การวิเคราะห์การแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกันในกุ้งกุลาดำ *Penaeus monodon* และศักยภาพในการประยุกต์ใช้คัดเลือกพ่อแม่พันธุ์

นางสาวนริศรา พูลสุข

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EXPRESSION ANALYSIS OF IMMUNE RELATED GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon* AND POTENTIAL APPLICATION IN BROODSTOCK SELECTION

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Thesis Title	EXPRESSION ANALYSIS OF IMMUNE RELATED GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon* AND POTENTIAL APPLICATION IN BROODSTOCK SELECTION
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นริศรา พูลสุข: การวิเคราะห์การแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกันในกุ้งกุลาดำ *Penaeus monodon* และศักยภาพในการประยุกต์ใช้คัดเลือกพ่อแม่พันธุ์. (EXPRESSION ANALYSIS OF IMMUNE RELATED GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon* AND POTENTIAL APPLICATION IN BROODSTOCK SELECTION) อ. ที่ปรึกษา: รศ.ดร.อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม: ดร.ศิราวุธ กลิ่นบุหงา, 196 หน้า. ISBN 974-17-3919-2

ศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกันของกุ้งกุลาดำจำนวน 11 ยีน จากเซลล์เม็ดเลือดของกุ้งกุลาดำที่ติดเชื้อวิบริโอฮาวิอาย และเชื้อไวรัสตัวแดงจุดขาวที่เวลาต่าง ๆ โดยอาศัยเทคนิค semi-quantitative RT-PCR ซึ่งยีนที่ศึกษาในครั้งนี้ประกอบด้วยยีนโปรฟีนอลออกซิเดส แอคติเวติง แฟคเตอร์ (PPAF), ไซโทโซลิก แมงกานีส ซูปเปอร์ออกไซด์ ดิสมิวเทส (SOD), กลูตาไธโอน เปอร์ออกซิเดส (GPx), ทรานสกลูตามิเนส (TG), เวย์ อะซิดิก โปรตีน (WAP), เซอไววิน ตัวยับยั้งอะโพโทซิส (survivin), โปรตีน พี 109 , ไซโคลฟิลิน 18, เปปติดิลโพรลิล ซีสทรานส ไอโซเมอเรส 5 (PPI), ชาเปอร์โรนิน คอนเทนนิ่ง ทีซีพี 1 สับยูนิต 4 (CCTP) และ โปรตีนฮีสช็อค 60 (HSP60) จากการกระตุ้นด้วยเชื้อแบคทีเรียวิบริโอ พบการแสดงออกของยีน PPAF, GPx, TG, WAP และ PPI เพิ่มขึ้นอย่างมีนัยสำคัญ ($p < 0.05$) และการแสดงออกของยีน SOD และ survivin ลดลงอย่างมีนัยสำคัญ ($p < 0.05$) ในขณะที่การกระตุ้นด้วยเชื้อไวรัสตัวแดงจุดขาว พบการแสดงออกของยีน PPAF, WAP และ HSP60 เพิ่มขึ้นอย่างมีนัยสำคัญ ($p < 0.05$) และการแสดงออกของยีน GPx, SOD และ survivin ลดลงอย่างมีนัยสำคัญ ($p < 0.05$) ได้คัดเลือกยีน PPAF และ WAP ซึ่งมีการแสดงออกเพิ่มขึ้นภายหลังการติดเชื้อแบคทีเรียและไวรัส มาศึกษาการแสดงออกด้วยเทคนิค Real-time RT-PCR พบว่าภายหลังการติดเชื้อวิบริโอและเชื้อไวรัสตัวแดงจุดขาว ยีน PPAF มีการแสดงออกเพิ่มขึ้นสูงสุด 4.13 และ 2.53 เท่า การติดเชื้อ 24 ชั่วโมง ขณะที่การแสดงออกของยีน WAP มีการแสดงออกสูงสุด 4.23 และ6.68 เท่า ภายหลังการติดเชื้อที่ 24 ชั่วโมงของทั้งสองเชื้อ ซึ่งยีนทั้งหมด 6 ยีน คือ PPAF, GPx, WAP, TG, PPI และ HSP 60 ที่มีการแสดงออกเพิ่มขึ้นภายหลังการติดเชื้อแบคทีเรีย และไวรัส โดยยีนเหล่านี้น่าจะมีบทบาทสำคัญในการป้องกันกุ้งกุลาดำจากการบุกรุกของเชื้อโรค

ในการศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกัน 6 ยีน ในกุ้งวัยอ่อนระยะแรก (นอเพลียส III, ซูเอีย II และ ไมซิส II), ระยะโพสลาวา (โพสลาวา 5 และ โพสลาวา 15) และกุ้งระยะ 1 เดือน (จูเวนไนล์) โดยใช้เทคนิค semi-quantitative RT-PCR พบว่ายีน PPAF, GPx, TG, PPI และ HSP60 มีการแสดงออกตั้งแต่ระยะนอเพลียส III ขณะที่ยีน WAP เริ่มพบการแสดงออกในระยะซูเอีย และที่น่าสนใจคือระดับการแสดงออกของยีนเหล่านี้ในระยะโพสลาวามีการแสดงออกสูงกว่าในระยะตัวอ่อนระยะแรก จากการศึกษาข้างต้นจึงได้เปรียบเทียบระดับการแสดงออกของยีน 6 ยีนที่ได้รับการคัดเลือกในกุ้งที่ได้จากฟาร์มที่มีการผลิตกุ้งเพื่อการค้าจากจังหวัดสุพรรณบุรี และ ฉะเชิงเทรา กับกุ้งที่ได้จากโครงการปรับปรุงพันธุ์ซึ่งเป็นกุ้งที่มีการตรวจสอบว่าปลอดเชื้อโรคจากนครศรีธรรมราช พบว่าระดับการแสดงออกของยีน PPAF, GPx, WAP, PPI และ TG ในตัวอย่างกุ้งจากโครงการปรับปรุงพันธุ์มีการแสดงออกสูงกว่ากุ้งที่ผลิตเพื่อการค้า ในขณะที่การแสดงออกของ HSP60 ในกุ้งจากทั้ง 3 ฟาร์มไม่แตกต่างกัน อาจจะกล่าวได้ว่ากุ้งจากโครงการปรับปรุงพันธุ์น่าจะมีสุขภาพดีกว่ากุ้งที่ผลิตเพื่อการค้า จากการศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกัน แสดงให้เห็นว่า ยีน PPAF, WAP, PPI และ TG มีศักยภาพในการใช้เป็นตัวบ่งชี้สุขภาพที่ดีของกุ้งเพื่อใช้คัดเลือกพ่อแม่พันธุ์กุ้งกุลาดำต่อไป

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต นริศรา พูลสุข

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม

##4572333423: MAJOR BIOTECHNOLOGY
KEY WORD: *Penaeus monodon* / BLACK TIGER PRAWN / SEMIQUANTITATIVE RT-PCR / IMMUNE-RELATED GENE / DEVELOPMENTAL STAGE

NARITSARA PULSOOK : EXPRESSION ANALYSIS OF IMMUNE-RELATED GENES IN THE BLACK TIGER SHRIMP *Penaeus monodon* AND POTENTIAL APPLICATION IN BROODSTOCK SELECTION. THESIS ADVISOR : ASSOC. PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR: SIRAWUT KLINBUNGA, Ph.D., 196 p. ISBN 974-17-3919-2.

The semi-quantitative RT-PCR technique was used to examine the expression levels of 11 immune-related genes from hemocytes of the black tiger shrimp in response to *Vibrio harveyi* and WSSV challenges at different time courses after injection. These defense-related genes obtained from the *Penaeus monodon* EST libraries, consist of prophenoloxidase activating factor (PPAF), cytosolic manganese superoxide dismutase precursor (SOD), glutathione peroxidase (GPx), transglutaminase (TG), whey acidic protein (WAP), apoptosis inhibitor survivin (survivin), P 109 protein, cyclophilin 18, peptidyl-prolyl cis-trans isomerase 5 (PPI), chaperonin containing t-complex polypeptide-1(CCTP) and heat shock protein 60 (HSP60). The mRNA expression in response to bacterial challenge during the time points of injection showed significant increase ($p<0.05$) of PPAF, GPx, TG, WAP and PPI however, the significant decrease ($p<0.05$) of SOD and surviving expression were observed. Upon the viral challenge, the expression levels of PPAF, WAP and HSP60 were significantly increased ($p<0.05$), whereas those of GPx, SOD and survivin were significantly decreased. Interestingly, both pathogens caused up-regulation of PPAF and WAP genes which were then relatively quantified by Real-time RT-PCR. The results showed that the highest expression level of PPAF gene after *V. harveyi* and WSSV challenges was 4.13 and 2.53 fold at 24 hpi, respectively. Furthermore, the highest expression level of WAP gene after *V. harveyi* and WSSV challenges was 4.23 and 6.68 fold at 24 hpi, respectively. The mRNA levels of PPAF, GPx, WAP, TG, PPI and HSP 60 were up-regulated after bacterial or viral challenge, indicating the probability of those genes in host defense against invading pathogens.

In addition, the expression of the 6 genes were determined in the early-larval stages (Nauplius III, Zoea II, Mysis II), post-larvae (PL5 and 15) and juvenile (1 month) of the shrimp *P. monodon*. RT-PCR analysis showed that transcripts of PPAF, GPx, TG, PPI and HSP60 were already present in the early stage of Nauplius III, whereas WAP mRNA were observed in the Zoea II and later through developmental stages tested. Interestingly, the expression levels of almost genes in post-larval stages were higher than in early-larval stage. The expression levels of 6 immune-related genes were examined by a semi-quantitative RT-PCR from hemocytes of juvenile *P. monodon* from 3 different shrimp farms: commercial farms (Suphunburi, Chachoengsao) and a domesticated farm (Nakhonsithammarat, specific-pathogen-free (SPF) shrimp). The results showed that the transcription levels of PPAF, GPx, WAP, PPI and TG were significantly higher ($p < 0.05$) in the domesticated *P. monodon* than those from the commercial farms, whereas that of HSP60 was not significant difference. Consequently, it was suggested that, the domesticated *P. monodon* might be healthy than commercial shrimp farms based on the level of immune-related genes. From expression analysis some immune related genes such as PPAF, WAP, PPI and TG can be candidate biomarkers for health monitoring and have potential used for broodstock selection.

Field of study Biotechnology Student's signature Naritsara Pulsook

Academic year 2005 Advisor's signature A. Tassanakajon

Co-advisor's signature S. Klinbunga

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Now, I would like to devote the final place here to my beloved family for being such a great support and the forever source of love and care to me. You are everything of mine. Thank you of being you.

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bp	base pair
^{o}C	degree Celcius
CCTP	chaperonin containing TCP1 subunit 4
SOD	cytosolic manganese superoxide dismutase precursor
DEPC	diethylpyrocarbonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EF	elongation factor-1-alpha gene
EtBr	ethidium bromide
GPx	glutathione peroxidase
HSP60	heat shock protein 60
hpi	hour-post injection
LPS	lipopolysaccharide
M	molar
ml	millilitre
MT	metric ton
$MgCl_2$	magnesium chloride
mg	milligram
mM	millimolar
ng	nanogram
O.D.	optical density
ORF	open reading frame
PPI	peptidyl-prolyl cis-trans isomerase 5 precursor
PCR	polymerase chain reaction
pfu	plaque forming unit
proPO	prophenoloxidase
PPAF	prophenoloxidase activating factor

RNA	ribonucleic acid
RT	reverse transcription
survivin	apoptosis inhibitor survivin
TG	transglutaminase
WAP	WAP four-disulfide core domain protein 5 precursor
WSSV	white spot syndrome virus
μg	microgram
μl	microlitre
μM	micromolar

CHAPTER I
INTRODUCTION

1.1 General introduction

Shrimps are one of the most economically important aquatic species in culture today. Due to their high world-wide demand and a gradually-experienced effort in the development of production technologies, they become very important export products for many countries along the Indo-Pacific coast. In Thailand, the shrimp species mainly produced is the black tiger shrimp, *Penaeus monodon*.

Thai shrimp farming started in the early 1980s and really began to expand in the mid 1980s. Since the early 1990s, Thailand has been the world leader for exporting shrimp products in terms of frozen and value-added products into several countries, e.g. Japan, USA and the European Union. The industry has been worth approximately 200,000-250,000 metric tons annually providing an income of nearly 60,000 million baht yearly export earnings for the country (Figure 1.1) (Source: Thai Customs Department cited in Shrimp Culture Newsletter).

Until 2001, the once successful shrimp farming industry has been seriously affected by many dramatically fuctor for example, the outbreaks of bacterial and viral diseases, the water quality problem, and the very rare high-quality broodstock.

Consequently, the shrimp farming has been switched to the other species namely, the Pacific White Shrimp (*Litopenaeus vannamei*) (Figure 1.2). Because it is a genetically-improved stain, *L. vannamei* contains very great advantages over the *P. monodon* including rapid growth rate, high stocking density tolerance, low salinities and temperatures tolerance, lower protein requirements (and therefore production costs), certain disease resistance (if specific-pathogen-free stocks are used), and high survival rate during larval rearing (50-60% comparing to 20–30% for *P. monodon*). As an alien species for Thailand, *L. vannamei* possibly acts as a carrier of various new pathogens to the culture areas, and a very important reason is the broodstocks must be imported from the stain stocking institute, mainly from the Hawaii Marine Institute. From the above reasons, shrimp farming of the native rather than the alien shrimp species should be considered as essential for this area. Although overall biological

systems of *P. monodon* are progressively studied at the molecular level, the knowledge of the immune system is also considered to intensive study in order to characterize how shrimp response during infected by the pathogens. The information will be applied to the selective breeding of healthy shrimp for the near future of shrimp production industry of *P.monodon* in Thailand.

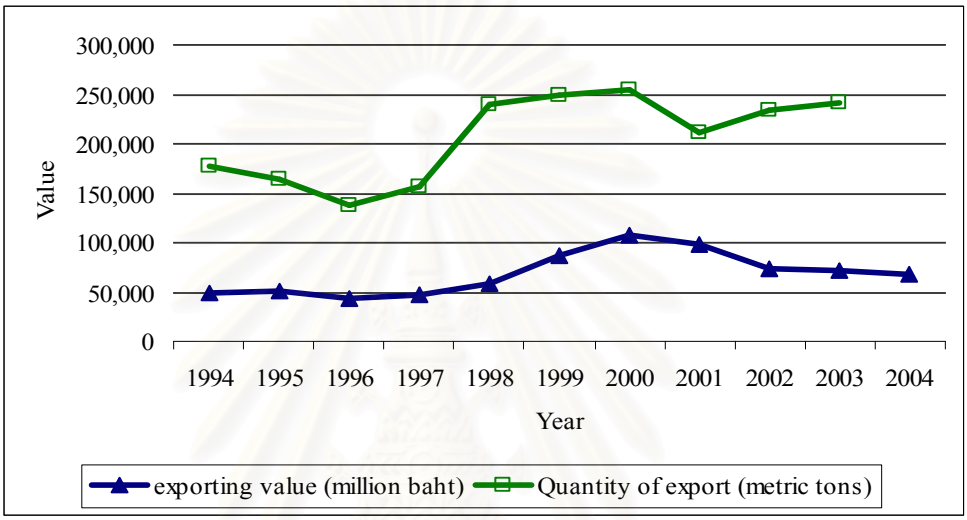

Figure 1.1 Cultured shrimp production in Thailand from 1994 to 2004 (Source: Thai Customs Department cited in Shrimp Culture Newsletter)

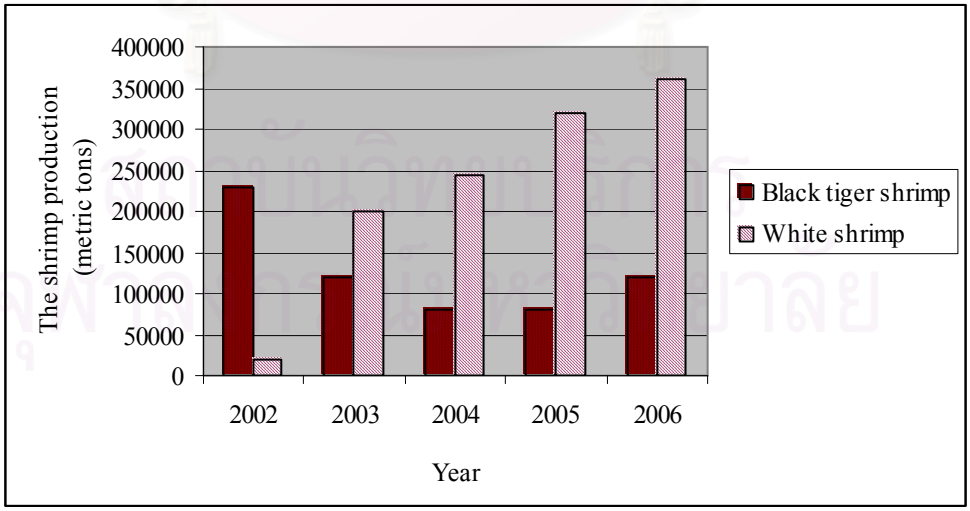

Figure 1.2 The black tiger shrimp and white shrimp production in Thailand from 2002 to 2004 and the predicted values for 2005-2006 (Source: http://www.shrimpcenter.com)

1.2 Taxonomy of *Penaeus monodon*

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

Phylum Arthropoda
Subphylum Crustacea
Class Malacostraca
Subclass Eumalacostraca
Order Decapoda
Suborder Natantia
Infraorder Penaeidea
Superfamily Penaeoidea
Family 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).

FAO Names: Giant tiger prawn, Crevette giante tiger, Camaron tiger gigante.

1.3 Distribution

The black tiger shrimp is principally distributed in the major part of the Indo-West Pacific region; the East and Southeast Africa, the Red Sea and Arabian Gulf, the Indian subcontinent, and throughout the Malaysian Archipelago to Northern Australia and Japan. It is a marine species that inhabits in mud or sand bottoms at all depths from shallows to around 110 meters (360 feet), so it can be caught from offshore or

and inshore as well as the tidal zones. This species is one of the most important aquaculture species in Asia (Rosenberry, 1997).

1.4 Morphology

From the external view, shrimp is basically divided into thorax and abdomen (Figure 1.3). The thorax (or head) is covered by a single, immobile carapace which protects internal organs and supports muscle origins. The eyestalks and eyes, the sensory antennules and the antennae arise rostrally. The pereiopods or walking legs are the thoracic appendages. Gills are formed from sac-like outgrowths of the base of the walking legs and sit in branchial chambers on ether side of the thorax. The carapace extends laterally to cover the gills completely.

The abdomen has the obvious segmentation of invertebrates. A swimming legs or pleopods are the abdominal appendages. A pair of pleopods arises from each of the G abdominal segments. A tail fan comprises of a telson which bears the anus, and two uropods attach to the last (6^{th}) abdominal segment. The telson has a 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 shrimp (Anderson, 1993).

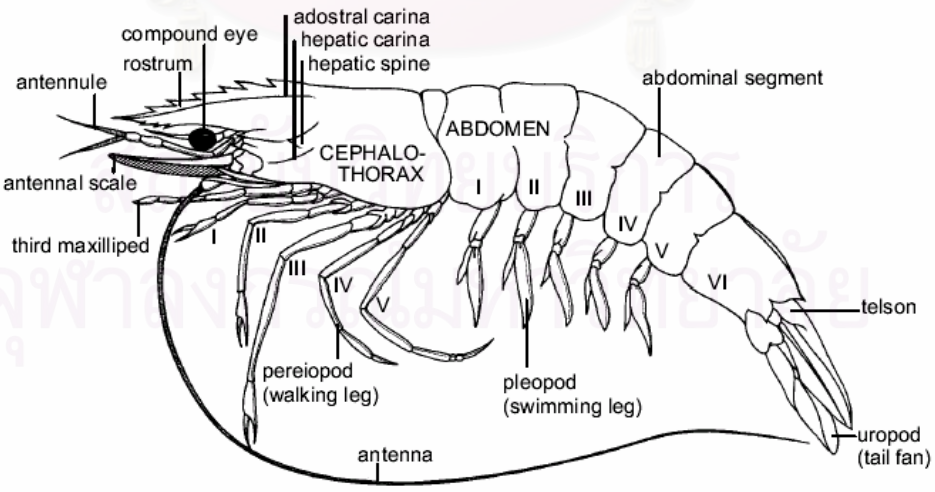

Figure 1.3 Lateral view of the external morphology of *Penaeus monodon* (Primavera, 1990).

