoferricin, dermaseptin S4 and nisin (Rollema et al., 1995; Rydlo et al., 2006). The structural features of alpha-helical antimicrobial peptides have been studied in detail including helicity, hydrophobic moment, hydrophobicity, net charge distribution and amphipathicity (Tossi et al., 1997). Many antimicrobial peptides require an alpha-helical conformation upon interactions with cell membranes to exert activity. Therefore, the helix stability plays an important role for the antimicrobial action of these peptides.

Under high salt or alkali conditions, the rALF*Pm*3 can not maintain its potency. The anti-*Vibrio* activity of rALF*Pm*3 protein was reduced when the salt concentrations were increased from 1% to 3%. The conditions of high salt concentration provide the high ionic strength in the solution that interferes binding between the rALF*Pm*3 peptide and negatively charged targets (LPS or endotoxin of *Vibrio*). Salt might also affect peptide organization in solution in terms of either secondary structure, salting out or induce aggregation. The helical content of the antimicrobial peptides was almost completely destroyed at salt concentrations over 200 mM leading to an 8-32-fold decrease in antimicrobial activity (Park et al., 2004). Changes in antibacterial activity of dermaseptin derivatives did not correlate with structural changes salt concentration since the peptides exhibited the highest helical structure at 6% NaCl where activity was significantly reduced (Rydlo et al., 2006)

Moreover, variations in pH can affect the peptides net charge, which in turn affects its secondary structure, binding properties, and cytotoxic activity. Hisidine-containing amphipathic helical peptides were reported to exhibit random coil structure at acidic pH levels and typical alpha-helix structure at basic conditions (Vogt and Bechinger, 1999). The acidic conditions destabilized ion pairs between acidic and basic side chains due to the protonated state of acidic residues, whereas at basic conditions, repulsive forces between basic side chains were limited due to deprotonation of positively charged residues, leading to helix stabilization. Thus, a peptide's pKa value can determine loss or gain of activity as a function of the environmental pH conditions (Rydlo et al., 2006). The anti-*Vibrio* activity of the rALF*Pm*3 protein under low acid-alkaline incubation (pH5-9) showed that high alkali pH resulted in lowering the rALF*Pm*3 antimicrobial activity. For pH5 to pH9 incubation, the MIC value showed that the anti-*Vibrio* activity of the rALF*Pm*3 was reduced 4 to 64 folds. According to the crystal structure of Limulus ALF and rALF*Pm*3, the positives charges of the LPS binding domain supposed to play an important role in antimicrobial activity (Hoess et al, 1993) by binding to the negative rough on membrane component. The changes of pH could affect the net charge of the molecules and also that of the amphiphatic loop. These might be the cause of the reduction of anti-*Vibrio* activity under the condition of pH near pI (about 10).

The effect of temperature on the anti-*Vibrio* activity of the rALF*Pm*3 was also investigated. Either incubation at various temperature (4, 30, 50 and 100^{o}C) for 2 hours and stored at room temperature for three months did not effect the molecule integrity and its anti-*Vibrio* activity. This revealed the stability of the rALF*Pm*3 in a wide-range of temperature. To investigate the degradation of the stored rALF*Pm*3 at 3 months, MALDI-TOF mass spectrometry was used to determine the molecular mass of the rALF*Pm*3. The size of major and minor product observed and corresponded to the previously study were about 11.3 kDa (100 amino acid residues) and 11.5 kDa (102 amino acid residues), respectively. These products were cleavaged at the STE13 cleavage site (Somboonwiwat et al, 2006). Because the rALF*Pm*3 protein containing α-factor mating signal sequence secreted into the culture medium. The signal sequence was proteolytically removed by KEX2 and STE13 gene products resulting in the mature rALF*Pm*3 protein (Cereghino and Cregg, 2000). Therefore, cleavage of the peptide might provide three sizes of the products which were 11.3, 11.5 and 11.7 kDa. Mass spectrometry showed another minor product of 11.7 kDa which has not been previous reported. The protein may contain additional six amino acid residues (Glu-Ala-Glu-Ala-Thy-Val) from mature peptide. These extra amino acid residues could result form an efficient KEX2 and inefficient SPE13 protease cleavage at the N-terminal part of the secreted rALF*Pm*3.