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

The black tiger shrimp has the following characteristic colorations: 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 brackish waters or cultured ponds, the color patterns are mostly changed to dark and blackish brown (Moton, 1981:cited in Solis, 1988).

1.5 Life cycle

The matured female spawns between 50,000-1,000,000 eggs per spawning (Rosenberry, 1997). The fertilized egg of *P. monodon* is spherical yellowish green in color and somewhat translucent, ranging from 0.27-0.31 mm in diameter. The development of 2-celled, 4-celled, morula and embryonic nauplius stages are observed within 0.5, 1, 1.8 and 11 hours after spawning, respectively. Before hatching, the embryonic nauplius is observed to move intermittently inside the egg.

1.5.1 Larvae

After fertilization, eggs hatch into the first larval stage, nauplius (6 stages in 2 days). Nauplii are about 0.3 mm long at hatching, exist entirely on their own egg yolk and are characterized as planktonic and positively phototoxic. The second stage, protozoeae (3 stages in 5 days), they begin to feed algae and metamorphose into myses (3 stages in 4-5 days). The myses have many characteristics of adult shrimp, feeding on algae and zooplankton. The final stage is the post larvae (6-35 days) (Solis, 1988), while a full complement of functioning appendages is present.

Occurring offshore, they are planktonic in behavior. The body of the post-larvae is transparent with a dark brown streak from the tip of the antennular flagellum to the tip of the telson. The 6th abdominal segment is relatively longer than the

carapace length. The carapace length of the post-larvae varies between 1.2 and 2.2 mm. *P. monodon* enters nursery grounds during the last sub-stage of the post-larvae.

1.5.2 Juvenile

During the earlier juvenile stage, the body is partly transparent with a dark brown streak on the ventral side similar to the post larvae. They differ from the post larvae as follows: relatively shorter 6th abdominal segment to the carapace length is still greater than in the adolescent, greater body and complete gill system.

In the early stage reaching about 2.7 mm in carapace length (CL), the body becomes blackish in color and the rostrum has 6 dorsal and 2 ventral spines. When it reaches about 3.7 mm CL, the body becomes more blackish and bulky and the rostrum has 7 dorsal and 3 ventral spines as found in adult. The carapace length varies from 2.2 to 11.0 mm. They use pereiopods for clawing and pleopods for swimming, the former become the main locomotive organ and the latter may be regarded as supplementary and used for rapid movement. Juveniles inhabit blackish water areas as nursery ground.

In natural, if shrimp have stability of body proportion and development of outer genitalia is called adolescent. The adolescent, the body proportion is almost the same as in the adult or slightly greater with the ratio of the length of about 0.58. The sexes can now be identified beginning at 11 mm CL. The carapace length of the adolescent varies between 11 to 34 mm. The minimum size of males possessing a jointed petasma is about 30 mm CL and the minimum size of females possessing adult-like thelycum is about 37 mm CL.

1.5.3 Subadult

This stage begins at the onset of sexual maturity i.e., minimum sized males possessing spermatozoa in terminal ampoules and minimum sized females possessing spermatozoa inside the thelycum through copulation.

A sex-size disparity occurs at almost 30 mm CL, and here after the size of females becomes greater than males. They migrate from nursery to spawning ground. During this stage, first copulation takes place between males with minimum CL of 37

mm and females of 47 mm in the estuarine or inner littoral areas before migrating to the deeper water.

1.5.4 Adult

This stage is characterized by the completion of sexual maturity males possess spermatozoa in the paired terminal ampoules, and in fact there are no sexual differences from subadult males apart from size increment and different habitat. Females start to spawn mostly offshore whereas some spawn in shallow water. A second and other copulation may occur in majority of individuals. Their major habitat is the offshore areas at depths of about 160 m.

The maximum size of males recorded is 71mm CL, whereas the maximum recorded length of females is 81 mm CL, reaching 270 mm in body length or 260 g in weight. Carapace length varies between 37 and 71 mm in males and 47 and 81 mm in females.

The life history phases of *P. monodon* are summarized in table 1.1 **(**Motoh H, 1981**)**, and the diagram of its life history is shown in Figure 1.4.

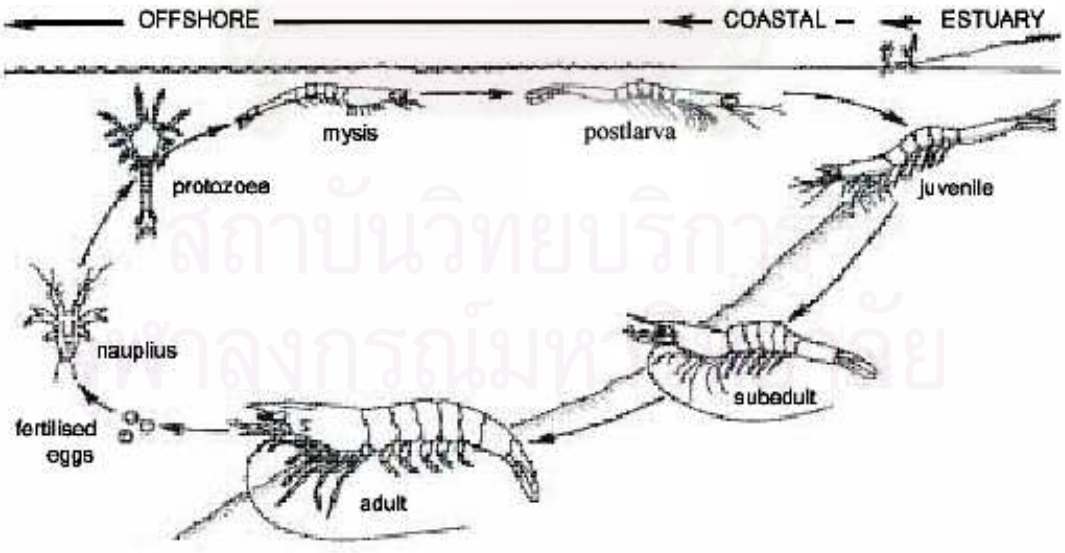

Figure 1.4 Diagrammatic representation of the life history of Penaeus *monodon* shrimp (Bailey-Brock and Mass,1992).

Table1.1 Life history phases of *P.monodon.*

Phase	Begin at	Duration
Embryo	Fertilization	12 hours
Larvae * nauplii * protozoeae * mysis * post-larvae	Hatching	20 days * 2 days * 5 days * 2-5 days * 6-35 days
Juvenile	Completion of gill system Stability of body proportion, development of outer genitalia	4-5 months
Subadult	Commencement of sex maturity , first copulation	4 months
Adult	Completion of sexual maturity	10 months

1.6 Shrimp diseases

Bacterial and viral diseases are known to be the major constraint for in the further progress of semi-intensive and intensive shrimp cultures throughout the world. The major disease outbreaks owing to both *Vibrio* spp. and viruses, have been detrimental to the industry in recent years.

Viral disease outbreaks often result from stress factors, such as overcrowding, abnormal temperatures or low dissolved oxygen. Most bacterial infections result from extreme stress. The most common bacterial infection in marine shrimp is *Vibrio. Vibrio* infections often occur following environmental stresses or viral diseases and are not the primary disease problem (Nash, 1990).

1.6.1 Viral disease

Recently, five direct viral pathogens are ordered descending from the greatest to the least economic impact to cultivated *P. monodon* in Thailand including, white spot syndrome virus (WSSV), yellow head virus (YHV), hepatopancreatic parvovirus (HPV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and monodon baculovirus (MBV). *P. monodon* have high mortality rate when infected with WSSV and YHV (Kiatpathomchai et al., 2004; Wongteerasupaya et al., 2003).

White spot syndrome (WSS)

White spot syndrome virus (WSSV) first appeared in northeast Asia in 1992-93, and rapidly spread to several shrimp farming countries in Asia and the Indo-Pacific. This is a lethal disease (90-100%, mortality) with potential to infect other crustacean species. WSSV is a pathogen on crustaceans with a wide host range. It can infect both freshwater and marine species, for instance shrimp, crab and crayfish (Chou et al., 1995; Lo et al., 1996; Wang et al., 1998). This viral infection has been observed in several commercial penaeid species, including *Penaeus monodon*, *Marsupenaeus japonicus*, *Penaeus chinensis*, *Penaeus indicus*, *Penaeus merguiensis*, *Litopenaeus vannemei*, *Penaeus stylirostris*, *Penaeus penicillatus*, and *Litopenaeus setiferus*. The virus has been given various names, including baculoviral hypodermal and haematopoietic necrosis virus (Huang et al., 1995), rod shaped virus of *M.*

japonicus (Takahashi et al., 1996), systemic ectodermal and mesodermal baculovirus (Wongteerasupaya et al., 1995; Sahul Hameed et al., 1998) and white spot baculovirus (WSBV) (Wang et al., 1995; Lo et al., 1996). These viruses have been collectively referred to as WSSV (Nunan and Lightner, 1997).

The virions of WSSV are enveloped and have an ovoid to bacilliform shape with a tail-like appendage at one end. The size of virions is approximately 250-380 nm in length and 70-150 nm in width. The virions enclose a rod shape nucleocapsid, which contains a DNA-protein core bounded by a distinctive capsid layer giving it a cross-hatched appearance. The size of nucleocapsid is 200-250 nm in length and 65-70 nm in diameter (Wongteerasupaya et al., 1995; Duran et al., 1997). WSSV is a large double-stranded DNA virus, which contains a genome of about 293 kb (Yang et al., 1997; van Hulten et al., 2001). Extensive characterization of WSSV genome is suggested to be a member of a new virus family,the *Nimaviridae*, and to the genus *Whispoviridae* (van Hulten et al., 2000; Tsai et al., 2000).

In shrimp, the disease caused by WSSV is characterized by the presence of white spots of about 5 mm on the cuticle and sometimes is accompanied with a reddish coloration on the body. Lethargy, a rapid reduction in food consumption; infected shrimp, swim slowly near the pond surface, and eventually sink to the bottom and die; high mortality rates (100%) occur 3 to 10 days after the first signs of this disease (Karunasasagas et al., 1997). Histopatology indicates that infection of WSSV is not organ or tissue specific. Infection of WSSV can be observed in several tissues; however, it does not infect hepatopancreatic epithelial cells or midgut epithelial cells even in moribund shrimp, which are considered to be refractory tissues for WSSV infection (Chang et al., 1996; Wang et al., 1999). The mechanism of infection and spread of WSSV in crustacean hosts is not clear. It is believed that the envelop protein of virus play important roles in virus infection (WU et al., 2005; Zhang et al., 2004). However, the experimental transmission of WSSV indicates that the white spot disease could be transferred by cohabitation with or ingestion of WSSV-infected animals (Kanchanaphum et al., 1998; Supamattaya et al., 1998). WSSV can be detected in early larvae stages of *P. monodon*; however, significant mortality was observed in post-larvae and juveniles shrimp. (Yoganandhan et al., 2003).

Chou et al (1995), exposed *P. japonicus* to WSSV by immersing the shrimp in the epidermal filtrate of diseased shrimp; the mortality reached 100% within 4–7 days. Chang et al (1996) reported that during a WSSV infection, WSSV-positive cells occur initially (16 h after oral infection) in the stomach, gill, cuticular epidermis and hepatopancreas, and spread rapidly to other organs. By 52–62 h most organs became heavily infected and necrotic, and shrimp died concurrently.

Detection of virus at an early stage is necessary to reduce damage from WSSV infection. Several diagnostic method have been described such as PCR (Tapay et al., 1999), in situ hybridization (Wang et al., 1998), observation of tissues subjected to fixation or negative staining (Inouye et al., 1994) and reverse passive latex agglutination (RPLA) method (Okumura et al., 2005)

To protect shrimp or other crustaceans against WSSV, the WSSV subunit vaccine, the WSSV envelop protein VP19 and VP28 was evaluated. The VP19 and VP28 fused to MBP showed significantly better survival than the control group (Witteveldt et al., in press).

Yellow-head (YH) disease

In Thailand the disease is called Hua leung (Chantanachooklin et al. 1993, Lightner, 1996). It has significant losses of cultured shrimp *P. monodon* throughout Asia (Chantanachooklin et al., 1993; Flegel, 1997). Extensive characterization of Yellow-head virus (YHV) genome (Sittidilokratna et al., 2002; Cowly and Walker, 2002; Jitrapakdee et al., 2003) has clearly shown that this virus is classified in a new genus *Okavirus* and family *Roniviridae* in the order *Nidovirales* (Mayo, 2002).

Yellow-head principally infects pond-reared black tiger shrimp *P. monodon*. This syndrome occurs in the juvenile to sub-adult stages of shrimp 5 to 15 grams in size, especially at 50-70 days of grow-out. It causes serious disease in juvenile stages (Lightner, 1996). At the onset of YHV shrimp have been observed consuming feed at an abnormally high rate for several days. Feeding abrubtly ceases and within 1 day, a few moribund shrimp appear swimming slowly near the surface at the pond edges. Affected shrimp exhibit light yellow coloration of the cephalothorax area and a generally pale or bleached appearance; they die within a few hours. By the following day, the number of similarly affected shrimp increases dramatically, and by

the third day after the first appearance of moribund shrimp, the entire crop is typically lost (Lightner, 1996, Chantanachooklin et al., 1993). Moribund shrimp with YHD generally appear pallid in color, with a yellowish, often swollen cephalothorax. Infected shrimp frequently exhibit whitish or pale yellowish to brown gills, and often a pale yellow hepatopancreas (Lightner, 1996).

Several diagnostic method to detect YHV infected shrimp, antibody (Lu et al., 1996; Nadala and Loh, 2000; Sithigorngul et al., 2000, 2002; Soowannayan et al., 2003), in situ hybridization (ISH) (Tang and Lightner, 1999; Tang et al., 2002; Spann et al., 2003), et al., conventional RT-PCR (Wongteerasupaya 1997; Tang and Lightner, 1999; Cowley et al., 1999, 2000) and real-time RT-PCR (Dhar et al., 2002). Of the antibody-based diagnostic techniques, immuno-histochemistry using monoclonal antibodies to a surface glycoprotein and the nucleocapsid protein of YHV (Sithigorngul et al., 2000, 2002) has recently allowed the detection and distinction of YHV infections (Soowannayan et al., 2003).

To neutralize virus, the mouse polyclonal antiserum raised against the YHV gp116 or gp64 structural glycoproteins could neutralize YHV infectivity as determined using an in vitro quantal assay in primary cultures of lymphoid organ cells was investigated. Anti-gp116 antiserum showed virus-neutralizing activity whereas anti-gp64 antiserum failed to inhibit infection. The results suggest that gpl16 antiserum blocks binding of virions to cellular receptors to facilitate YHV entry into lymphoid organ cells. (Assavalapsakul et al., 2005).

1.6.2 Bacterial disease

Species of *Vibrio* are commomly found in aquatic environments as the bacterial flora and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). The virulence of this species has been recognized in a small but expanding list of penaeids cultured in Asia and Australia (Vandenberghe et al., 1998). *Vibrio* spp., especially the luminous *Vibrio harveyi*, has been implicated as the main bacterial pathogens of shrimps (Baticados et al., 1990).

V. harveyi, is considered as the most devasting that causes extreme losses of cultured *P. monodon* in hatcheries and shrimp farms. This bacterial outbreak causes

mortality of the affected shrimps up to 100%, whether they are larvae, post-larvae, juveniles, sub-adults or adults (Lightner, 1983).

V. harveyi is a rod shape, Gram-negative bacterium with 0.5-0.8 μm in width and 1.4-2.6 μm in length. This bacterium is able to emit a blue-green color light. Presumptive diagnosis is made on the basis of clinical signs and culture of the suspensions of hepatopancreas or blood on trytic plate supplemented with 2% (w/v) NaCl. After incubation at 30°C overnight, colonies of *V. harveyi* show strong luminescence in dim light.

The gross signs of localized infection in the cuticle or sub-cuticle also give the names shell disease or black/brown spot disease. These superficial infections can develop into systemic infections under some circumstances and can cause mortality. Other gross features of the infected shrimps are the milky white body and appendages, weakness, disoriented swimming, lethargy and loss of appetite. Eventually it leads to death.

Antibiotics have been used in attempts to control these bacteria, but it has lead to the problems of drug resistance. The development and use of probiotics, a marine bacterial strain *Pseudomonas* I-2 can produced a compound with inhibitory property against shrimp pathogenic *Vibrios,* which is used for control shrimp pathogenic Vibrios in aquaculture system (Chythanya et al., 2001). The *in vitro* and *in vivo* antagonistic effect of *Bacillus subtilis* BT23 against the pathogenic *Vibrios* was studied (Vaseeharan et al., 2003). Cell free extracts of *Bacillus subtilis* BT23 showed greater inhibitory effect against the growth of *V. harveyi* isolated by agar antagonism assay from *P. monodon* with black gill disease. The Marine bacterium *Pseudoalteromonas* species strain X153 isolated from a pebble collected at St. Anne du Portzic (France) was purified and partial identified. This bacterium protected bivalve larvae against mortality, following experimental challenges with ichthyopathogenic *Vibrio*. *Pseudoalteromonas* sp. X153 may be useful in aquaculture as a probiotic bacterium (Longeon et al., 2005).

1.7 The invertebrate immune system

Evolution of the immune system can be classified into two types adaptive (acquired) immunity and innate (natural) immunity. Vertebrates possess both adaptive and innate immune system, whereas invertebrates involve only in innate immunity.

Consequently, invertebrates survive in a pathogen-laden environment without an adaptive immune system. Innate immune responses are characterized by their rapidity, which the first line of defense that helps to limit infection at an early stage and relies on germ line encoded receptors that recognise conserved molecular patterns present on microorganisms (Janeway, 1998). However they do not improve upon repeated stimulation. It has no "memory". In contrast, the adaptive immune system has developed more sophisticated and complicated mechanisms. It containing T and B cells, does have memory. The antibodies made by B cells the second time around to coat and kill the microbial or foreign substances are more effective than the antibody made in the initial response. The T cells fighting the second time will also generally be more effective.

In invertebrates, these innate immune systems are very effective. It is including haemolymph coagulation, complement activation, cell agglutination, the use of RNA interference (RNAi), pattern-recognition receptor (PRRs), hydrolytic enzymes, antimicrobial peptides (AMPs), phagocytotic cells, production of active oxygen and nitrogen metabolites, and melanization pathways. Their defense systems are activated by the recognition of common epitopes on the surface of pathogens, such as bacterial lipopolysaccharide (LPS), peptidoglycan and β-1, 3-glucan (Figure1.5).

1.8 The crustacean immune system

The major defense systems of crustaceans are an innate immune response, which based on humoral and cellular components of the circulatory system. After pathogen infection, the recognition molecules may interact with and activate the hemocytes. Haemocytes are the effectors of the cellular immune response and they are also involved in the synthesis of the majority of humoral effectors (Figure 1.5). The actions with direct participation of blood cells are understood by the term cellular response as demonstrated in phagocytosis, encapsulation, cell-mediated cytotoxicity

and clotting. Whereas, the humoral factors comprise molecules that act in the defense without direct involvement of cells, although many of the factors are originally synthesized and stored in the blood cells such as clotting proteins, agglutinins (e.g. lectins), hydrolytic enzymes and antimicrobial peptides.

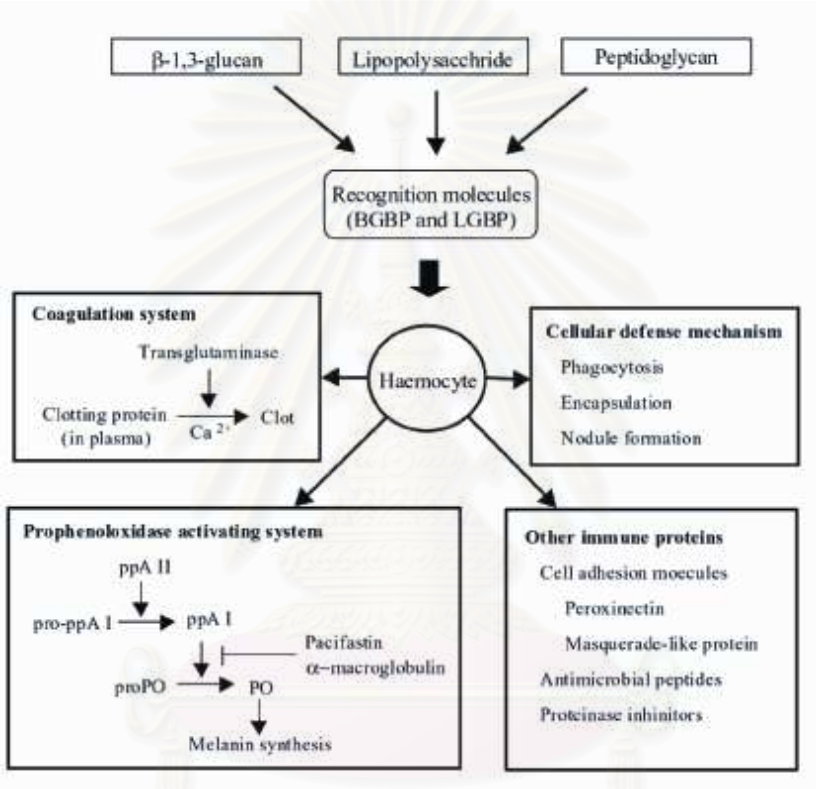

Figure 1.5 Schematic overview of crayfish defense reactions.

1.8.1 Blood cells

In general, crustacean circulating haemocytes are important in immune system, which morphologically can be grouped into three subpopulations: hyaline cells, semigranular cells, and granular cells. Indeed, they are also described in penaeid shrimp and freshwater crayfish.

Hyaline cells are the smallest of the hemocytes, lack cytoplasmic granules, and compose only 5-10% of the circulating hemocytes, which involved in phagocytosis (Söderhäll et al., 1986) and coagulation (Omori er al., 1986)

Semigranular cells are the most abundant type of hemocyte (75% of all haemocytes) and contain a variable number (1-40) of small (S) granules (0.4 μm diameter), which display some phagocytic capacities, would be specialized in particle encapsulation. Semigranular cells appear to be the most sensitive ones and react first during an immune response, by degranulation.

Granular cells compose 10-20% of the haemocytes and contain a large number of secretory large (L) granules (0.8 μm diameter). Both granular cells and semigranular cells store the components of prophenoloxidase activating system and are capable of cytotoxic reaction, which is an important component of the cellular defense reaction. (Smith and Söderhäll 1983b; Söderhäll et al., 1985).

1.8.2 Pattern recognition proteins

When the foreign substances attack animals, the first immune process is the recognition of a broad spectrum of factors that are released or are present on the surface of invading microorganisms, which is mediated by the haemocytes and by plasmatic protein. There is little knowledge about the molecular mechanisms that mediate recognition. However, in crustaceans, several types of modulator proteins have been described that recognize cell wall components of pathogens. The target recognition of innate immunity is the so-called “pattern recognition molecules (PRMs)” shared among groups of pathogens. Host organisms have developed the response to these PRMs by a set of receptors referred to as “pattern recognition proteins or receptors (PRPs or PRRs)”. These patterns include the lipopolysaccharides (LPS) of Gram negative bacteria, the glycolipids of mycobacteria, the lipoteichoic acids of Gram positive bacteria, the mannans of yeasts, the β–1,3-glucan of fungi and double-stranded RNA of viruses (Hoffmann et al., 1999). Most current research has emphasized the possible role of non-self recognition molecules in the vertebrate and the invertebrate immune system.

Recognition of carbohydrates is important because they are common constituents of microbial cell wall and have structures that are distinct from those of

carbohydrates of eukaryotic cells. Therefore, LPS or/and β-1,3-glucan binding proteins (LBP, βGBP, or LGBP), peptidoglycan recognition protein (PGRP), several kinds of lectins, and haemolin have been found in a variety of invertebrates and different biological functions have been proposed for these molecules following their binding to their targets (Lee et al., 2002).

In shrimp, the LPS-binding protein has been reported as a multivalent carbohydrate-binding agglutinin that, besides its bacterial agglutination ability, increases the phagocytic rate (Vargas-Albores, 1995). A second protein involved in the recognition of microbial products and the activation of cellular functions is the β-glucan binding protein. It is apparently monovalent and dose not induce agglutination, but activates degranulation and the proPO system. Thus, these recognition proteins are capable of activating cellular activities only after reaction with the microbial carbohydrates (LPS peptidogycan or glucan) (Vargas-Albores et al., 2000). Roux et al. (2002) proposed that shrimp LGBP is an inducible acute-phase protein that may play a critical role in shrimp WSSV interaction and that the WSSV infection regulated the activation and/or activity of the proPO cascade in a novel way.

1.8.3 Cell-mediated defence reactions

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.

1.8.4 The prophenoloxidase (proPO) system

The proPO activating system consists of several proteins involved in leading to melanin production, cell adhesion, encapsulation, and phagocytosis (Söderhäll et al., 1998; Sritunyalucksana and Söderhäll., 2000).

In vitro studies have shown that phenoloxidase (PO) exists as an inactive precursor, prophenoloxidase (proPO), which is activated by a stepwise process involving serine proteases activated by microbial cell wall components, such as low quantities of lipopolysaccharides or peptidoglycans from bacteria and β-1,3-glucans from fungi, through pattern-recognition proteins (PRPs) (Ariki et al., 2004). An enzyme that is able to activate the proPO *in vivo* is termed prophenoloxidase activating enzyme (factor) (ppA, PPAE, PPAF) (Figure 1.6). In crayfish, ppA is a trypsin-like proteinase present as an inactive form in the haemocyte granules. After degranulation, the enzyme is released together with proPO and becomes an active form in the presence of microbial elicitors. The active ppA will convert proPO to an active form, phenoloxidase (PO) (Aspán and Söderhäll, 1991; Aspán et al., 1995). PO is a copper-containing protein and a key enzyme in melanin synthesis (Söderhäll and Cerenius. 1998; Shiao et al., 2001). It both catalyses o-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones, which can polymerise non-enzymatically to melanin. PO is a sticky protein and can adhere to the surface of parasites, which will lead to melanisation of the pathogen. Melanisation is usually observed by blackening of the parasite in the hemolymph or black spots on the cuticle. The melanin and intermediates in the melanin formation can inhibit growth of microbial parasites, such as the crayfish plague fungus, *Aphanomyces astaci* (Söderhäll and Ajaxon, 1982). The production of forming insoluble melanin deposits involving in the process of sclerotisation, wound healing and encapsulation of foreign materials (Theopold et al., 2004). To prevent excessive activation of the proPO cascade, proteinase inhibitors are needed for its regulation.

The prophenoloxidase activating enzyme (PPA) is a zymogenic protein (proppA). The C-terminal half of the proppA is composed of a typical serine proteinase domain, with a sequence similar to other invertebrate and vertebrate serine proteinases. The N-terminal half contains a cationic glycine-rich domain, a cationic proline-rich domain and a clip-domain, in which the disulfide-bonding pattern is likely to be identical to those of the horseshoe crab big defensin and mammalian β-defensins. The clip-domains in proppAs may function as antibacterial peptides (Wang et al., 2001).

In the penaeid shrimp, enzyme of the proPO system are localized in the semigranular and granular cell (Perazzolo and Barracco, 1997). This is in agreement with a recent study showing that *P.monodon* proPO mRNA is expressed only in the haemocytes (Sritunyalucksana et al., 2000).

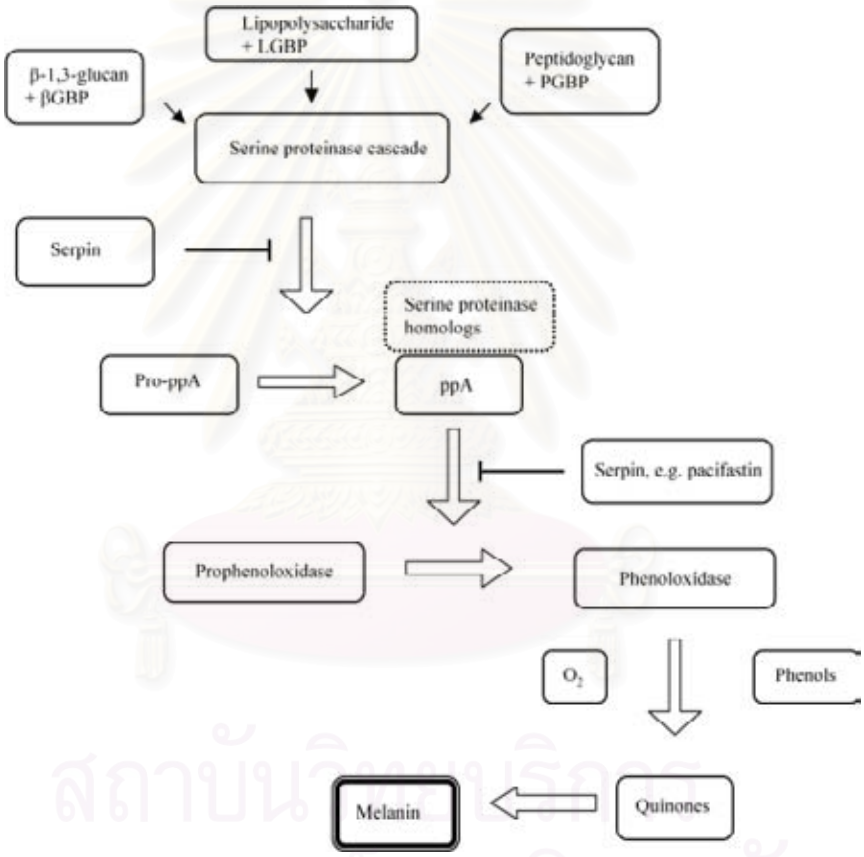

Figure 1.6 Overview of the arthropod prophenoloxidase (proPO)-activating system.

1.8.5 The coagulation system/ the clotting system

Hemolymph coagulation defense response of crustaceans that prevents both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al., 1991). It is a proteolytic cascade and is

activated by microbial cell wall components. The coagulation system involves a plasma clotting protein (CP) and a haemocyte-derived transglutaminase (TG) (Kopácek et al., 1993; Yeh et al., 1998).

The crayfish CP is a dimeric protein, which subunit has both free lysine and glutamine. They are recognized and become covalently linked to each other by TGases. CP synthesized in the hepatopancreas and released to hemolymph. In crustaceans, CP were found in several species; the freshwater crayfish (Kopacek et al., 1993), *P. monodon* (Yeh et al., 1998), and in the lobster, *Panulirus interruptus* (Doolittle and Fuller, 1972).

Transglutaminase (TG) are Ca^{2+}-dependent enzymes capable of forming covalent bonds between the side chains of free lysine and glutamine residues on certain proteins of the clotting protein molecules in the presence of calcium ion to form a soft gel at the wound sites (Wang et al., 2001) during the final stage of coagulation (Huang at al., 2004). In spite of, they have assumed a wide variety of functions during development, differentiation and immune responses or post-translational protein remodeling (Greenberg et al., 1991; Aeschlimann and Paulsson, 1994).

TG sequence of *P.monodon* was similar to crayfish and other vertebrate and invertebrate TG. TG gene, whose sequence is homologous with that of factor XIIIa that involved in coagulation system.

In shrimp, TG is important for blood coagulation and post-translation remodeling of proteins. Synthesized and stored in young haemocytes (hyaline and semigranular cell), TG facilitates the instant release of TG protein and blood clotting following injury (Aono and Mori, 1996; Huang at al., 2004). TG activity was greatest in the hepatopancreas, then the heart, haemocytes and other organs (Huang at al., 2004). It sequence is homologous with that of factor XIIIa that involved in coagulation system. Recently, a shrimp second TG (STG II) was found from the tiger shrimp hemocyte cDNA. It differed from STG I, STG II was characterized as a hemocyte TG that is involved in coagulation (Chen MY et al., 2005).

1.8.6 Reactive oxygen

In animal, the synthesis of reactive oxygen species (ROS) has important roles in inflammation and host defense. Reactive oxygen species (ROS) produced by 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 (Bachère et al., 1995; Muñoz 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, 1993), immunity (Holmbland and Soderhall, 1999; 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-Cordora 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; Ho et al., 1889).

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.

1.8.7 Antimicrobial peptides (AMPs)

Animal peptide antibiotics are defined as anti-microbial agents made by an animal, including humans, with a function that is important for the innate immunity of that animal.

The most of AMPs are small in size, generally less than 150-200 amino acid residues, amphipathic structure and cationic property. However, the anionic peptides also exist. Their small size makes them easy to synthesize without dedicated cells or tissues and they rapidly diffuse to the point of infection. For many of these peptides, there is evidence that one of the targets for the peptide is the lipid bilayer of the membrane. This is because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it

is possible that the peptide induces complete lysis of the organism by rupture of the membrane or that it perturbs the membrane lipid bilayer, which allows for leakage of certain cellular components as well as dissipating the electrical potential of the membrane.

AMPs are active against a large spectrum of microorganisms; bacterial and filamentous fungi. In addition, some AMPs have antiviral or antiparasitic activities (Hancock and Diamond, 2000; Murakami et al., 1991; pan et al., 2000) and may also exhibit an anti-tumor property (Cruciani et al., 1991).

There are few reports on antimicrobial peptides in shrimp. Penaeidins, a new family of antimicrobial peptide which acting against Gram positive bacteria and fungi were reported in penaeid shrimp *L. vannamei* (Destoumieux et al., 1997). cDNA clones of penaeidin isoform were also isolated from the haemocytes of *L. vannamei*, *P. setferus* (Gross et al., 2001) and *P. monodon* (Supungul et al., 2004). Crustins, an antimicrobial peptide were identified from 2 species of *Penaeid* shrimp, *L. vannamei* and *L. setiferus*. Several isoforms of crustins were observed in both shrimp species. Like the 11.5 kDa antibacterial protein from *Carcinus maenas*, crustins from shrimp show no homology with other known antibacterial peptides, but possess sequence identity with a family of proteinase inhibitory proteins, the whey acidic protein (WAP). Peptide derived from the hemocyanin of *L.vannamei, P. stylirostris* and *P. monodon* posses antiviral activity has been also identified (Destoumieux-Garzon et al., 2001; Patat et al., 2004; Zhang et al., 2004). Recently, the histones and histone derived peptides of *L. vannamei* has been reported as an innate immune effector because they can inhibit growth of Gram-positive bacteria (Patat et al., 2004).

1.8.8 Proteinase inhibitor

Proteinase inhibitors, also produced by the haemocytes, are necessary to protect host from microbial proteinase and regulate the proteinase cascades (the proPO and coagulation system).

Proteinases function in many pathogenic fungi to aid in penetrating the cuticle of their arthropod hosts. Proteinases also contribute to the virulence of bacterial pathogens. Some of the proteinase inhibitors in hemolymph may defend the

host against such microbial proteinases. For example, the silk worm (*Bombyx mori*) serine proteinase inhibitor is active against proteinases from fungal pathogens (Eguchi et al., 1993). Several of *Manduca sexta* serpin gene-1 variants inhibit bacterial and fungal serine proteinases (Jiang et al., 1997). Proteinase inhibitors in the cuticle or at the surface of the integument might also function in protection against fungal infection. An external secretion from grasshoppers has been shown to contain proteinase inhibitors with a wide range of specificity (Polanowski et al., 1997).

Injury and microbial infection in vertebrates lead to activation of the blood coagulation and proPO systems. Both of these systems employ cascades of serine proteinases to amplify an initial signal (wounded tissue or the presence of microbial polysaccharides) resulting in rapid and efficient responses to the threats to health (Whaley et al., 1993; O'Brien et al., 1993). Blood clotting and phenoloxidase activation can also be harmful to the host if they are not limited as local and transient reactions. For this reason the proteinases in these systems are tightly regulated by proteinase inhibitors.

Like blood clotting, phenoloxidase activation is normally regulated in vivo as a local reaction of brief duration. Also comparable to blood clotting, the regulation may be due in part to serine proteinase inhibitors in plasma (Kanost et. al., 1996). Pacifastin and, to the lesser degree, α-macroglobulin inhibit crayfish PPO activation (Aspán et al., 1990). Among the low molecular weight inhibitors from insect hemolymph, Kunitz family inhibitors from *M. sexta, Sarcophaga bullata* and *B. mori* (Sugumaran et al., 1985; Saul et al., 1986 and Aso et al., 1994) and the 4 kDa locust inhibitors (Boigegrain et al., 1992) can interfere with PPO activation. Serpin-1J from hemolymph of *M. sexta* inhibits the activity of a serine proteinase linked to prophenoloxidase activation (Jiang et al., 1997). Recently, the *M. sexta* serpin-6 was isolated from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3) (Wang and Jiang, 2004). In addition, its structure and function was further characterized by cloning and expression in *E. coli* expression system (Zou and Jiang, in press). The results suggest that serpin-6 plays important roles in the regulation of immune proteinases in the hemolymph. It appears likely that each proteinase in the PPO cascade is regulated by one or more specific inhibitors present in plasma or in haemocyte granules.

Interesting, the WAP domain (InterPro code IPR002221) is a 50-residue protein with four disulfide bonds, conforming to a conserved tightly packed structure (Grutter et al., 1988; Ranganathan et al., 1999; Tsunemi et al., 1996), named four disulfide core (4-DSC). Many of the proteins containing a 4-DSC domain bind to membrane-bound receptors and have protease inhibitor activities (Simpson et al., 2000; Ranganathan et al., 2000; Kirchoj et al., 1991).

1.8.9 Heat shock protein

Heat shock protein (HSPs) is a family of highly conserved proteins, constitutively expressed and upregulated in thermal, oxidative stress, nutritional deficiencies, ultraviolet irradiation, chemicals, ethanol, viral infection, and ischaemia-reperfusion injury (Lathangue and Latchman, 1988; Donati et al., 1990; Fincatoet al., 1991; Nover, 1991; Welch, 1993; Parsell and Lindquist, 1993; Chouchane et al., 1994; Morimoto, 1998; Ritossa, 1962; Downs et al., 2001). Essential in the folding of newly synthesized proteins, maintenance of structural proteins, refolding of misfolded proteins, translocation of proteins across membranes and into various cellular compartments, prevention of protein aggregation, degradation of unstable proteins, play a critical role in parasitic signalling (signal transduction) and cell division (cell cycle regulation). The principal heat shock proteins range in molecular mass from ~ 15 to 110 kDa and are divided into groups based on both size and function. HSP60 (60 kDa) are refolds proteins and prevents aggregation of denatured proteins, highly immunogenic, part of the PfHsp90 multi-chaperone complex. In addition, HSP 60 have been shown to induce the production of pro-inflammatory cytokines by monocytes, macrophages, and dendritic cells in a manner similar to that of lipopolysaccharide (LPS) and bacterial lipoproteins (Basu et al., 2000; Panjwani et al., 2002; Wang et al., 2002; Flohe et al., 2003). Immune responses to HSP 60 are also frequently found in microbial infections. They are known to induce very strong humoral and cellular immune responses in numerous infections, which HSP 60 act as a T cell carrier peptide in the induction of specific T cell immunity against infectious agents (Zu¨gel et al., 1999).

Besides these, Hsp60 is involved in the activation of apoptosis, which Hsp60 helping to induce apoptosis by acting as a chaperone to procaspase-3 and

aiding in its maturation into active caspase-3 (Lim et al., 2001; Samali et al., 1999; Xanthoudakis et al., 1999).

1.8.10 Apoptotic and tumor

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 (Kerr et al., 1977; 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 beimplicated in shrimp death so apoptosis inhibitor is necessary. For example, both survivin and P109 protein involved in apoptosis inhibitor (Liston et al., 1996; Uren et al., 1996; Tambunan et al., 1998). Besides, survivin is involved in regulation of cell division during HIV-1 infection (Zhu et al., 2003).