Previously, the LALF was also found to retain its activity even boiling. It is highly stable on heat treatment in an acidic medium, and no loss of the anti-LPS factor activity was observed (Tanaka et al., 1982). Low temperatures of incubation may affect the peptides solubility, leading to formation of activity-limiting aggregates. Maisnier-Patin et al. (1996) found that the antimicrobial activity of the bacteriocin EFS2 on *L. innocua* was highest at 35^{o}C, with activity loss at 15^{o}C due to low solubility (Maisnier-Patin et al., 1996). Thermal stability of the rALF*Pm*3 suggests its potential application for prevention of shrimp disease.

Studying the susceptibility of shrimp to *Vibrio* infection, the median lethal dose, LD_{50} figures are used as a general indicator of acute toxicity (Trevan., 1927). Hameed et al. (1995) demonstrated that the LD_{50} values of *V. campbellii*-like bacteria varied among three species of shrimps (*P. monodon*, *P. indicus* and *P. semisulcatus*). The bacterial

suspension used for the bath challenge was high, with a final concentration of $3.5x10^6$-$3.5x10^9$ CFU/ml, whereas de la Pena et al. (1995) succeeded in infecting *P. japonicus* prawns with low number of a *V. penaeicida* isolate (10^3-10^4 CFU/animal) orally administered. In *P. stylirostris* infection, the LD_{50} of *V. alginolyticus* was $6x10^4$ CFU/shrimp (15 g) used for intramuscular injection (De La Pen?a et al., 1995). In this study, *Vibrio* infection was performed by intramuscular injection of *V. harveyi* 639 (isolated in Thailand) at the second abdominal segment of *P. monodon*. The LD_{50} of *V. harveyi* 639 effects on *P. monodon* showed was $4.3x10^4$ CFU per gram of shrimp. Therefore 10^6 CFU of *V. harveyi* 639 was used for 15-20 gram shrimp in the mortality study. Vibriosis in penaeids is generally recognized as a secondary infection influenced by factors such as stress, environmental failures, and high numbers of potentially pathogenic bacteria (Chen and Nan, 1992).

Thiosulfate citrate bile salt sucrose (TCBS) agar is a selective medium used for isolation of *V. cholerae* and *V. parahaemolyticus* as well as other vibrios. Spreading of the hepatopancreas on TCBS agar was shown to identify the *Vibrio* infectious shrimp; however, it was not specific to *Vibrio harveyi* species. The other vibrios species may be grown on TCBS agar plate as well. Because of the very close phylogenetic relationship of the other *Vibrio* species such as *V. parahaemolyticus*, *V. alginolyticus*, *V. campbellii* and *V. carchariae* (Kita-Tsukamoto et al., 1993), identification of *V. harveyi* by conventional biochemical techniques is not accurate. Therefore, a PCR method that could target nucleotide sequences unique to *V. harveyi* may facilitate its detection and differentiation from closely related *Vibrio* species. From the previous report, *V. campbellii* was accumulated in the lymphoid organ, the gills and hepatopancreas (Burgents et al., 2005). To investigate the *V. harveyi* infection, the gills of shimp were collected for the detection of *V. harveyi*. The PCR method was preformed using A2B3 primers that can amplify the gyrB gene of *V. harveyi* NICA, isolated from shrimp in Thailand. The size of PCR product was 363 bp. Seventeen other *Vibrio* species showed no cross reaction except for *V. carchariae* which is a synonym for *V. harveyi.* The primers could detect *V. harveyi* at a level of 15 cells/ml (Thaithongnum et al., 2006). Consequently, A2B3 primers were used for detection of *V. harveyi* infectious shrimp in this study. To study the protection effect

of the rALF*Pm*3 on *V. harveyi* injected shrimp, the shrimp was challenged with 10^6 CFU of *V. harveyi* 639 injection and then detected for the *Vibrio* infection at 6-48 hours. The shrimps were successfully infected with *V. harveyi* 639 by muscularly injection. The PCR product was approximately 363 bp which is similar to the positive control band.

The development of strategies for prophylaxis and control of Vibriosis in shrimp have been reported in a few studies. Extracellular products (ECP) of *Vibrio* secretion were of interest for toxicity neutralization and protection in *P. monodon* and they were investigated by using rabbit antisera to the formalinized extracellular products (RaECP) and formalinized bacterial cells (RaBC) of luminescent *V. harveyi* strain 820514. Passive immunization by pre-injection of RaBC or RaECP protected against a lethal dose challenge of bacteria (Lee et al., 1997). In our study, we investigate the potential use of the antimicrobial peptide for the Vibriosis protection. The rALF*Pm*3 protein was characterized the anti-*Vibrio* activity and stability in vitro. Interestingly, *in vivo* neutralization activity of the rALF*Pm*3 protein on *V. harveyi* 639 demonstrated that the cumulative mortality was reduced when high concentration of the rALF*Pm*3 peptide was used. The lowest inhibition concentration of the rALF*Pm*3 that can neutralize the effect of *V. harveyi*, resulting in 100% survival rate, was found to be 6.25 μM where as the 50% inhibitory concentration (IC_{50}) of rALF*Pm*3 was found to be 2 μM. The high neutralization activity of rALF*Pm*3 on *V. harveyi* suggested a potential use of the protein in Vibriosis prevention. However, we further investigated the protective effect of rALF*Pm*3 by injection of the protein to the shrimps for a certain period of time prior *V. harveyi* injection.

A reduction of cumulative mortality was observed form 60% to 25%. Decreasing of cumulative mortality showed the efficacy and the persistence of passive protection by rALF*Pm*3 protein in *P. monodon.* Antimicrobial peptides are known to interact with cell membranes through electrostatic forces leading to insertion and performing phenomena (Huang, 2000). The Limulus ALF (LALF) possesses the ability to bind and neutralize LPS which is particularly attractive, because it can directly block the primary stimulus for the proinflammatory cytokine cascade (Vallespi et al., 2003) and may reduce lethality in bacterial sepsis. It has been shown that LALFs are effective inhibitors for decreasing

mortality before and after administration of LPS challenge in rabbits (Danenberg et al., 1992). Furthermore, the synthetic shrimp anti-lipopolysaccharide factor peptide ($SALF_{55-76}$ cyclic peptide) was tested against bacterial clinical isolates and showed broad-spectrum antimicrobial activity. When mice were treated with the $SALF_{55-76}$ cyclic peptide before bacterial challenge with *P. aeruginosa*, the peptide highly protected mice against death by sepsis. In addition, the syntheses of inflammatory cytokines were significantly upregulated after $SALF_{55-76}$ cyclic peptide treatment (Pan et al., 2007). Therefore, the rALF*Pm*3 could be useful in future studies on disease control of Vibriosis in aquaculture.

Because hemocytes are an important tissue in the response to immune stimulation, thus, the hemocytes were used for analysis of immune-related genes. To examine if the injection of rALF*Pm*3 could affect the immune response in the shrimps, the mRNA expression level of several immune genes were compared between shrimp injected with rALF*Pm*3 and those injected with normal saline (control).

The expression analysis of mRNA quantification was detected by various methods such as northern blotting and in *situ* hybridization (Parker and Barnes, 1999), RNAse protection assays (Hod, 1992), the reverse transcription polymerase chain reaction (RT-PCR) (Tan and Weis, 1992) and the cDNA arrays (Bucher, 1999). Semi-quantitative RT-PCR was chosen in this study for evaluation of transcriptional levels of immune-related genes because it is a highly sensitive, specific and easy to set up.

Immune-related genes represent the different shrimp immune reactions including those involved in Pro-PO system, phagocytosis, antimicrobial effector, oxidative stress and apoptosis effector were selected. Expression levels of five putative genes including ALF, PAP, PPA, SOD and survivin in hemocytes of *P. monodon* were analyzed after rALF*Pm*3 injection at 0 and 24 h (-24 h), followed by *V. harve*yi injection at 6, 24 and 48 hr.

Interestingly, the different immune genes showed different responses to the injection of rALF*Pm*3 followed by *V. harveyi* infection. The expression of ALF*Pm*3 showed no difference of mRNA expression level comparing to the control group at 0 and 24 hours after the rALF*Pm*3 injection. Injection of *V. harveyi* resulted in up-regulation of ALF*Pm*3 transcript in both groups that were injected with rALF*Pm*3 or the saline

solution. However, the delay of response was detected in the shrimp group that was injected with rALF*Pm*3. Up-regulation of ALF*Pm*3 transcript was observed at 24 h instead of 6 h. Tharntada et al. (2008) showed a rapid response of ALF*Pm*3 transcript at 6 h post injection with *V. harveyi* (Tharntada et al., 2008).