1.8.11 Other immune molecules

Cyclophilins are highly conserved proteins first identified as the main binding proteins for cyclosporin A (CsA), an immunosuppressive (Fischer et al., 1989; Schonbrunner et al., 1991). They were later identified as peptidyl-prolyl cis/trans isomerases (PPIase) and have been proposed to be involved in protein folding (Galat, 1993). Cyclophilins are found in all cells of all organisms studied. Different members of the cyclophilin family have been described. They all contain a conserved core domain, carrying both the CsA binding and isomerase sites, flanked by distinct N and C termini accounting for their specificities (Gething and Sambrook, 1992). The prototype of this family is the abundant cytosolic 18-kDa form now named cyclophilin A (CyPA) or cyclophilin 18 (Handschumacher et al, 1984). Cyclophilin B (CyPB) or Cyclophilin 5 or Peptidyl-prolyl cis-trans isomerase 5, PPI 5 (7) and cyclophilin C (CyPC) (Spik et al., 1991) are closely related. A mitochondrial form called cyclophilin D (Bergsma et al., 1991) and a second larger cytosolic form named cyclophilin 40 (Kieffer et al., 1992) have also been described.

Cyclophilins are involved in cellular processes and have many clinical applications such as cell signaling (Mattila et al., 1990), apoptosis (Montague et al.,1997) oxidative stress ((Jaschke et al., 1998), heat shock and hypoxia (Andreeva et al., 1997). For example, CyPA is predominantly cytosolic [but can be secreted by macrophages in response to stimulation with bacterial endotoxin (Sherry et al., 1992) or by vascular smooth muscle cells in response to oxidative stress (Jin et al., 2000). CyPB is found within the endoplasmic reticulum and is secreted into milk and plasma (Spik et al., 1991; Allain et al., 1995; Arber et al., 1992). CyPB was shown to enhance platelet adhesion to collagen (Allain et al., 1999) whereas Cyclophilin A has been shown to help protect cells from oxidative stress (Jaschke et al., 1998).

A shrimp cyclophilin was also identified from *L. vannamei and L. setiferus* (Gross et al., 2001). Cyclophilins have diverse regulatory functions in mammalian cells, but it is interesting to note that they can be involved in viral attachment to cells (Saphire et al., 1999) and in the stress response to oxygen depletion (Santos et al., 2000).

Recently an antifungal protein with its N-terminal sequence manifesting remarkable similarity to cyclophilins was first isolated from mung bean seeds (Ye and Ng, 2000). It exhibited an anti-mitogenic activity on mouse splenocytes but was incapable of inhibiting human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (Ye and Ng , 2000).

Thrombospondins (TSPs) are multidomain, calcium-binding extracellular glycoproteins of animals that have cell- and context-specific effects on cell adhesion, growth, survival, differentiation, and motility (for reviews see Adams et al., 1995; Roberts, 1996; Bornstein et al., 2000; Lawler, 2000). Most attention has focused on establishing their properties and functions in vertebrates, including human and mouse. TAPs are believed to be involved in platelet aggregation, inflammatory response and the regulation of angiogenesis during wound repair and tumor growth (reviewed in Lawler, 2000; Bornstein et al., 2000). In null mice, TSP is importance in the processes of collagen fibrillogenesis and angiogenesis (Kyriakides et al. 1998b). An invertebrate TSP was recently discovered in leukocytes of Japanese flounder, *Paralichthys olivaceus* with infected with *Hirame rhabdovirus* (NamB-H et al., 2000).

Lectins are sugar-binding proteins that agglutinate cells and/or precipitate glycoconjugate molecules with a carbohydrate portion like polysaccharide, glycoproteins, glycolipids and other. Many different lectins, for example tachylectins from hemolymph plasma of the horseshoe crab *Tachypleus tridentatus* (Gokudan S et al.,1999). They are involved in a variety of processes, including the innate immune response critical for the detection and elimination of infectious micro-organisms (Weis et al., 1998; Kilpatrick, 2000).

Generally they recognize sugar or carbohydrate structures on the surfaces of pathogens that are not present on host cell. Koizumi et al (1999) and Jomori and Natori (1992) have been reported that lectins have an LPS- binding property. The biological function of these LPS binding proteins was shown to have bacterial clearance activity and an opsonic effect. Lectins are responsible for promoting phagocytosis and stimulate the proPO system (Yu et al., 1999; Yu and Kanost, 2000). Tachylectins were found to have hemagglutinating and antibacterial activities which are important in the immune system (Kawabata and Iwanaga, 1999).

1.9 Gene expression analysis by quantification methods

Quantitative RT-PCR is the method of choice used to quantify the mRNA expression. It is the most sensitive and accurate of the quantification methods (Wang and Brown, 1999). Since the discovery of PCR numerous applications have been described to quantify the results, such as semi-quantitative and quantitative competitive RT-PCR and its latest innovation quantitative “real-time” RT-PCR.

1.9.1 Reverse Transcription-PCR (RT-PCR)

RT-PCR is a rapid and quantitative method in analyzing the level of expression of gene. The RNA cannot serve as a template for PCR, reverse transcription was combined with PCR to make RNA into a complementary DNA (cDNA) suitable for PCR. The combination of both techniques is colloquially referred to as RT-PCR (Figure 1.7). The necessity to reverse transcribe mRNA into a cDNA prior to subjecting the RNA template to PCR is given by the fact that the polymerase used in PCR is a DNA-dependent polymerase. Reverse transcription of mRNA

requires choosing a reverse transcriptase, a means of priming the mRNA to initiate polymerization and supplying optimal condition for the enzymatic reaction. Reverse transcriptase are RNA-dependent DNA polymerases which have been used predominantly to catalyze first strand synthesis (synthesis of a complementary DNA-cDNA), but are also capable of synthesizing a DNA strand complementary to a primed single stranded DNA. The RT-PCR can be used for semi-quantitative (also known as relative, so don't get confused with the different terms) or quantitative analysis.

The semi-quantitative RT-PCR method is based on the use of an internal control, which is included in the polymerase chain reaction with the gene specific primers. In the majority of cases, the internal control is a housekeeping gene expressed at a very high level, which is assumed to be expressed at a constant level throughout all samples analyzed. Also, it is assumed that the expression levels of the control RNA are not altered by the experimental conditions, thus acting as an experimental control. Semi-quantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. Common internal controls are β-actin and GAPDH mRNA and also 18s rRNA. The PCR products (including the internal control) are then separated with agarose gel electhophoresis, stained with ethidium bromide and analyzed to observe relative expression of the target transcript. However, Semi-quantitative RT-PCR is only able to tell you that one transcript is expressed at a higher level than the other.

1.9.2 Real-time reverse transcription polymerase chain reaction (real-time PCR)

Real-time reverse transcription (Real-time RT-PCR) is followed by polymerase chain reaction (PCR) is a highly sensitive method for the detection and quantification of gene expression levels, in particular for low abundance mRNA. Real-time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection.

Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. Agarose gel results are obtained

from the end point of the reaction. Endpoint detection is very time consuming. Results may not be obtained for days. Results are based on size discrimination, which may not be very precise. As seen later in the section, the end point is variable from sample to sample. While gels may not be able to resolve these variabilities in yield, real-time PCR is sensitive enough to detect these changes. Agarose gel resolution is very poor, about 10 fold. Real-time PCR can detect as little as a two-fold change. Some of the problems with end point detection were poor precision, low sensitivity, low resolution, non automated, ethidium bromide for staining is not very quantitative and post PCR processing.

Limitation of classical endpoint RT-PCR could be overcome by the kinetic or real-time RT-PCR assay. The truly linear part of the amplification can be easily observed with the developed fluorescence based PCR technique together with the specialized detector and software. Real-time RT-PCR can be used to compare and quantitate expression of selected genes in different biological samples. It is especially useful for confirming differential expression of candidate genes identified by other means.

Real-time RT-PCR data analysis methods may be broadly classified as absolute quantitation, based either on standard curve method or relative based on the comparative threshold method. In the standard curve method, a sample of known concentration is used to construct a standard curve. Since the cycle at which PCR enters log linear amplification is directly proportional to the amount of starting template, one determines the concentration of an unknown sample by comparing it to such standard curve. Absolute quantification should be performed when determination of the absolute transcript copy number is required.

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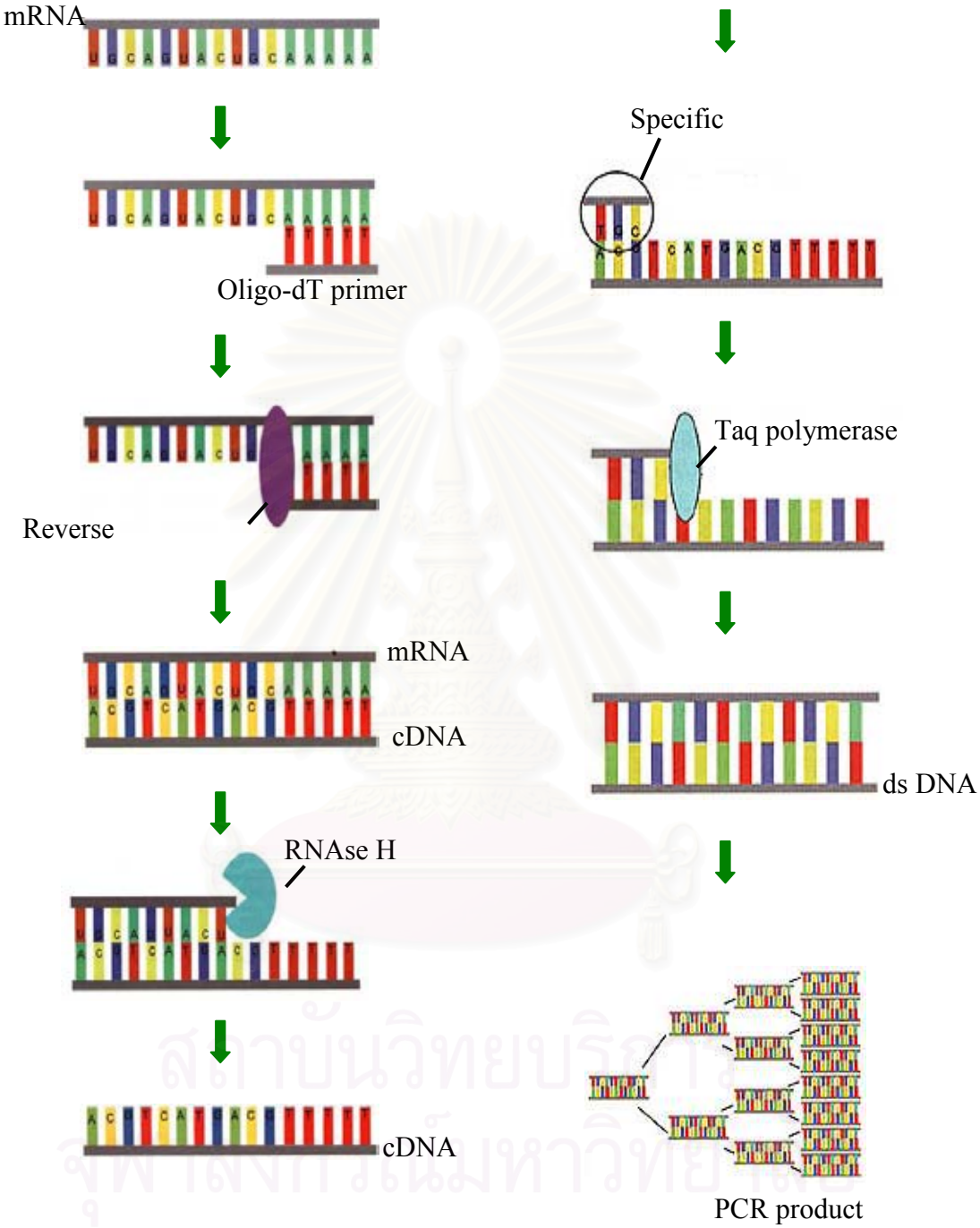


Figure 1.7 Overview of the RT-PCR technique

(http://ccm.ucdavis.edu/cpl/Tech%20updates/RT-PCR%20folder/RT-PCRweb.jpg)

The comparative threshold method was used to report the changes in expression of interested genes relative to reference gene in a given treatment. Pfaffl reports a mathematical model for relative quantification determined from real-time PCR experiments without calibration curve. Expression of target genes was normalized with reference genes of housekeeping genes like glyceraldehydes-3-phosphate dehydrogenase (G3PDH or GAPDH), albumin, actins, tubulins, cyclophilin, 18S rRNA or 28S rRNA were applicable. Housekeeping genes are present in all nucleated cell types since they are necessary for basis cell survival. In shrimp, elongation factor 1-alpha gene (EF 1α) was identified as constitutively expressed gene and was used as reference gene for real-time RT-PCR analysis (Astrofsky et al., 2002). The relative expression ratio (R) of a target gene is computed, based on its real-time PCR efficiencies (E) and the crossing point (CP) difference (Δ) of an unknown sample versus a control ($\Delta CP_{control\text{-}sample}$), and expressed in comparison to a reference gene;

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

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

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

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

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

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

The specific of the amplification of the amplified product is monitored by its melting curve. Since the melting curve of product is dependent upon its GC content, length, and sequence composition, specific amplification can be distinguished from nonspecific amplification by examining the melting curve (Ririe et al., 1997).

The amplified products can be detected by fluorescent dyes that are specific for double-stranded DNA (dsDNA) or by sequence-specific fluorescent oligonucleotide probes. The first dye used for this purposes was ethidium bromide. A dsDNA-specific dye frequently used in real-time PCR today is SYBR Green I. It is an asymmetric cyanine dye which binds sequence independently to the minor groove of dsDNA (Morrison et al., 1998). It does not bind to ssDNA. The SYBR Green dye is excited at a 485-nm wavelength, and the emission is measured at a 520-nm wavelength. The binding affinity is more than 100 times higher than that of ethidium bromide. It is suitable for monitoring accumulation of the product during PCR without a separate assay to detect this product, because the fluorescence of the bound dye is more than 1,000 fold higher than of free dye. However, the biggest disadvantage of SYBR is that it binds to any dsDNA; the specific product, non-specific products and primer dimmers are detected equally well. There are a number of ways to handle this problem. Careful optimization of the PCR reaction can usually reduce primer dimmers to a level that is only important for very low copy detection.

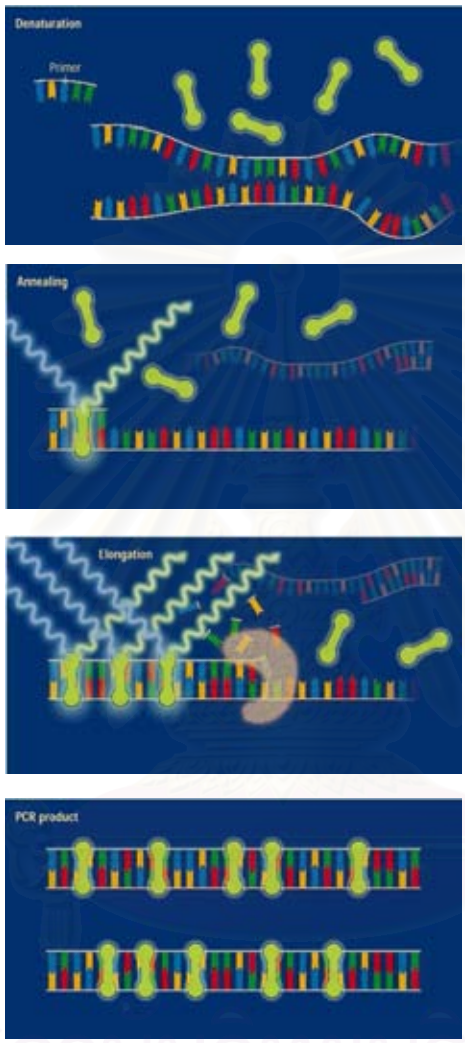


Figure 1.8 Detection of amplified products by SYBR Green (www.med.mcgill.ca/cancer/Course%20Material/516-607/Lecture%203.ppt)

1.10 Previous studies

Gross et al. (2001) studied the immune-related genes using the expressed sequence tag (EST) method of hemocytes and hepatopancreas in two sister litopenaeid shrimps *L. vannamei* and *L. setiferus*. A total of 268 expressed sequence tags (ESTs) were found that corresponded to 44 immune function genes. The most common immune-function ESTs (172) were antimicrobial peptides, which were restricted to the hemocyte libraries. Lectins were the largest group of immune-function ESTs found in the hepatopancreas.

Rojtinnakorn et al. (2002) analysed the gene expression in hemocytes of kuruma prawn, *P. japonicus*, in response to infection with WSSV by EST approach and found 152 new deduced proteins and 28 types of these proteins were involved in biodefence. These includes gene involved in prophenoloxidase cascade, protease inhibitors (Kazal and Kunitz-type inhibitors), antimicrobial peptides, apoptotic and tumour proteins and putative defence related proteins. All ESTs representing protease inhibitors and tumour-related proteins were found only in the WSSV-infected library. Those encoding for apoptotic peptides were expressed at high levels in infected library.

Dhar et al. (2003) used cDNA microarray analysis to compare the gene expression patterns in the hepatopancreas tissues of healthy and WSSV-infected *P. stylirostris* shrimp. Microarray analysis showed that immune genes such as the lipopolysaccharide and β-1,3 glucan binding protein (LGBP) gene, serine protease, C-type lectin, macrophage mannose receptor, and low density lipoprotein receptor were overexpressed in WSSV-infected shrimp.

He et al. (2004) identified genes in hemocyte of *P. japonicus* following microbial challenge by suppression subtractive hybridization (SSH), and found that the most abundant gene was Kunitz-type protease inhibitor. Some genes that encoding signaling molecules, were found for the first time in the shrimp such as Ras-related nuclear protein (Ran), growth factor receptor bound protein (Grb), TGF-β receptor interacting protein, integrin binding protein and interferon receptor bound protein. Besides, cDNAs of chaperonin (chaperonin containing TCP1 (CCT)), and antioxidant (cytochrome *c* oxidase and NADH dehydrogenase) were also expressed at a higher level after the challenge.

Bo et al. (2005) proposed that the expression of antimicrobial peptides genes in hemocytes of Chinese shrimp (*Fenneropenaeus chinensis*) in response to virus and infections bacterial are completely different. The expression level of penaeidin was increased after vibrio infection, whereas it was decreasde after virus infection.

Furthermore, He et al. (2005) identified genes differentially expressed in hemocytes between the normal and White Spot Syndrome Virus-resistant shrimp (P. japonicus). It was found that some other components such as redox-related factors (NADH dehydrogenase; glutathione peroxidase and AP1), C-type lectins and cytoskeleton-related protein-cofilin were highly expressed in the virus-resistant shrimp, suggesting that these proteins may contribute to virus resistance.

Pan et al. (2005) studied differential gene expression profile in hepatopancreas of WSSV-resitant shrimp (*P. japonicus*), found that βGBP is the most abundant gene in the subtractive library. Furthermore, and a number of genes encoding apoptotic-related proteins and antioxidant enzymes were expressed at a higher level in the virus-resistant shrimp. Besides, they demonstrated that the hepatopancreas is a crucial organ in the immune system of these shrimp differential gene expression.

Munoz et al. (2003) studied the expression of penaeidin antimicrobial peptides in early larval stages of the shrimp *P. vannamei*, and found penaeidin transcripts and peptides in a few haemocytes of larvae from mysis II stage using in situ hybridisation and immunohistochemical analyses, but transcripts are already present in the early stage of nauplius V by RT-PCR analyses.

At present, the large-scale of expressed sequence tags (ESTs) project of *P. monodon* is conducting at the Shrimp Molecular Biology and Genomics Laborory, Chulalongkorn University (http://pmodon.biotec.or.th). The EST clones were generated from different tissues of shrimp *P. monodon* under normal or stress conditions to identify tissue-specific genes and genes responded to infection and stress. Initially, 12 different standard cDNA libraries prepared from eyestalk, hematopoietic tissue, hepatopancreas, heat-stressed hemocytes, lymphoid organ, *Vibrio harveyi*-challenged lymphoid organ, YHV-challenged lymphoid organ, and ovary, WSSV-challenged hemocytes, *V. harveyi*-challenged lymphoid organ, *V. harveyi*-challenged hemocytes and two normalized libraries from hepatopancreas and lymphoid organ were constructed. From 10,100 clones analyzed, 5,272 clones

(52.2%) showed significant homology (E-values < 10^{-4}) to known genes whereas 4,828 clones (47.8%) showed no significant homology with any genes in the GenBank. The percentages of matched ESTs in each library ranged from 32.4% to 71.8%. Six hundreds and thirty nine sequences representing 6.3% of the total, are putative immune-related genes and categorized into 7 different subgroups (Table 1.2).