For the response of PAP mRNA to *V. harveyi*, no difference in the transcription level between the groups injected with rALF*Pm*3 was found comparing to the control group injected with normal saline. These results suggested that the administer of rALF*Pm*3 prior to *V. harveyi* injection did not stimulate phagocytosis. While the injection of *V. harveyi* caused a decrease in PAP transcript at 6 h followed by induction at 24 h and returned to the basal level within 48 h. Deachamag et al. (2006) found that the expression of PAP in the haemolymph of *P. monodon* was induced via the injection of immunostimulants of inactivated *V. harveyi* (Deachamag et al., 2006).

On the contrary, shrimps that were pre-injected with rALF*Pm*3 before challenge with to *V. harveyi* injection showed significant difference in the expression of PPA transcript comparing to the group injected with saline solution. The results suggest that the rALF*Pm*3 has an effect on the Pro-PO system. PPA mRNA expression level of the control shrimps and the shrimps pre-injected with the rALF*Pm*3 was decreased at 6 hours. Subsequently, the expression level of the control shrimps significantly increased at 48 hours. The results correspond to the proPO-activating system (proPO system) triggered by the presence of minute amounts of compounds of microbial origins, such as β-1,3-glucans, lipopolysaccharides, and peptidoglycans (Cerenius and Soderhall 2004). However, a much lower stimulation of the PPA expression was detected in the shrimps that received rALF*Pm*3 prior to *V. harveyi* infection. The lower response to *V. harveyi* observed in the study may be due to the lower number of *V. harveyi* in circulatory haemocytes due to the protection effect of rALF*Pm*3.

Cytosolic manganese superoxide dismutase (SOD) is an important enzyme in the antioxidant defense pathway responses to oxidative stress produced by activated phagocytes (Fridovich, 1998). The mRNA expression level of SOD of the shrimps injected with the rALF*Pm*3 was decreased since 0 hour post injection of rALF*Pm*3 comparing with the control group. The results suggested that rALF*Pm*3 had effect on

oxidative stress response. Moreover, the mRNA level of SOD in the control group was significantly decreased at 6 hour after *V. harveyi* stimulation, similar to the enzymatic activity reported in *Palaemonetes argentinus* after infected with *Probopyrus ringueleti* (Neves et al., 2000). A lower level of SOD might sensitize the hemocyte due to an increasing of accumulation of superoxide anion (O_2^-), hence superoxide anion are potentially deleterious to microorganism (Thornqvist and Soderhall, 1993; Mun?oz et al., 2000). Nevertheless, the effective and rapid elimination of reactive oxygen species is essential to the proper functioning and survival of organisms (Homblad and Soderhall, 2000). From the reason, the host cell might protect themselves from free superoxide anion by increasing the expression level of SOD gene. Shrimp pre-injected with rALF*Pm*3 protein before challenge with *V. harveyi* showed a rapid response in SOD expression by decreasing the synthesis of SOD thus, increasing the toxic superoxide anions to defense themselves. These shrimp rapidly destroyed the injected bacteria and within 48 h the SOD expression was up-regulated in order to control the excess of free superoxide anion.

Survivin is an inhibitor of apoptosis protein regulating apoptosis at cell division (Liston et al., 1996; Uren et al., 1996). Shrimps received rALF*Pm*3 prior to *V. harveyi* injection first showed down regulation of survivin within 24 h indicated that apoptosis was highly promoted to eliminate any infected cells. The lower expression of survivin at early phase of injection might promote the apoptosis in infected-cells to eliminate microbial cell from spreading (Anggraeni and Owens, 2000). After that the up-regulation of survivin was required for suppression of apoptosis in shrimp hemocytes (Sahtout et al., 2001).

In addition, several lines of evidence have shown that AMPs are signaling and chemotactic molecules that connect the innate and adaptive immune responses (Tang et al., 2005) For example, alpha-defensins enhance phagocytosis by macrophages, induce degranulation of mast cells and induce bronchial epithelial cells to release IL-8 that is chemotactic and leads to neutrophil influx (Tomita and Nagase, 2001) and cathelicidins are multifunctional cationic peptides that play an important role in regulating host

defences (Zanetti, 2004). In application, the protection of mytilin reduced the shrimp mortality on WSSV infectious disease in palaemonid shrimp (Dupuy et al., 2004). The LALFs are effective inhibitors for decreasing mortality before and after administration of LPS challenge in rabbits (Alpert et al., 1992). The ALF of *P. monodon* was highly protected mice against *Pseudomonas aeruginosa* (Pan et al., 2007). Our results suggest that the rALF*Pm*3 appears to have promising therapeutic potential for future developments.

In summary, our results clearly demonstrated the protection of rALF*Pm*3 against *V. harveyi* infection in shrimps resulting in a higher survival rate of the animals. In addition, rALF*Pm*3 exerts its effects different immune reactions including antimicrobial action, proPO system, antioxidant defence pathway and apoptosis. These results suggest that rALF*Pm*3 has high potential applications for use in disease control in the aquaculture.

CHAPTER V

CONCLUSIONS

The stability of the recombinant protein of anti-lipopolysaccharide factor isoform 3 (rALF*Pm*3) under extreme conditions was tested. A rALF*Pm*3 can not maintain its potency at high the salt concentrations and alkaline condition. Salt concentration at 3% (w/v) NaCl and high alkaline pH (pH $\geq$ 8) resulted in an increase in the MIC value against *V. harveyi*. However, the rALF*Pm*3 is thermal stable. Either incubation at various temperature (4, 30, 50 and 100^{o}C) for 2 hours and stored at room temperature for three months did not effect the molecule integrity and its anti-*Vibrio* activity.

Neutralization of *V. harveyi* 639 with the rALF*Pm*3 protein resulted in 100% shrimp survival. Study on protective effect of the rALF*Pm*3 showed a reduction of cumulative mortality from 60% to 25% when shrimps were injected with rALF*Pm*3 prior to *V. harveyi* injection. Decreasing of cumulative mortality showed the efficacy and the persistence of passive protection. Our findings described herein confirm the therapeutic potential of the rALF*Pm*3 peptide for prevention and the control of diseases in shrimp aquaculture. Furthermore, the analyses of the pattern of expression of the effectors encoding immune-related genes during the response to infections suggested that rALF*Pm*3 could affect several immune reactions such as proPO system, antimicriobial action, antioxidant defence pathway and apoptosis.

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APPENDICES

Appendix A

Defined medium for *P. pastoris* cultivation (Invitrogen company). In one litre medium contains:

component	Amount
Basal salt medium	
85% H_3PO_4	26.7 ml
$Ca_2SO_4.2H_2O$	1.176 g
K_2SO_4	18.2 g
$MgSO_4.7H_2O$	14.9 g
KOH	4.13 g
*Glycerol	20 g
15% NH_4OH	Adjust to pH 5
water	To 1L
**PTM Trac salts (1L)	
Cupric sulfate.$5H_2O$	6.0 g
Sodium iodide	0.08 g
Manganese sulfate.H_2O	3.0 g
Sodium molybdate.$2H_2O$	0.2 g
Boris acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	20.0 g
Ferrous sulfate.$7H_2O$	65.0 g
Biotin	0.2 g
Sulfuric acid	5.0 ml
water	To 1 L

Note: * Methanol was added instead glycerol during the production phase. It was added after the other components were sterilized and mixed.

** Filter sterilized and stored at temperature and added afterwards.

Appendix B

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.

2. **SDS-PAGE**

12 % Seperating gel

Distilled water	3.3	ml
30 % Acrylamide solution	4.0	ml
1.5 M Tris (pH 8.8)	2.5	ml
10% SDS	0.1	ml
10% $(NH_4)_2S_2O_8$	0.1	ml
TEMED	4	µl

5.0 % Stacking gel

Distilled water	2.1	ml
30 % Acrylamide solution	0.5	ml
1.0 M Tris (pH 6.8)	0.38	ml
10% SDS	0.03	ml
10% $(NH_4)_2S_2O_8$	0.03	ml
TEMED	3	µl

3. 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 minutes. in boiling water before loading to the gel.