From ESTs of *P. monodon*, Supungul et al. (2002) studied the expression of some immune gene in hemocyte of *V. harveyi* challenged *P. monodon*. The expression level of ALF, HSP 90 and lysozyme were significantly increased whereas the expression level of penaeidin and crustin were significantly decreased after *V. harveyi* injection. No change in the expression level of SPI, proPO and HSP 70 was observed. Besides Somboonwiwat et al. (2005) analyzed the gene expression level in the hemocytes of *V. harveyi* infected *P. monodon* by a real-time RT-PCR and found that expression level of caspase 3B, SERPINB 3, profiling, lysozyme, glucose transporter 1 and interferon-related developmental regulator 1 gene were increased after *V. harveyi* injection.

In this study, the expression level of some immune-related genes in *V. harveyi* and WSSV challenged hemocytes and at different developmental stages of *P. monodon* were for selection examined in order to identify suitable biomarkers of healthy shrimp, which will be used as bloodstocks shrimp.

Table 1.2 Some immune related genes from EST libraries of *P. monodon*

Groups of immune genes	Putative genes
Antimicrobial molecules	anti-lipopolysaccharide factor, crustins, lysozyme, penaeidin
ProPO systems	prophenoloxidase activating factor , prophenoloxidase , haem peroxidase
Oxidative enzyme	cytosolic manganese superoxide dismutase-precursor, thioredoxin peroxidase, glutathione peroxidase
Proteinases and inhibitors	whey acidic protein (putative Protease inhibitor), serine proteinase inhibitor, proteinase inhibitor, serine proteinase,
Heat shock proteins	heat shock protein 70, heat shock protein 60
Apoptotic and tumor Related proteins	P109 protein, survivin, caspase, translationally controlled tumor protein
Other immune molecules	cyclophilin 18, cyclophilin 5, transglutaminase chaperonin containing t-complex Polypeptide 1, peptidyl-prolyl cis-trans isomerase 5, ferritin, lectin C-type, Fc fragment of IgE

1.11 Objectives of the thesis

- To analyze expression levels of some immune related genes in hemocyte of *Penaeus monodon* in response to Gram-negative bacteria, *Vibrio harveyi* and White spot syndrome virus (WSSV) infection.
- To analyze expression level of selected immune genes in different developmental stages of *P. monodon*.
- To examine expression level of selected immune genes in different populations of shrimp.
- To identify candidate genes for use as biomarkers for application in broodstock selection.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

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

Automatic micropipettes P10, P100, P200, and P1000 (Gilson®, France)

-20°C Freezer (Whirlpool)

-80°C Freezer (ThermoForma)

Hot plate (CERAMAG Midi, IKA® WORKS, USA)

Incubator 37°C (Memmert)

Laminar Airflow Biological Safety Cabinets Class II Model NU-440-400E (NuAire, Inc., USA)

Microcentrifuge tubes 0.6 ml and 1.5 ml (Bio-RAD Laboratories, USA)

Minicentrifuge (Costar, USA)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

PCR thin wall microcentrifuge tubes 0.2 ml (Axygen® Scientific, USA)

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

Pipette tips 10, 20, 100 and 1000 µl (Axygen® Scientific, USA)

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

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Spectrophotometer DU650 (Beckman, USA)

2.1.2 Chemicals and Reagents

Absolute ethanol, C_2H_5OH (BDH)

Agarose (Sekem)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Bromophenol blue (Merck, Germany)

Chloroform, $CHCl_3$

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

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

Ethidium bromide (Sigma)

Formaldehyde (BDH)

Formamide (Gibco BRL, technologies, Co., USA)

Isopropanol (Merck)

Sodium chloride (Carlo Erba)

Sodium hydroxide, NaOH (Eka Nobel)

Tryptic soy broth (Difco)

Trizol reagent (Gibco BRL)

2.1.3 Bacterial strains

Vibrio harveyi 1526

White spot syndrome virus (WSSV)

2.1.4 Kits

ImProm-II™ Reverse Transcription system kit (Promega)

2.1.5 Animals

Larvae (NaupliusIII, Zoea II, Mysis II) and post-larvae were obtained from hatcheries of the Province of Chonburi and juvenile *P. monodon* were obtained from pond-reared farms of the Province of Chachoengsao, Thailand. The animals were acclimated for 1 week in in indoor tanks, water temperature ranged between 26 and 32 °C, salinity at 10-12 parts per thousand before experimentation.

Juvenile of *P. monodon* (approximately 2-3 month-old, 14-18g of body weight) were obtained from Suphunburi, Chachoengsao and Nakhonsithammarat (N=15), Thailand. Specific-pathogen-free (SPF) juvenile *P. monodon* was obtained from the *P. monodon* Domesticated Progran in Nakhonsithammarat

2.2 Method

2.2.1 Challenge experiments

For the experimental infection, SPF shrimps were divided into two groups, one group was the challenged shrimp, which SPF shrimps were injected with normal saline (0.85% NaCl(w/v)) containing *Vibrio harveyi* at 10^6 colony forming unit per ml (CFU/ml) or lobster hemolymph medium (LHM) containing a $1{:}10^3$ dilution of white spot syndrome virus (WSSV) stock solution and another group was the control shrimp, which SPF shrimps were injected with normal saline or LHM medium. During the experiment, water temperature ranged between 26 and 32 °C, salinity at 10-12 parts per thousand and the shrimps were fed twice daily with compound shrimp diet based on 5% of body weight.

Hemolymph was collected from an individual shrimp at different time points (0, 3, 6, 12, 24, 48 and 72 hr) after injection. Each time point, hemolymph was collected from a 9 individual shrimps, 3 female and 6 male. In the control group, hemolymph was collected from 3 individual shrimp (1 female and 2 male)

After the first stranded cDNA was synthesized from total RNA, cDNA was pooled into three groups from 3 individual shrimps (1 female and 2 male) in challenge groups and one group (1 female and 2 male) in the control group at different time points after injection.

2.2.2 Preparation of *V. harveyi* infected shrimp

V. harveyi 1526 (kindly provided by Charoenpokphand Group of Companies) was cultured in the tryptic soy agar (TSA supplemented with 1% NaCl)

at 30 °C. A single colony of *V. harveyi* 1526 was inoculated in TSB supplemented with 1% NaCl at 30 °C for 8 hours. The culture was then diluted 1:100 with a sterile normal saline solution (0.85% NaCl, w/v) (modified from Roque et al., 1998). The titer of this dilution was monitored by a plate count method in TSA supplement with 1% NaCl (modified from Austin, 1988). 100 µl of the 10^6 CFU/ml diluted cultures were intramuscularly injected into the 4th abdominal segment with a 1-ml tuberculin syringe, whereas the control group was injected with 100 µl of normal saline.

2.2.3 Diagnosis of *V. harveyi*

The suspensions of hepatopancreas were spread on TSA plates supplemented with 1% NaCl (w/v) and incubated at 30 °C overnight. Colonies of *V. harveyi* 1526 from infected shrimps showed strong luminescence in the dark.

2.2.4 Preparation of WSSV infected shrimp

WSSV stock for challenge experiments was kindly provided by Charoenpokphand Group of Companies. The stock solution of virus was kept in LHM buffer at -80 ^{o}C and thawed at 4 ^{o}C before use. Shrimps were injected into the ventral 4th abdominal segment with 100 µl of a 1:10^3 dilution (LHM) of the viral stock solution, whereas the control group was injected with 100 µl of LHM buffer.

2.2.5 Diagnosis of WSSV

Shrimp was checked for WSSV-infection using the PCR technique (modified from Kiatpathomchai et al.,2001). This procedure detects the presence and the severity of WSSV 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 min before immediate incubation on ice. After a brief centrifugation at 7,500 rpm for 5 min, 1 µl of the supernatant was used as the template for PCR amplification. The 25 µl PCR reaction contained 1 µl of cDNA template, 1.25 mM of each dNTPs, 1.5 mM MgCl2, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 1 U of Taq DNA polymerase, 10 pmole of the F1 (5'AGAGCCCGAATAGTGTTTCCTCAGC3') and R3 (5'AACAC AGCTAACCTTTATGAG3') primers, amplifying a 250-bp fragment of WSSV DNA. The PCR reaction was followed by 2 successive cycling protocols of (1) 90 °C for 3 min, 60 °C for 30 s and 72 °C for 30 s, followed by (2) 35 cycles of 90 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and the final extension was 72 °C 5 min. Following PCR, 10 µl of the reaction solution was examined following electrophoresis through a 2% w/v agarose gel and ethidium bromide staining.

2.2.6 Sample collection

Hemolymph was taken from the ventral sinus of each shrimp using a 27 G/1/2 inch needle fitted onto a 1.0 ml syringe, with 200 µl anticoagulant (10% sodium citrate, w/v) preloaded. Hemolymph was immediately centrifuged at 800xg for 10 minutes at 4 °C to separate hemocytes from the plasma.

For differential stage of shrimp experiment, larval, post-larval and juvenile shrimp were frozen immediately in liquid nitrogen and transported to the laboratory for further processing. Shrimp were crushed in liquid nitrogen.

2.2.7 Total RNA preparation

Add in 1 ml of ice-cold Trizol reagent (Gibco BRL) per 50 mg of powdered tissue in the mortar. Homogenize the tissue with a pestle, until the creamy solution is liquefied and clear. Transfer the clear solution to a 1.7 ml tube (1ml / tube). For hemocyte pellet from individual shrimp was homogenized with 1 ml of Trizol reagent (Gibco BRL). The homogenate was incubated at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. After that, it was extracted twice with 200 μl of chloroform then mixed gently for 15 seconds and incubated at room temperature for 3 min. The sample was further centrifuged at 12,000xg for 15 minutes at 4 °C and collected upper aqueous phase containing total RNA to new tube. RNA was precipitated by the addition of 500 μl of isopropanol then incubated at room temperature for 10 min and centrifuged at 12,000xg for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol. The RNA pellet was kept under 75% ethanol until used. When required, the samples were centrifuged at 7,500xg for 10 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 diethyl pyrocarbonate (DEPC)-treated water.

The total RNA concentration was determined by UV spectrophotometer 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)

Protein had a maximum absorption at 280 nm. Determining the ratio of $A_{260/280}$, The relative purity of the sample could be estimated. RNA sample should not have an $A_{260/280}$ ratio below 1.6. Then RNA was dissolved in RNase-free water and stored at -70°C until used.

2.2.8 Formaldehyde-agarose gel electrophoresis

After total RNA was extraction, RNA quality was assessed by formaldehyde-agarose gel electrophoresis as described below

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

Ten to twenty micrograms of total RNA in 3.5 μl of DECP-treated H2O, 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; 1mmol/l EDTA, pH 8.0, 0.5%, w/v bromophenol blue) was added to each sample. The sample was loaded to the 1.0 % agarose gel containing formaldehyde. The RNA marker was used as a standard RNA marker. Electrophoresis was carried out in 1x MOPS buffer at 50 volts, until bromophenol 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.).

2.2.9 First stranded cDNA synthesis

The first stranded cDNA was synthesized from 1 μg of total RNA using an ImProm-II™ 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 0.5 μl. The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. After that, 4 μl of 5x reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of dNTP Mix (10 mM each), 20 units of Ribonuclease inhibitor and 1 μl of ImProm-II reverse transcriptase were added and gently mixed. The 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.

2.2.10 Primer designation

PCR primers were designed from nucleotide sequencing of the selected EST clones using the Oligo version 4 program. Each pair of upstream and downstream primers had closely similar Tm values, and they were checked for minimal self-priming and upper/lower dimer formation.

2.2.11 PCR

Amplification reaction was performed in 15 μl mixture containing 5 μl of a 1:50 dilution of cDNA template, 1.25 mM of each dNTP, 1x PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 1 U of *Taq* DNA polymerase, the primers (0.2-0.08μm), $MgCl_2$ concentration (1-3 mM) and the number of cycle (21-36 cycles). The amplification was consisted of predenaturation at 95 °C for 2

min followed by 30 cycle of denaturation at 94 °C for 30 sec, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and a final extension at 72 °C for 5 min.

2.2.12 Gel electrophoresis and quantitative analysis

PCR products were determined by electrophoresis on 2% agarose gels. Two per cent (w/v) of agarose gel was prepared using 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 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 2 µl of the 10x loading dye (0.25% bromophenol blue and 25% Ficoll in water) before loading into the well. A DNA ladder (100 bp marker) was used as standard DNA markers.

Electrophoresis was carried out in 1 x TBE buffer at 100 volts until the bromophenol blue dye marker migrated about ¾ of the gel length. After electrophoresis, the gel was stained in a 2.5 µg/ml ethidium bromide (EtBr) solution for 5 minutes and destained to remove unbound EtBr by submerged in distilled water for 15 minutes. Fractionated PCR product was visualized under a UV transilluminator and photographed.

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

2.2.13 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 $p < 0.05$.

2.2.14 Quantitative real-time RT-PCR

Real-time RT-PCR analysis was performed on iCycler-iQ^{TM} system (Bio-Rad Laboratories) by SYBR Green I dye detection. The amplifications were performed in a 96-well plate in a 20 μl reaction volume containing 10 μl of 2X SYBR Green supermix (Bio-Rad), the final concentration of each forward and reverse primer was 0.15μM. The sequences of the primers used in SYBR Green real time RT-PCR are given in Table 2.1. The 5 μl of 1:50 diluted cDNA of each reverse transcription reaction used as template. The thermal profile for SYBR Green real-time RT-PCR was 95 ^{o}C for 5 min followed by 40 cycles of denaturation (95 ^{o}C for 30 sec) annealing (as indicated in Table 2.1) and extension (72 ^{o}C for 30 sec). We verified the specificity of PCR by measuring the melting curve of the PCR product at the end of reaction. The reaction was incubated at 95 ^{o}C for 1 min and subsequently 50 ^{o}C for 1 min, followed by 80 repeats of heating for 10 seconds staring at 50 ^{o}C with 0.5 ^{o}C increments. Fluorescent data are specified for collection during primer extension. The relative cDNA ratio was calculated using the value of threshold cycles. Each sample had 3 replicates in each plate. Sterile-water replaced template as the negative control.

2.2.15 Data analysis of real-time RT-PCR

The amplified products were detected by fluorescent dyes. Fluorescence signal was analyzed by the data analysis software of iCycler iQ^{TM} Real-time Detection system (Bio-Rad) using PCR base line Subtracted curve fit method. For each sample, the cycle number at which the fluorescence crosses the arbitrary line called the threshold. The threshold should be in the line part of the reaction and higher than the background signal to ensure that the reaction cross the line due to amplification rather than noise. This crossing point, Ct, is also known as the threshold cycle or Ct value. The obtained Ct values were used to calculate the relative expression ratio (R).

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

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

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

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

$\Delta CP_{\text{target}}$ is the CT deviation of control (saline-injected) - sample (*V. harveyi*-injected) of the target gene transcript;

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

2.2.16 Determination of PCR efficiency

As target and reference gene had different sequences and amplicon lengths, it was probable that they would show different PCR efficiency. PCR efficiency of each gene amplified with specific pair of primers was determined by constructing a standard curve. The cDNA was made from hemocytes of normal *P. monodon* by the same procedure as described above. This cDNA was used for creating all standard curve of both target and reference genes. These standard cDNA were diluted in five steps from 5 to 5×10^4. The amplification was performed in triplicate including a negative control, used water as template, for each run. A calibration curve plotting Ct values against input quantities (log scale) was constructed for both reference (EF-1α) and each target genes. In each plot, a linear graph should have an excellent correlation coefficient (certainly more than 0.999).

PCR efficiency was equal to $10^{-1/slope}$. These efficiencies were taken into account in relative quantification.

Table2.1 Primer pair and condition for real-time PCR.

Gene name*	Primer	Annealing Temperature (°C/sec)	Product Size (bp)
PPAF	5' TACGTACTCATTGATATCAGGTTTGG 3' (F) 5' GCCTCGTTATCCTTGAATCCAGTGA 3' (R)	56/30	283
WAP	5' TTGCTCTTGCTTTACCGAT 3' (F) 5' ACTTCTGTCTGCCCCTACACT 3' (R)	58/30	291
EF	5' GGTGCTGGACAAGATGAAGGA 3' (F) 5' CGTTCCGGTGATCATGTTCTTGATG 3'(R)	55/30	150

* PPAF; Prophenoloxidase activating factor, WAP; WAP four-disulfide core domain protein 5, EF; Elongation factor-1-alpha gene

CHAPTER III

RESULTS

3.1 Selection of immune-related genes and optimization of PCR

In this study, selected immune-related genes of *Peneaus monodon* were analyzed the expression levels in order to identify candidate markers for the application in broodstock selection. Fourteen immune-related genes were chosen from the EST libraries of the *P. monodon* EST project (http://pmodon.biotec.or.th). These EST libraries were prepared from normal hemocytes, *V. harveyi*-challenged hemocytes, hepatopancreas, and *V. harveyi*-challenged lymphoid organ (Table 3.1). The selected genes consisted of prophenoloxidase activating factor (PPAF), transglutaminase (TG), WAP four-disulfide core domain protein 5 precursor (WAP), glutathione peroxidase (GPx), cytosolic manganese superoxide dismutase precursor (SOD), chaperonin containing TCP1 subunit 4 (CCTP), heat shock protein 60 (HSP60), apoptosis inhibitor survivin (survivin), P 109 protein, peptidyl-prolyl cis-trans isomerase 5 precursor (PPI), cyclophilin 18, thrombospondin 4, techylectin-5B and lectin C-type. Sequence analysis using the Genetyx-Win showed that only whey acidic protein (WAP) and cytosolic manganese superoxide dismutase (SOD) genes contained the complete open reading frame (ORF).

Fifteen primer sets were designed for the amplification of the interesting genes in *P. monodon* hemocytes based on the nucleotide sequence of the EST clones. Sequences of designed primers were shown in Table 3.2. In addition, primers for amplification of an elongation factor-1α (EF) were designed for use as an internal control in a semi-quantitative PCR. The RT-PCR of all primers was initially performed at the same annealing temperature of 55 °C. The amplification products were 154, 145, 291, 145, 166, 262, 295, 320, 166 and 310 bp for PPAF, TG, WAP, GPx, SOD, CCTP, HSP 60, survivin, PPI and cyclophilin,

respectively. No PCR product was observed from the amplification using specific primers for thrombospondin, techylectin-5B and lectin C-type which suggested that these genes did not expressed in shrimp hemocytes. Non-specific products were observed from the amplification using P109 primers. A touchdown PCR was then used to performed the amplification of P109 as follows, beginning with the initial annealing temperature of 66 °C and 2 °C decreasing each cycle for 8 cycles after that the remaining cycles were run at the annealing temperature of 57 °C. Finally, 11 genes including PPAF, TG, WAP, GPx, SOD, CCTP, HSP 60, survivin, P109, PPI and cyclophilin were selected for further study.

3.1.1 Determination of the optimal $MgCl_2$ concentration

Determination of the optimal $MgCl_2$ concentration for each primer set was performed using different $MgCl_2$ concentrations ranging from 1.0 to 3.0 mM using the standard PCR reaction as described above. The PCR products were then analyzed by agarose gel electrophoresis and the concentration of $MgCl_2$ that resulted in the highest yield of amplified product was chosen to be used in the expression determination (Table 3.3). The PCR products of HSP 60 and cyclophilin primers showed the highest yield at 1 mM (Fig. 3.1 g and k) while the PCR products given from TG, SOD and PPI primers exhibited the highest yield at 2 mM (Fig. 3. b, e and j). For the remaining genes (PPAF, WAP, GPx, CCTP, survivin, P109 and EF) the highest yield was found at 1.5 mM $MgCl_2$ (Fig. 3.1 a, c, d, f, h, iand l).

Table 3.1 ESTs homologues of *P. monodon* : Defense and homeostasis.

Clone No.	Sequence length (bp)	Genes	Closest species	Accession No.	Score	E value	Identities %	Positives %
HC-V-S01-0412-LF	568	prophenoloxidase activating factor	*Callinectes sapidus*	AAS60227.1	227	6.00E-18	64	45 / 70
HC-N-S01-0262-LF	719	transglutaminase	*Penaeus monodon*	AAO33455.1	235	6.00E-61	100	115 / 115
HC-V-S01-0446-LF	746	WAP four-disulfide core domain protein 5 precursor	*Homo sapiens*	Q8TCV5	57	4.00E-07	33	42 / 127
HC-N-S01-0194 -LF	331	glutathione peroxidase	*Homo sapiens*	A45207	91	3.00E-18	62	76 / 69
HC-V-S01-0153-LF	286	cytosolic manganese superoxide dismutase precursor	*Callinectes sapidus*	AAF74771.1	330	9.00E-90	82	152 / 185
HC-N-S01-0313-LF	800	chaperonin containing TCP1, subunit 4 (delta)	*Homo sapiens*	NP_006421.2	263	2.00E-71	66	131 / 196
HPa-N-S01-0194-LF	547	heat shock protein 60	*Culicoides variipennis*	AAB94640.1	72	3.00E-30	65	36 / 55
LP-V-S01-0346-LF	720	apoptosis inhibitor survivin	*Sus scrofa*	NP_999306.1	89	9.00E-17	43	43 / 100
LP-V-S01-0367-LF	539	P109 protein	*Bombyx mori*	T00207	117	1.00E-25	34	73 / 310
HC-V-S01-0051-LF	667	peptidyl-prolyl cis-trans isomerase 5 precursor (PPIase)	*Caenorhabditis elegans*	NP_493624.1	235	5.00E-61	75	116 / 153
HC-N-S01-0533-LF	576	cyclophilin 18	*Oryctolagus cuniculus*	AAF22215.1	241	2.00E-62	71	116 / 162
LP-V-S01-0388-LF	539	thrombospondin 4	*Penaeus monodon*	AAN17670.1	75.9	7.00E-25	94	34 / 36
LP-V-S01-0587-LF	566	techylectin-5B	*Tachypleus tridentatus*	BAA84189.1	91.3	1.00E-17	39	52 / 133
HPa-N-S01-0076-LF	505	lectin C-type	*Homo sapiens*	NP_919430.1	88	7.00E-07	23	35 / 150

Table 3.2 Primers used for semiquantitative RT-PCR of immune-related gene expression and internal control (EF).

Target transcripts	Primer	Annealing Temperature (°C)	Product Size (bp)
PPAF	5' TTGGTCTTGCTTCCCTCTAC 3' (F)	55	154
	5' TTATTTTGTATCTCCTGCTCG 3' (R)		
TG	5' ACCTTCAACTCCAGCCAGC 3' (F)	55	145
	5' TAGCATTCTTCAAAATCCTTCC 3' (R)		
WAP	5' TTGCTCTTGCTTTACCGAT 3' (F)	55	291
	5' ACTTCTGTCTGCCCCTACACT 3' (R)		
GPx	5' AACTGGCTTCCTCCGCTATC 3' (F)	55	145
	5' TGAGTTGGTTCATCAGGTGG 3' (R)		
SOD	5' AATGTTGGCTCTGGTGTAGG 3' (F)	55	166
	5' TGGATTTAACCGAAGAGACTG 3' (R)		
CCTP-1	5' CTCTGACTCCCTCCTCCATTT 3' (F)	55	262
	5' TACCACTACAGTCTCCCCAA 3' (R)		
HSP60	5' GAAGGGCAAGGGTAATACTA 3' (F)	55	295
	5' AAGCAGGTAAGGCAACGAAT 3' (R)		
Survivin	5' GGGAGGAGCACAAAAACCAT 3' (F)	55	320
	5' ACAAGAACAGAGGAGTGAA 3' (R)		
P109	5' AACCTGGGATTGTGTAAGCA 3' (F)	Touchdown	183
	5' GAGCAAGTCATCAACCTGAA 3' (R)		
PPI	5' ATCTTCCATCGTGTGATTCC 3' (F)	55	166
	5' TGTCCTTTCCAGCATTAGCC 3' (R)		
Cyclophilin 18	5' AAAAAGGTGTGGGCGGCTAA 3' (F)	55	310
	5' ACAGTCAATCTACGGCAACA 3' (R)		
Thrombospondin	5' TTGGATGGACCCCTAAAGTT 3' (F)	55	187
	5' GGAGAAGCAGAAGAGACCT 3' (R)		
Techylectin-5B	5' CGTCAAACACTGCCTCAAGA 3' (F)	55	233
	5' CACAGTCCAAACCTACACAC 3' (R)		
Lectin	5' CTGGTCTCTTCTTCCTCATA 3' (F)	55	229
	5' GCCACTTCCATACGCCTTC 3' (R)		
EF	5' GGTGCTGGACAAGATGAAGGA 3' (F)	55	150
(internal control)	5' CGTTCCGGTGATCATGTTCTTGAT G 3' (R)		

Touchdown* was a 2 °C decreasing from 66 °C every cycle to the fixed annealing temperature at 57 °C.

3.1.2 Determination of cycling parameters

To determine semi-quantitatively the PCR product of RT-PCR, it is necessary to select the most appropriate number of amplification cycles that the amplified product can be detected at the late exponential phase before reaching the process of plateau amplification phase. The amplification product showing a sharp DNA band on an agarose gel could then be correctly quantified.

In this experiment, to avoid the plateau phase of PCR product, a number of PCR cycles were determined in a range of 21 to 36. At which cycles that gave the highest band intensity before the product reached a plateau phase was chosen for further analysis. From the primers used, cycle numbers showing relatively abundance of PCR products for WAP and EF primers were at 21 cycles (Fig. 3.2 c, l), whereas TG and SOD were found at 24 cycles (Fig. 3.2 b, e). As well as the PPAF, GPx, CCTP, HSP 60, surviving, P109, PPI and cyclophilin were at 27 cycles (Fig. 3.2 a, d and f - k). Therefore, the PCR cycles of 21, 24 and 27 were selected for further investigation of each gene transcript (Table 3.3).

The optimal conditions for RT-PCR of those immune genes were shown in Table 3.3 . The optimal primer concentration varied between 0.08 – 0.2 μM.

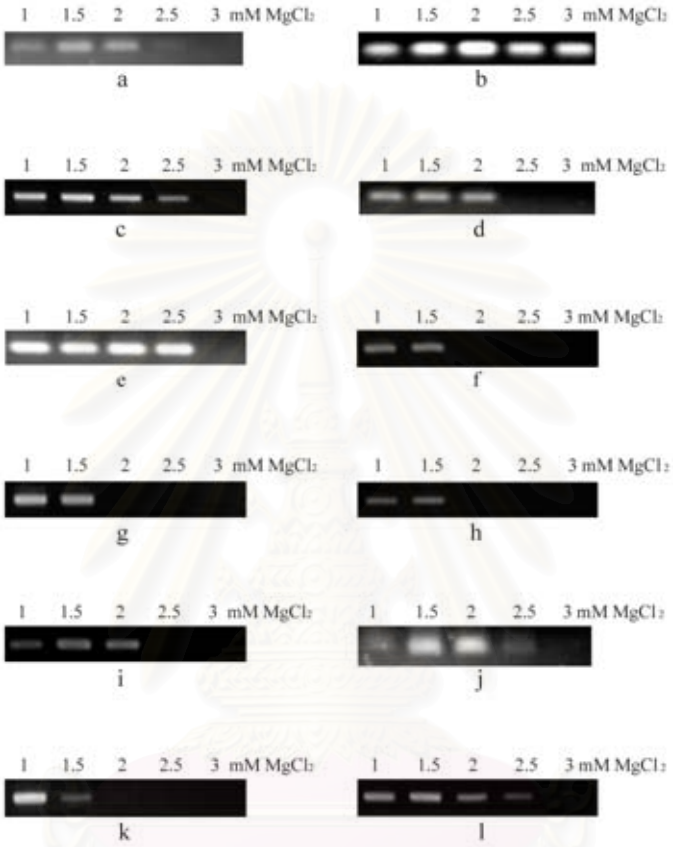


Figure 3.1 Optimization of $MgCl_2$ concentration for RT-PCR amplification by varying concentration of $MgCl_2$ from 1 to 3 mM.

(a) PPAF	(b) TG	(c) WAP	(d) GPx
(e) SOD	(f) CCTP	(g) HSP 60	(h) Survivin
(i) P109	(j) PPI	(k) Cyclophilin	(l) EF

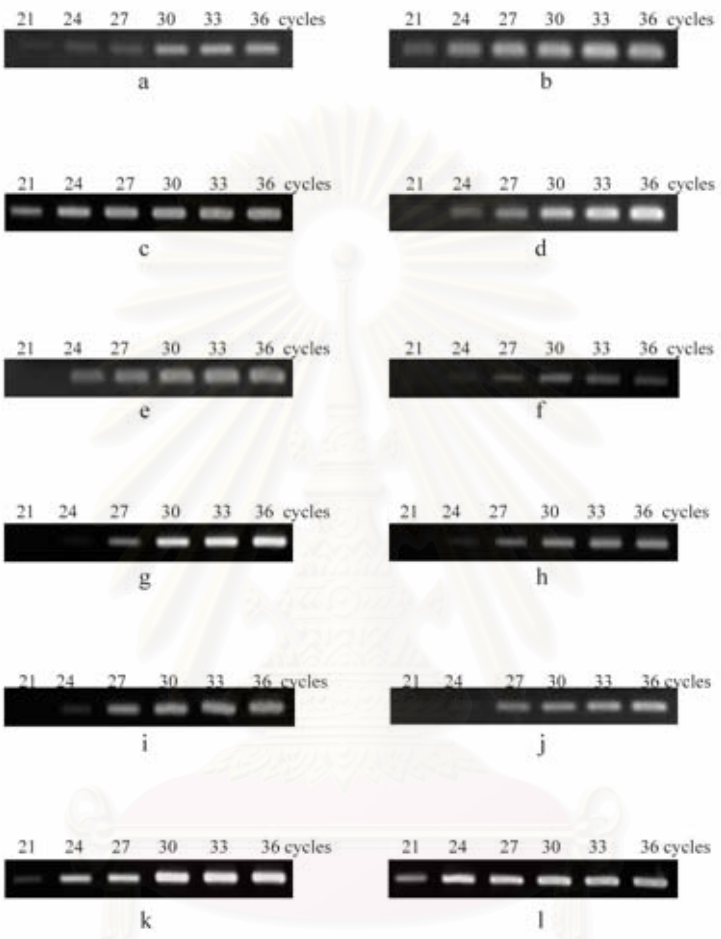


Figure 3.2 Optimization of number of PCR cycles for RT-PCR amplification by varying numbers of amplification cycles from 20-36 cycles.

(a) PPAF	(b) TG	(c) WAP	(d) GPx
(e) SOD	(f) CCTP	(g) HSP 60	(h) Survivin
(i) P109	(j) PPI	(k) Cyclophilin	(l) EF

Table 3.3 Optimal condition for RT-PCR of immune–related gene expression in *P. monodon*.

Gene transcrip	Primer Conc. (µm)	$MgCl_2$ Conc. (mM)	Cycles	Annealing Temp (°C)
PPAF	0.20	1.5	27	55
TG	0.20	2.0	24	55
WAP	0.20	1.5	21	55
GPx	0.20	1.5	27	55
SOD	0.20	2.0	24	55
CCTP1	0.08	1.5	27	55
HSP60	0.08	1.0	27	55
Survivin	0.08	1.5	27	55
P109	0.20	1.5	27	Touchdown*
PPI	0.20	2.0	27	55
Cyclophilin	0.08	1.0	27	55
EF	0.20	1.5	21	55

Touchdown PCR* was initially performed by starting the annealing temperature at 66 °C and decreasing by 2 °C every second cycle to 58 °C after that remaining cycles was set the constant annealing temperature at 57 °C.

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

3.2.1 RNA preparation

The three-month age SPF shrimps (14-18g of body weight) were used in this experiment. Hemolymph was collected from the challenged SPF shrimps injected with *V. harveyi* 1526 or WSSV. The control shrimps for *V. harveyi* 1526 were injected with 0.85% NaCl (w/v) and those for WSSV were injected with LHM medium. Approximately 1 ml of hemolymph was obtained from individual shrimp. The hemolymph was then centrifuged to separate hemocytes from plasma and the hemocytes were used to prepare total RNA using a Trizol reagent. The A_{260}/A_{280} ratio of total RNA which was prepared by this method was 1.5-1.8 indicating acceptable quality of total RNA. The average total RNA obtained was approximately 18 μg per individual shrimp. 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. 3.3).

3.2.2 Luminescent detection of *V. harveyi*

The suspensions of hepatopancreas from normal (or control) shrimps and *V. harveyi*-injected shrimps at different time points (0, 3, 6, 12, 24, 48 and 72 hour post-injection (hpi.)) were spread on TSA plates as described in method 2.2.3.2. The result showed that *V. harveyi* could be detected at 6 hpi in the challenged shrimps. However, no luminescence was detected in normal shrimp (data not show)

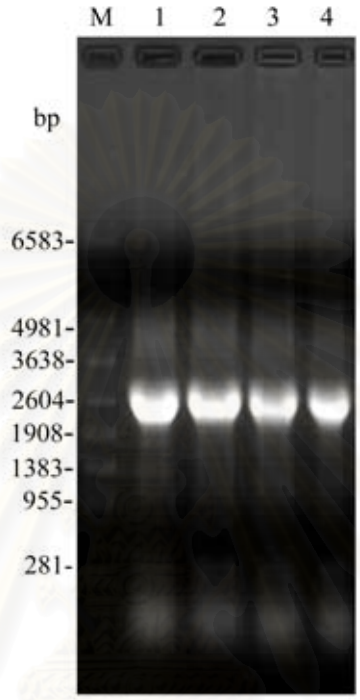


Figure 3.3 Total RNA from hemocyte of control and challenged shrimps electrophoresed on a 1% formaldehyde agorose gel.

Lane M : RNA marker

Lane 1 : shrimp injected with 0.85% NaCl(w/v)

Lane 2 : shrimp injected with LHM medium

Lane 3 : shrimp injected with *V. harveyi* 1526

Lane 4 : shrimp injected with WSSV

3.2.3 PCR detection of WSSV

Shrimp gills were collected from normal shrimp and WSSV-injected shrimp at different time points (0, 3, 6, 12, 24, 48 and 72 hpi.). A gill of shrimp was extracted by lysis buffer and the extracts were subjected to PCR The result showed that a 250 bp WSSV- specific band could be detected in shrimp gill at early time after injection. The positive DNA band was observed at 3 hpi (Fig. 3.4).

Figure 3.4 Ethidium bromide staining of a 250 bp PCR product amplified from Gill DNA of individual shrimp after injection with WSSV at various time post-injection on a 2% agarose gel.

Lane M	:	100 bp ladder
Lane P	:	positive control
Lane N	:	negative control
Lane 1	:	crude DNA extracted from normal shrimp
Lanes 2-8	:	crude DNA extracted from the WSSV-injected shrimp at 0, 3, 6, 12, 24, 48 and 72h post injection

3.2.4 Time course analysis of immune gene expression in the *V. harveyi*-, WSSV-challenged shrimp

The previously optimized conditions of the semi-quantitative PCR were used for a time course analysis of mRNA expression level of the 11 selected immune genes in shrimps challenged with *V. harvei* 1526 or WSSV and the control shrimp.

Sub-adult shrimps were injected with 10^5 CFU/shrimp of *V. harveyi* or $1.76x10^5$ copies/shrimp of WSSV. Hemolymph was collected at 0, 3, 6, 12, 24, 48 and 72 hpi. At each time point, hemolymph was collected from 3 individuals in the control sample (1 female and 2 male) and 9 individuals in the challenged sample (3 female and 6 male) and total RNAs were extracted. After the first stranded cDNAs of the challenged animals were synthesized, the cDNAs were pooled into three groups each group contained cDNAs from 3 individual shrimps (1 female and 2 male) and one group of cDNA of control sample at different time points after injection. The pooled-cDNAs were used as the templates for the semi-quantitative RT-PCR technique. The amplification products of the target gene and the control gene (elongation factor-1-alpha, EF) were run on the same 2% of agarose gel and a ratio of band intensity of the target gene and the control gene was analyzed using Genetools analysis software (Syngene). The expression was determined as the signal ratio of the interested gene : EF while the expression of EF was normalized to 100 and then normalized with signal expression of the control shrimp.

3.2.4.1 A time course analysis of mRNA expression level against infection of V. haeveyi

Upon challenge with *V. harveyi*, the expression levels of PPAF (Fig. 3.5A), GPx (Fig. 3.5B), TG (Fig. 3.5C), WAP (Fig. 3.5D) and PPI (Fig. 3.5E) were significantly increased, when compared with the relative expression at 0 hpi ($p<0.05$). The results of RT-PCR and data analysis were shown in appendix A (I) and C (I-X), respectively. The expression

levels of PPAF were significant increased within 24 hpi and returned to normal since 48 hpi. The fold change at 24 hpi of PPAF was 1.78 compared with relative expression at 0 hpi. Likewise, significant increase in the expression levels of GPx was observed within 6 hpi. ($p < 0.05$) and returned to normal since 48 hpi At 12 hpi, the highest expression level of GPx was 1.19-fold above that of the relative expression at 0 hpi. Whereas, significant increase in the expression levels of TG were observed since 12 hpi. ($p < 0.05$) and was not significantly lowered until 48 hours after injection. At 12 hpi, the highest level of TG was 1.33-fold above that of relative expression at 0 hpi. Likewise, the expression of WAP and PPI was significantly increased after challenged with *V. harveyi*, which was observed since 6 hpi. ($p < 0.05$). At 12 and 6 hpi., the highest level of WAP and PPI was 1.75- and 1.86-fold above that of relative expression at 0 hpi, respectively. The expression of WAP was not significant different until 24 hpi, whereas that of PPI still increased during the study period (72 hpi.).

In contrast, upon challenge with *V. harveyi*, a significant decrease in the expression of SOD (Fig. 3.6A) and survivin (Fig. 3.6B) was observed ($p<0.05$), The results of RT-PCR and data analysis were shown in appendix A (II) and C (XI-XIV), respectively. The expression level of SOD was significantly decreased since 3 hpi and returned to normal at 72 hpi. At 12 hpi, the lowest transcriptional level was observed 0.06-fold below that of relative expression at 0 hpi. Although, the expression of survivin was found as significant decrease during 3 - 24 hpi ($p < 0.05$), the lowest expression was observed at 3 hpi (0.43 -fold below that of relative expression at 0 hpi.). After reaching the lowest level, the expression of survivin was dramatically increased at 48 hpi until the end of the assay at 72 hpi the expression level was significantly higher than that of 0 hpi.

No significant difference ($p<0.05$) in the expression level of P109, cyclophilin, CCTP and HSP 60 was observed after challenging shrimps with *V. harveyi* (Fig. 3.7A, B, C and D) and the results of RT-PCR and data analysis were shown in appendix A(III) and C(XV-XXII), respectively). However, the expression of HSP 60 at 6 hpi was significant higher than that at 72 hpi (1.27-fold). The relative expressions of the 11 immune related genes in V.

harveyi challenged shrimps were summarized in Table 3.4 and the data analyses were shown in appendix C.