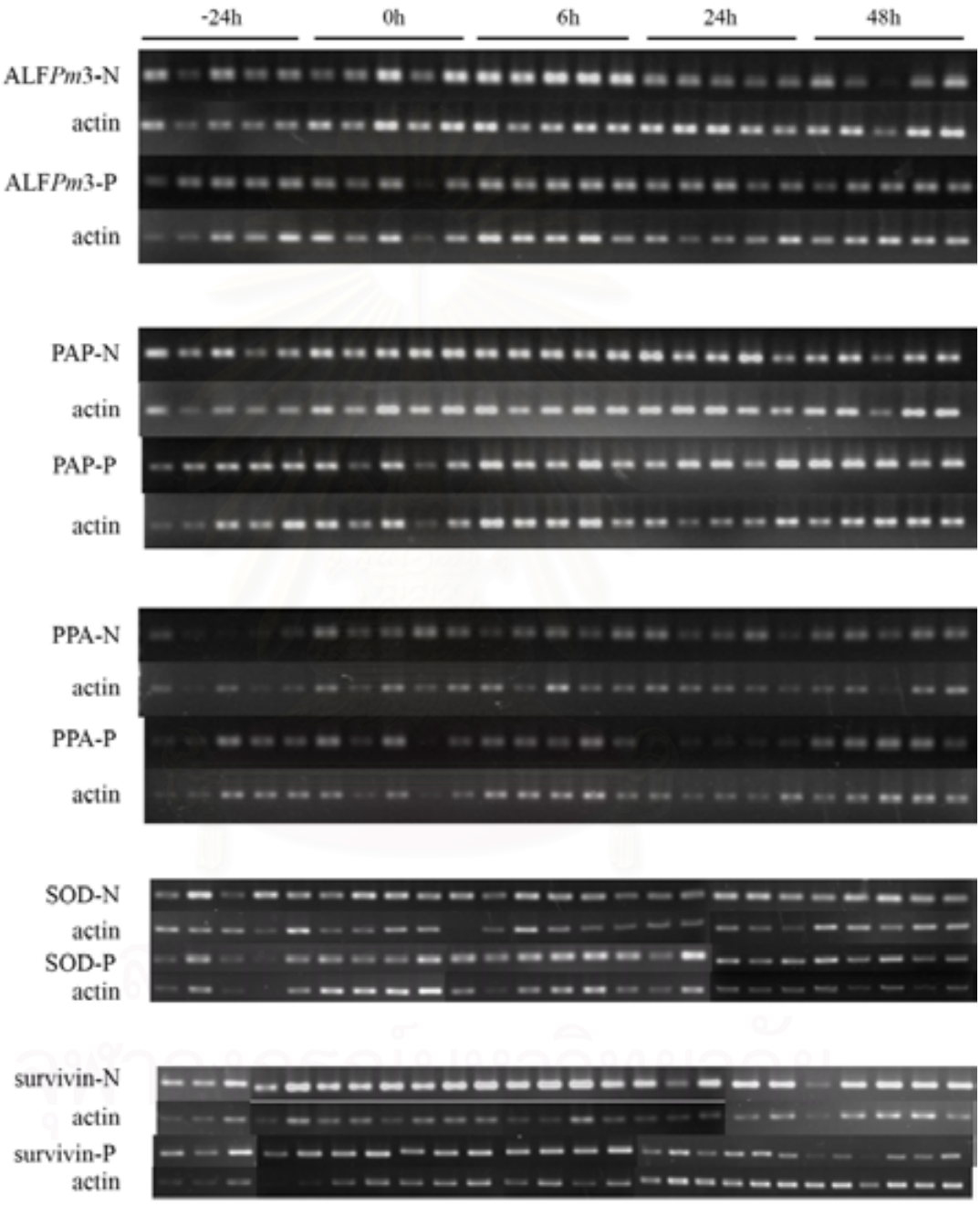
4. Electrophoresis buffer, 1 litre

(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 C



Expression analysis of immune-related genes, ALF*Pm*3, PAP, PPA, SOD and survivin were observed at 0 and 24 h after 0.85% NaCl or rALF*Pm*3 (-24 h) injection and at 6, 24 and 48 h post injection with *V. harveyi*.