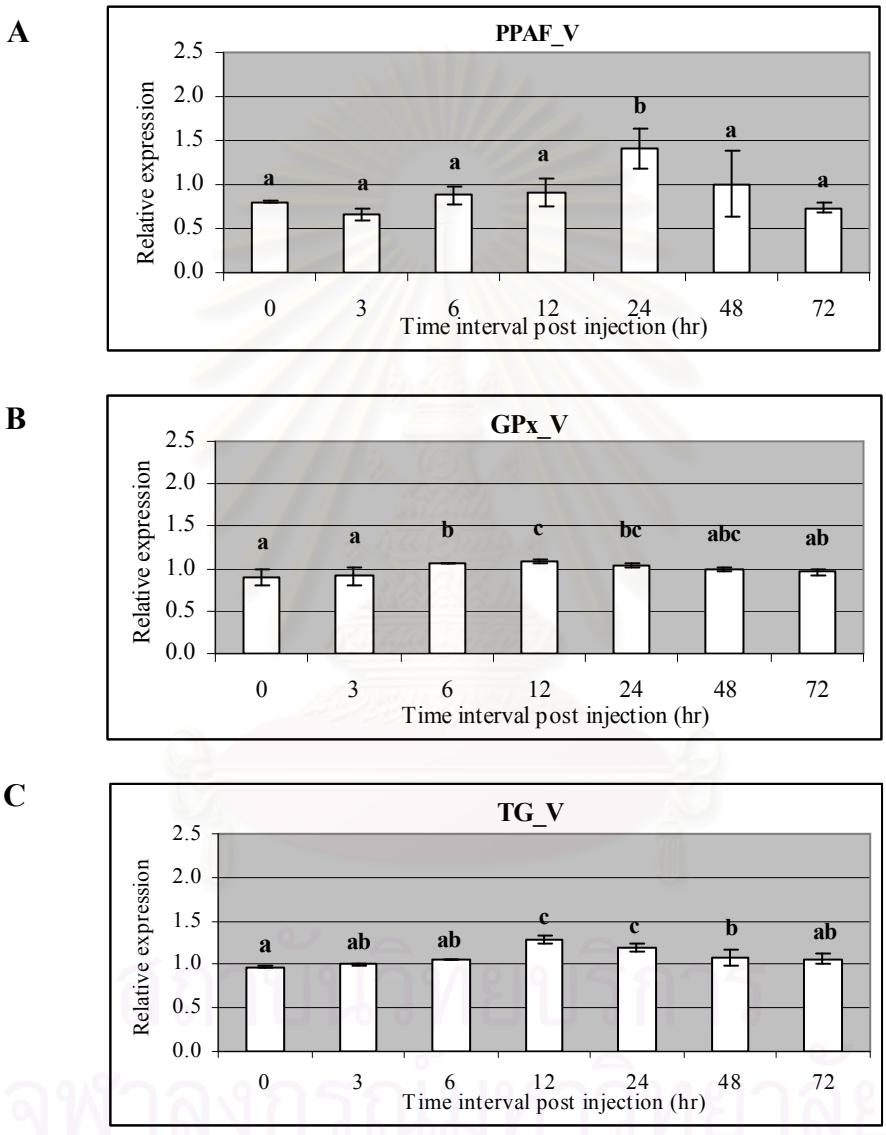
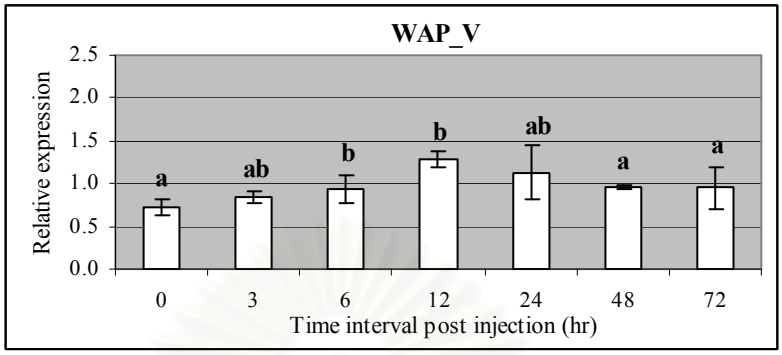


Figure 3.5 Relative expression levels of PPAF (A), GPx (B) and TG (C) at different time intervals after injected with *V. harveyi.* Different letters indicate significant ($p < 0.05$) difference in the mean expression level of the immune-related gene.

D



E

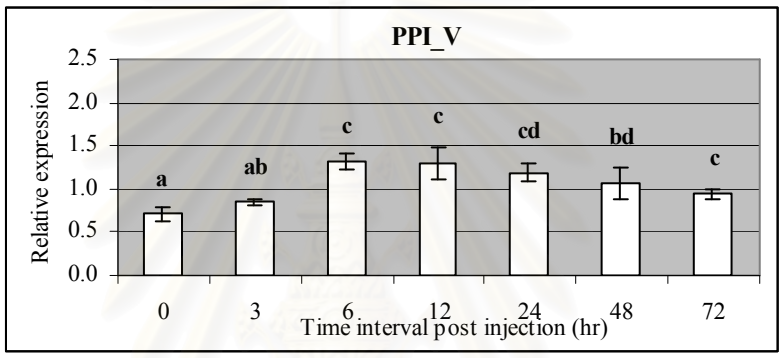


Figure 3.5 (continued) Relative expression levels of WAP (D) and PPI (E) at different time intervals after injected with *V. harveyi.* Different letters indicate significant ($p < 0.05$) difference in the mean expression level of the immune-related gene.

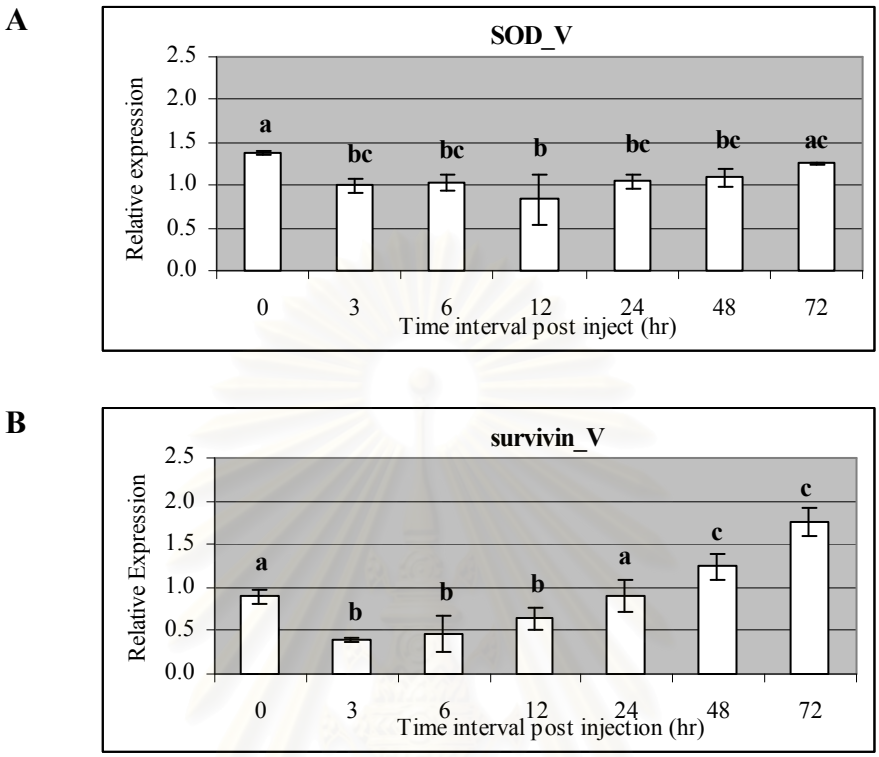


Figure 3.6 Relative expression levels of SOD (A) and survivin (B) at different time intervals after injected with *V. harveyi*. Different letters indicate significant ($p < 0.05$) difference in the mean expression level of the immune-related gene.

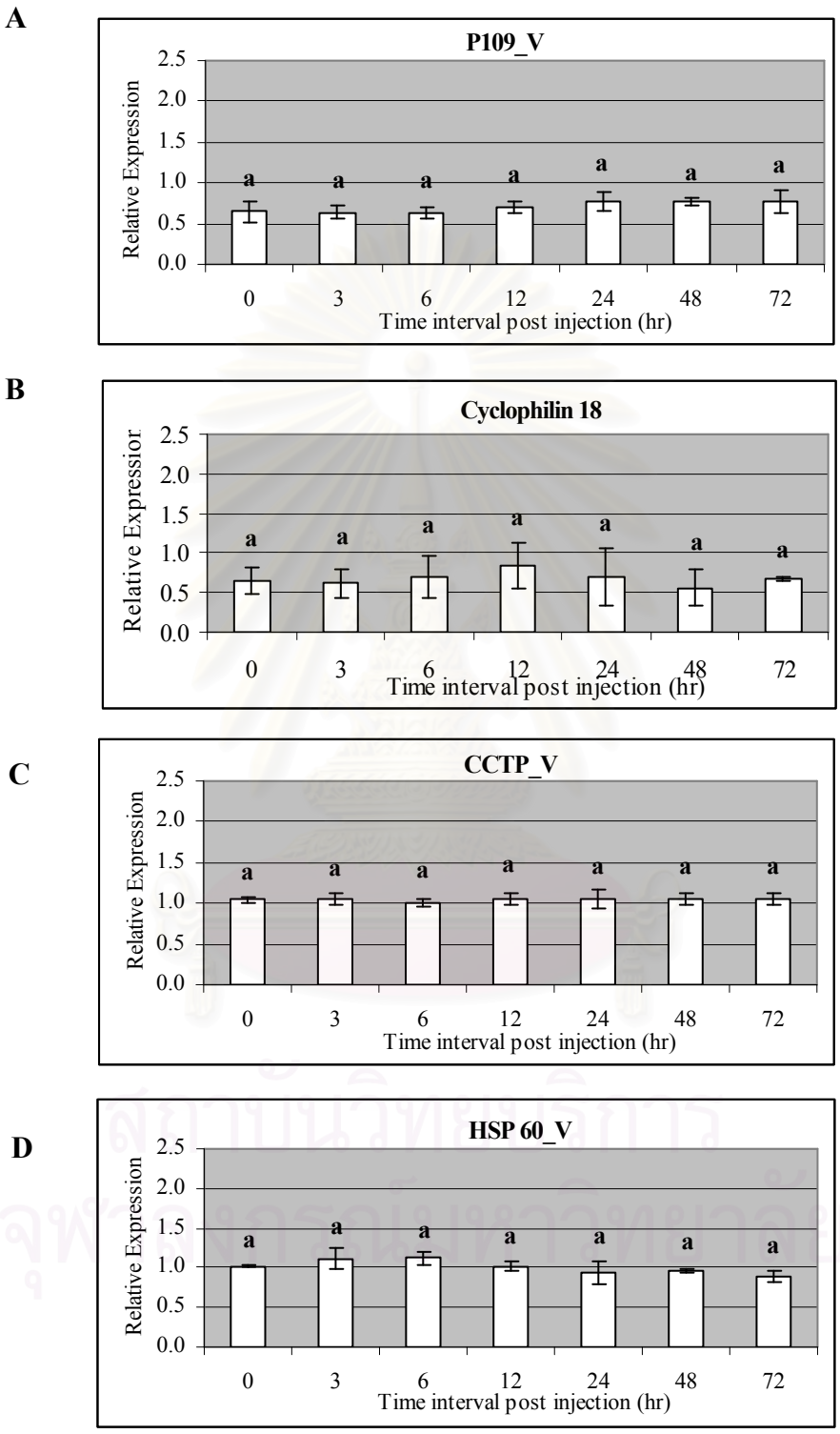


Figure 3.7 Relative expression levels of P109 (A), cyclophilin (B), CCTP (C) and HSP 60 (D) at different time intervals after injected with *V. harveyi*. Different letters indicate significant ($p < 0.05$) difference in the mean expression level of the immune-related gene.

Table 3.4 Expression levels of the 11 immune-related genes in *V. harveyi* challenged shrimp determined by using a semiquantitative RT-PCR.

Genes	Relative Expression						
	0 hr	**3 hr**	**6 hr**	**12 hr**	**24 hr**	**48 hr**	**72 hr**
PPAF	0.79^{a} (±0.01)	0.66^{a} (±0.07)	0.88^{a} (±0.10)	0.91^{a} (±0.16)	1.41^{b} (±0.23)	1.00^{a} (±0.37)	0.73^{a} (±0.06)
SOD	1.38^{a} (±0.02)	1.00^{bc} (±0.08)	1.02^{bc} (±0.10)	0.83^{b} (±0.31)	1.04^{bc} (±0.09)	1.09^{bc} (±0.11)	1.26^{ac} (±0.01)
Gpx	0.91^{a} (±0.10)	0.91^{a} (±0.10)	1.06^{b} (±0.01)	1.08^{c} (±0.00)	1.04^{bc} (±0.02)	0.99^{abc} (±0.03)	0.96^{ab} (±0.04)
TG	0.97^{a} (±0.01)	1.00^{ab} (±0.02)	1.05^{ab} (±0.00)	1.29^{c} (±0.09)	1.21^{c} (±0.05)	1.09^{b} (±0.11)	1.06^{ab} (±0.06)
WAP	0.73^{a} (±0.09)	0.83^{ab} (±0.07)	0.93^{b} (±0.16)	1.28^{b} (±0.09)	1.13^{ab} (±0.32)	0.96^{a} (±0.02)	0.95^{a} (±0.24)
Survivin	0.90^{a} (±0.08)	0.39^{b} (±0.02)	0.46^{b} (±0.20)	0.64^{b} (±0.13)	0.90^{a} (±0.18)	1.24^{c} (±0.16)	1.76^{c} (±0.16)
P109	0.65^{a} (±0.13)	0.64^{a} (±0.09)	0.63^{a} (±0.07)	0.70^{a} (±0.08)	0.77^{a} (±0.11)	0.77^{a} (±0.05)	0.77 (±0.14)
PPI	0.71^{a} (±0.07)	0.87^{ab} (±0.04)	1.32^{c} (±0.09)	1.30^{c} (±0.18)	1.19^{cd} (±0.11)	1.07^{bd}(0.18)	0.94^{b} (±0.05)
Cyclophilin	0.65^{a} (±0.16)	0.62^{a} (±0.18)	0.70^{a} (±0.27)	0.84^{a} (±0.29)	0.70^{a} (±0.36)	0.56^{a} (±0.23)	0.68 (±0207)
CCTP	1.04^{a} (±0.04)	1.05^{a} (±0.06)	1.01^{a} (±0.05)	1.04^{a} (±0.07)	1.05^{a} (±0.11)	1.04^{a} (±0.07)	1.06 (±0.07)
HSP60	1.02^{abc} (±0.01)	1.11^{bc} (±0.13)	1.12^{c} (±0.08)	1.01^{bc} (±0.06)	0.94^{ab} (±0.14)	0.96^{abc} (±0.02)	0.88^{a} (±0.07)

* Data in the same row having different letters are significantly different ($P<0.05$) in a mean expression level of the immune-related gene.

3.2.4.2 A time course analysis of mRNA expression level against infection of WSSV

Upon challenging with WSSV, expression levels of PPAF (Fig. 3.8A), WAP (Fig. 3.8B) and HSP 60 (Fig. 3.8C) were significantly increased when compared with the relative expression at 0 hpi ($p<0.05$). The results of RT-PCR and data analysis were shown in appendix B (I) and D (I-VI), respectively. Significant increase in the expression levels of PPAF was observed since 12 hpi and become no significant differences until 48 hpi. The highest level observed at 12 hpi was 1.38-fold above that of the relative expression at 0 hpi. Whereas, significant increase in the expression levels of WAP was observed at 24 hours after injection ($p < 0.05$), and reached the highest level (3.45-fold above that relative expression at 0 hpi.). The expression of WAP gene returned to normal level at 48h. The expression of HSP 60 was significantly increased since 3 hpi ($p < 0.05$) until 12 hpi. The highest level of HSP 60 mRNA was observed at 3 hpi. (1.23 folds above the relative expression at 0 hpi).

On the contrary, upon challenging with WSSV, a significant decrease in the expression of GPx (Fig. 3.9A), SOD (Fig. 3.9B) and survivin (Fig. 3.9C) ($p<0.05$) was observed. The Results of RT-PCR and data analysis were shown in appendix B (II) and D (VII-XII), respectively. Significant decrease in the expression levels of GPx was observed at 12 hpi, and reached the lowest level (0.78-fold below the relative expression at 0 hpi). The expression levels of SOD were significantly decreased since 3 hpi until the end of the assay at 72 hours. The lowest expression level of SOD was observed at 24 hpi. (0.36-fold below that of the relative expression at 0 hpi.). Although the expression of survivin was significantly decreased after 3 hpi, the lowest expression was observed at 12 hours after WSSV injection (0.71-fold below that of the relative expression at 0 hpi).

No significant ($p > 0.05$) difference in the expression levels of TG, CCTP, P109, PPI and cyclophilin was observed after challenge with WSSV (Fig. 3.10A, B, C, D and E and the results of RT-PCR and data analysis were shown in appendix B(III) and D(XIII-

XXII), respectively. The relative expression of the 11 immune relative genes in WSSV challenged shrimp were summarized in Table3.5.

In this study, it was found that both pathogens caused up-regulation of PPAF and WAP genes. The expression of GPx was significantly increased ($p < 0.05$) after shrimp was challenged with *V. harveyi* but there was significantly decreased ($p < 0.05$) after challenged with WSSV. The expression of TG and PPI transcripts were significantly increased ($p < 0.05$) after challenge with *V. harveyi* but both genes showed no response to WSSV infection. On the contrary, the expression of HSP 60 was significantly increased ($p < 0.05$) upon WSSV challenge but no response to *V. harveyi* challenge. From those results the up-regulated genes including PPAF, GPx, WAP, TG, PPI and HSP 60 were selected for further study in the next experiments.

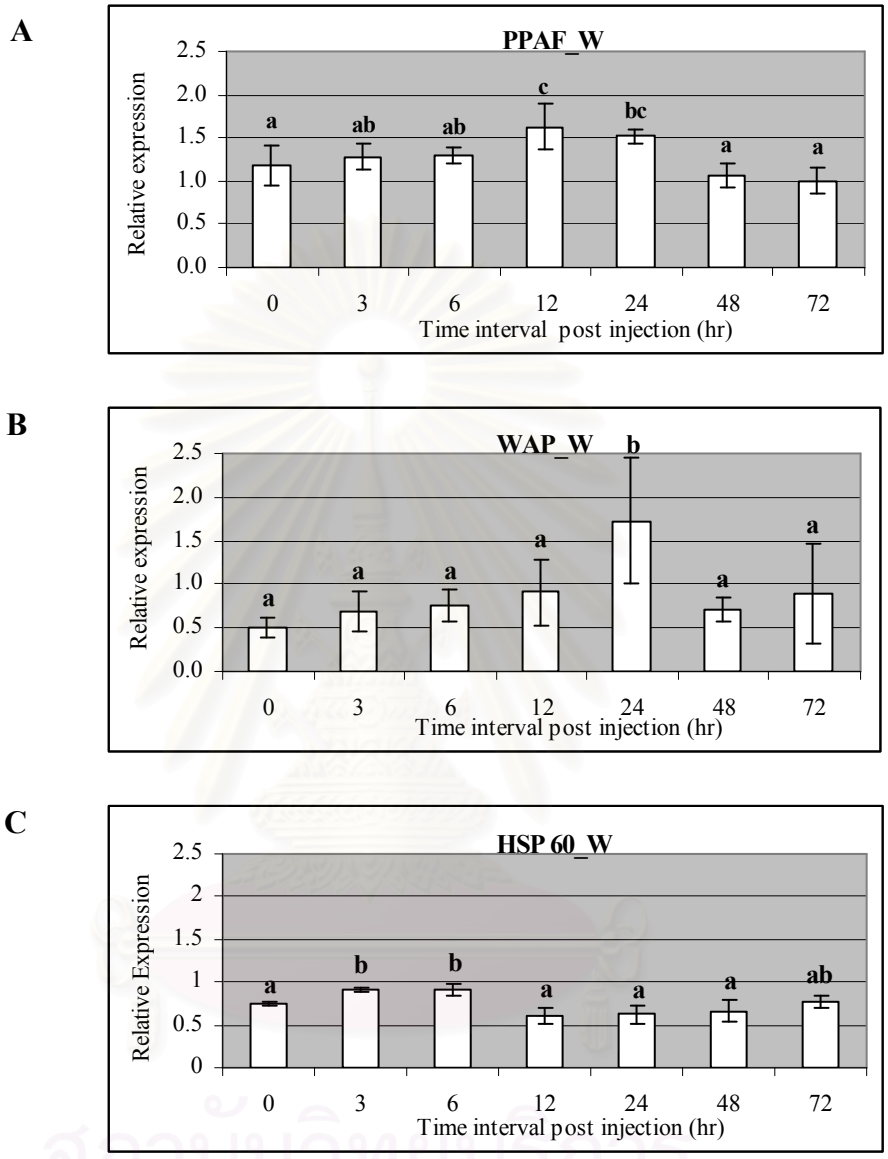


Figure 3.8 Relative expression levels of PPAF (A), WAP (B), and HSP 60 (C) at different time of intervals after injected with WSSV. Different letters indicate significant ($p < 0.05$) difference in a mean expression level of the immune-related gene.