Appendix D

I. Anova test of ALF*Pm*3 expression in *V. harveyi* challenged *P. monodon*

ALF*Pm*3

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.747	9	.527	9.084	.000
Within Groups	2.323	40	.058		
Total	7.069	49			

II. Dancan test of ALF*Pm*3 expression in *V. harveyi* challenged *P. monodon*

Duncan

	N	Subset for alpha = .05				
hours	1	2	3	4	5	1
24.10	5	.5460				
48.10	5	.5720	.5720			
.20	5	.7180	.7180	.7180		
48.20	5	.7980	.7980	.7980		
.10	5	.8780	.8780	.8780		
6.20	5		.8980	.8980		
-24.20	5			1.0480	1.0480	
-24.10	5				1.2920	1.2920
6.10	5				1.3040	1.3040
24.20	5					1.5040
Sig.		.057	.061	.058	.120	.197

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.000.

III. Anova test of PAP expression in *V. harveyi* challenged *P. monodon*

PAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.968	9	.219	8.926	.000
Within Groups	.980	40	.024		
Total	2.948	49			

IV. Dancan test of ALF*Pm*3 expression in *V. harveyi* challenged *P. monodon*

Duncan

	N	Subset for alpha = .05				
hours	1	2	3	4	5	1
6.20	5	1.1820				
48.20	5		1.4340			
6.10	5		1.4640	1.4640		
.20	5		1.6080	1.6080	1.6080	
.10	5			1.6580	1.6580	
48.10	5				1.6860	
-24.10	5				1.7200	1.7200
24.20	5				1.7320	1.7320
-24.20	5				1.7760	1.7760
24.10	5					1.9200
Sig.		1.000	.104	.070	.144	.071

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.000.

V. Anova test of PAP expression in *V. harveyi* challenged *P. monodon*.

PPA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.765	9	1.863	38.380	.000
Within Groups	1.941	40	.049		
Total	18.706	49			

VI. Dancan test of PAP expression in *V. harveyi* challenged *P. monodon*

	N	Subset for alpha = .05					
hours	1	2	3	4	5	6	1
24.20	5	.4000					
6.20	5	.6520	.6520				
-24.20	5	.8440	.8440				
6.10	5	.8960	.8960				
48.20	5	.9560	.9560				
24.10	5		1.1360	1.1360			
-24.10	5			1.2760	1.2760		
.10	5				1.5420		
.20	5				1.5420		
48.10	5						2.5760
Sig.		.078	.051	.061	.321	.078	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.000.

VII. Anova test of SOD expression in *V. harveyi* challenged *P. monodon*.

SOD

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.628	9	1.848	61.709	.000
Within Groups	1.198	40	.030		
Total	17.826	49			

VIII. Dancan test of SOD expression in *V. harveyi* challenged *P. monodon*

Duncan

	N	Subset for alpha = .05						
hours	1	2	3	4	5	6	7	1
-24.20	5	.6920						
6.20	5		1.1420					
48.10	5		1.2680	1.2680				
24.20	5			1.4020				
24.10	5				1.7340			
.20	5				1.8460			
6.10	5				1.8760			
-24.10	5					2.1040		
.10	5						2.4220	
48.20	5							2.6940
Sig.		1.000	.256	.228	.229	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.000.

IX. Anova test of survivin expression in *V. harveyi* challenged *P. monodon*

survivin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.953	9	.884	47.312	.000
Within Groups	.747	40	.019		
Total	8.700	49			

X. Dancan test of survivin expression in *V. harveyi* challenged *P. monodon*

Duncan

VAR00001	N	Subset for alpha = .05			
	1	2	3	4	1
6.20	5	.9860			
48.20	5	1.0160			
48.10	5	1.1100	1.1100		
.20	5	1.1320	1.1320		
6.10	5		1.2120		
-24.10	5			1.6000	
-24.20	5			1.6220	
24.10	5			1.6620	
.10	5				1.9960
24.20	5				2.1700
Sig.		.131	.273	.505	.051

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.000.

Biography

Miss Sirikwan Ponprateep was born on July 10, 1981 in Bangkok. She graduated with the degree of Bachelor of Science from the Department of Microbiology, King Mongkut University of Technology Thonburi in 1999. She has studied for the degree of Master of Science at the department of Biochemistry, Chulalongkorn University.