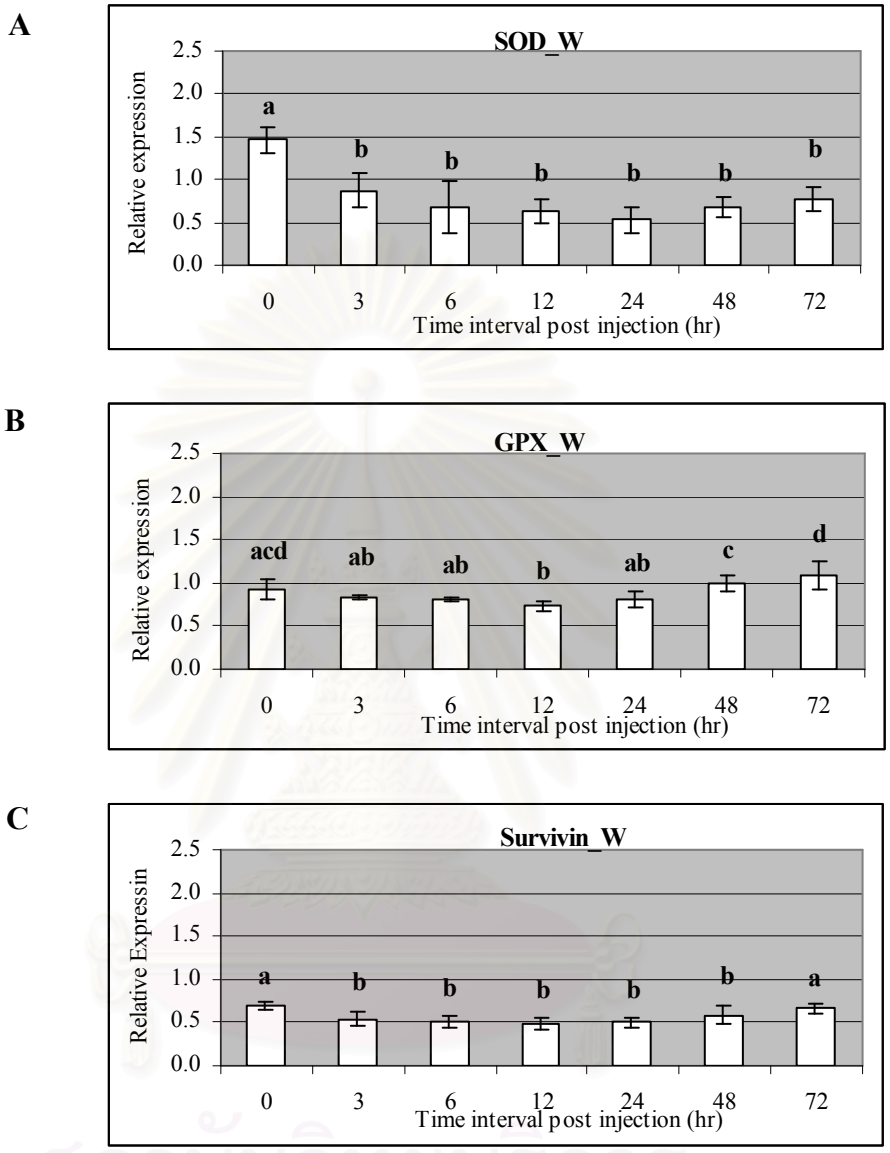


Figure 3.9 Relative expression levels of SOD (A), GPx (B) and Survivin (C) at different time intervals after injected with WSSV. Different letters indicate significant ($p < 0.05$) difference in the mean expression level of the immune-related gene.

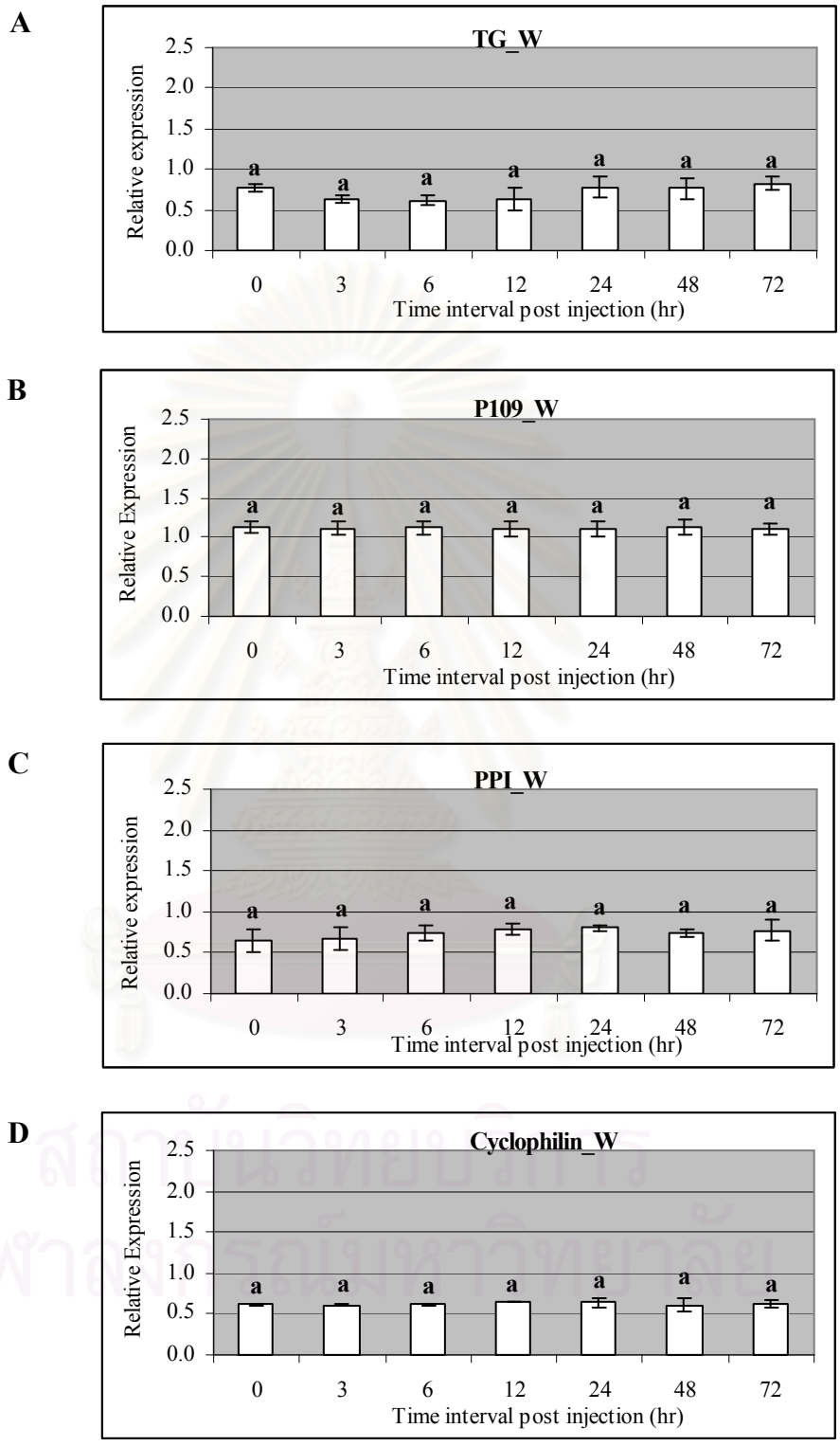


Figure 3.10 Relative expression levels of TG (A), P109 (B), PPI (C) and Cyclophilin (D) at different time intervals after injected with WSSV. Different letters indicate significant ($p < 0.05$) difference in the mean expression level of the immune-related gene.

E

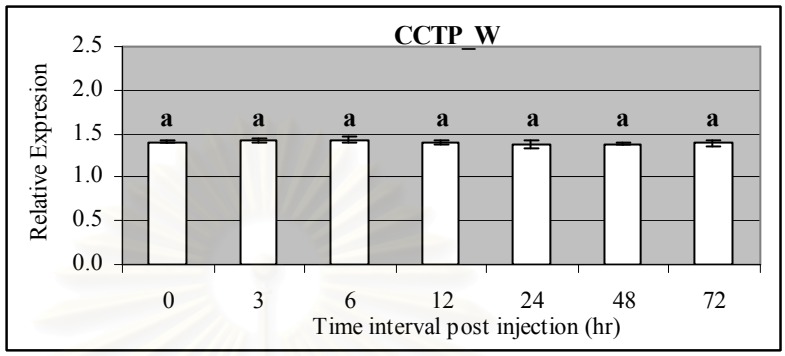


Figure 3.11 (continued) Relative expression levels of CCTP (E) at different time intervals after injected with WSSV. Different letters indicate significant ($p < 0.05$) difference in the mean expression level of the immune-related gene.

Table 3.5 Expression levels of the 11 immune-related genes in WSSV challenged shrimp determined by using a semiquantitative RT-PCR.

Genes	Relative Expression						
	0 hours	**3 hours**	**6 hours**	**12 hours**	**24 hours**	**48 hours**	**72 hours**
PPAF	1.18^{a} (±0.23)	1.28^{ab} (±0.15)	1.30^{ab} (±0.10)	1.63^{c} (±0.27)	1.52^{bc} (±0.08)	1.06^{a} (±0.14)	1.00^{a} (±0.15)
SOD	1.47^{a} (±0.15)	0.87^{b} (±0.30)	0.67^{b} (±0.30)	0.63^{b} (±0.15)	0.53^{b} (±0.15)	0.67^{b} (±0.12)	0.77^{b} (±0.15)
Gpx	0.93^{acd} (±0.10)	0.83^{ab} (±0.0.2)	0.81^{ab} (±0.02)	0.73^{b} (±0.06)	0.81^{ab} (±0.09)	0.99^{c} (±0.09)	1.09^{d} (±0.16)
TG	0.77^{ab} (±0.04)	0.63^{a} (±0.04)	0.61^{a} (±0.06)	0.63^{a} (±0.14)	0.78^{ab} (±0.12)	0.76^{ab} (±0.12)	0.82^{b} (±0.07)
WAP	0.51^{a} (±0.12)	0.69^{a} (±0.22)	0.75^{a} (±0.18)	0.91^{a} (±0.38)	1.76^{b} (±0.72)	0.71^{a} (±0.14)	0.89^{a} (±0.58)
Survivin	0.69^{a} (±0.04)	0.54^{b} (±0.08)	0.51^{b} (±0.07)	0.49^{b} (±0.07)	0.50^{b} (±0.05)	0.59^{b} (±0.10)	0.66^{a} (±0.05)
P109	1.12^{a} (±0.07)	1.11^{a} (±0.08)	1.12^{a} (±0.08)	1.10^{a} (±0.09)	1.10^{a} (±0.10)	1.13^{a} (±0.09)	1.10 (±0.07)
PPI	0.64^{a} (±0.14)	0.67^{a} (±0.14)	0.74^{a} (±0.10)	0.79^{a} (±0.07)	0.80^{a} (±0.04)	0.74^{a} (±0.04)	0.77 (±0.13)
Cyclophilin	0.62^{ab} (±0.01)	0.61^{a} (±0.02)	0.62^{a} (±0.01)	0.65^{b} (±0.01)	0.64^{ab} (±0.05)	0.61^{a} (±0.08)	0.63^{ab} (±0.05)
CCTP	1.41^{a} (±0.02)	1.42^{a} (±0.02)	1.43^{a} (±0.03)	1.40^{a} (±0.03)	1.38^{a} (±0.05)	1.38^{a} (±0.01)	1.39 (±0.04)
HSP60	0.74^{a} (±0.02)	0.91^{b} (±0.03)	0.90^{b} (±0.07)	0.61^{a} (±0.10)	0.62^{a} (±0.10)	0.66^{a} (±0.13)	0.77^{ab} (±0.08)

* Data in the same row having different letters are significantly different ($P<0.05$) in a mean expression level of the immune-related gene.

3.3 Quantitative analysis of some immune related genes after expression in response to *V. harveyi* and WSSV challenge by a real-time RT-PCR

Our results showed that PPAF and WAP genes may play important roles in shrimp immunity against both *V. harveyi* and WSSV. Thus, the time course analysis of mRNA expression level against infection of *V. harveyi* and WSSV were verified and quantitatively analyzed by a real-time RT-PCR using SYBR Green chemistry. In this study, the housekeeping gene, elongation factor 1-alpha (EF) was used as a reference gene. The pooled-cDNAs were prepared from total RNA of hemocyte of the challenged shrimp (*V. harveyi* 1526 or WSSV injected shrimp) and the control shrimp (0.85% NaCl(w/v) or LHM medium injected shrimp) at 0, 3, 6, 12, 24, 48 and 72 hpi., the cDNAs were used as templates for a real-time RT-PCR.

To determine the amplification efficiency of the target genes, serial dilutions of pooled cDNAs of normal animal were made 5 to 5×10^4 dilutions for PPAF, WAP and EF. Real time PCR efficiency was calculated from the slope, obtained from the curve plotted between five dilutions of cDNA (log scale) of normal animal and the threshold cycle (Ct) (Fig. 3.11), using the equation, $E = 10^{[-1/slope]}$. The real-time PCR efficiencies, correlative coefficient and melting temperature are shown in Table 3.6. Real-time PCR efficiencies of selected immune genes varied between 1.85 and 1.96, however, as these efficiencies were not exactly 2.00 (representing 100% amplification efficiency at each cycle), which calculated relative expression ratios of certain gene using an equation to correct for differences in efficiency as described by Pfaffl.

A mathematical model described by Pfaffl was used to determine the relative expression ratio according to the equation:

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

E_{target} is the real-time PCR efficiency of target gene transcript;
E_{ref} is the real-time PCR efficiency of reference gene transcript;
ΔCP_{target} is the CT deviation of control (saline-injected) - sample (*V. harveyi*-injected) of the target gene transcript;
ΔCP_{ref} is the CT deviation of control (saline-injected) - sample (*V. harveyi*-injected) of the reference gene transcript.

The specificities of the products amplified by SYBR Green PCR were monitored by analyzing the corresponding dissociation curve of each amplicon. Figures 3.12 provided examples of the corresponding dissociation curves of PPAF, WAP and EF control gene after challenged with *V. harveyi* (V) and WSSV (W). Dissociation curve of each gene showed the single peak at expected melting temperature indicating that the gene of interest was specifically amplified and there was no non-specific amplification or primer-dimer.

The mRNA expression levels of each gene were determined by normalizing the Ct values of the pooled sample of *V. harveyi*-injected shrimp hemocytes with saline-injected shrimp hemocytes and WSSV-injected shrimp hemocytes with LHM-injected shrimp hemocytes. The expression ratios of certain gene at each time point after injection were calculated relative to EF and shown in Fig. 3.13 and Table 3.7 and 3.8.

The temporal expression of the PPAF gene in *V. harveyi*-challenged shrimps is shown in Fig. 3.13A. The expression of PPAF was decreased at 3 and 12 hours of challenged with *V. harveyi* ($p < 0.05$), the lowest expression was observed at 12 hpi. (0.33 time compared with relative expression at 0 hpi.). After reaching the lowest level, the expression level of PPAF was increased between 24 to 48 hours. The highest expression was observed at 24 hpi (4.13 times

compared with relative expression at 0 hpi.). The temporal expression of the PPAF gene in WSSV-challenged shrimps was shown in Fig. 3.13B. After WSSV challenge, the PPAF gene expression level at the stage from 6 to 24 h was higher ($p < 0.05$) than at other times, which there was no difference between the periods from 0 to 3 h and between 48 to 72 h. The highest expression was observed at 24 hpi. (2.53 times compared with relative expression at 0 hpi.).

The temporal expression of the WAP gene in *V. harveyi*-challenged shrimps was shown in Figure 3.13C, which was increased until 24 hpi. and fall down at 48 hpi. Before climbed up again at 72 hpi.($p < 0.05$). The highest expression was observed at 24 hpi. (4.23 times compared with relative expression at 0 hpi.). The temporal expression of the WAP gene in WSSV-challenged shrimps is shown in Fig. 3.13D, the increase expression level of WAP was observed since 3 hpi. ($p < 0.05$) and still increased during the study period. The highest expression level of WAP was observed at 24 hpi. at 6.68 times compared with relative expression at 0 hpi.

Expression pattern of PPAF and WAP against infection of *V. harveyi* and WSSV were analyzed by semi-quantitative PCR as the same pattern as verified by real-time RT-PCR. The transcription level of PPAF and WAP were significantly increased between 12 to 24 hpi ($p < 0.05$).

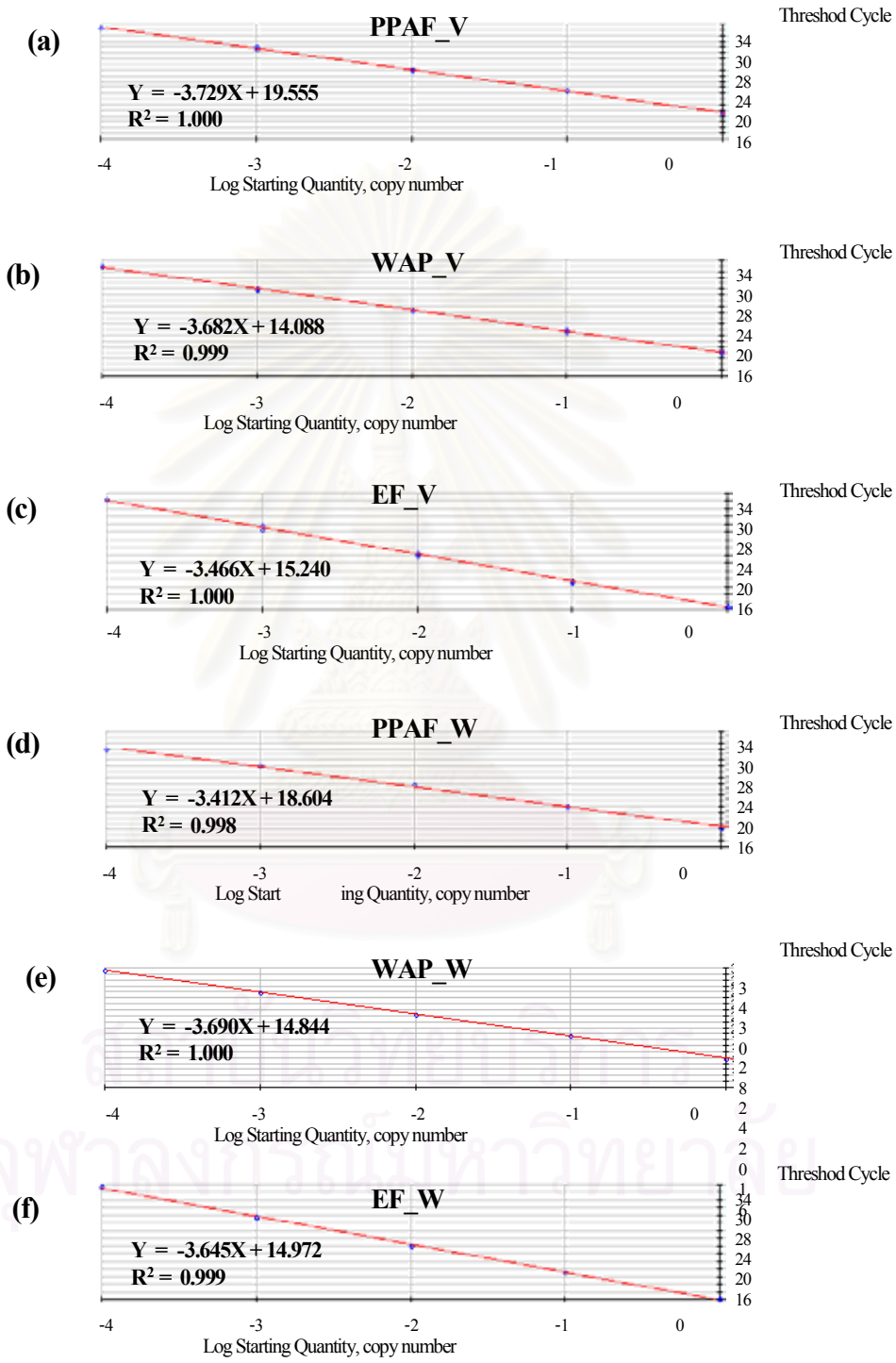


Figure 3.11 Amplification efficiency curves of immune-related genes; PPAF(a,d) and WAP (b, e) , and reference gene EF-1α (c, f) after injected With *V. harveyi* (a, b, c) or WSSV (d, e, f)

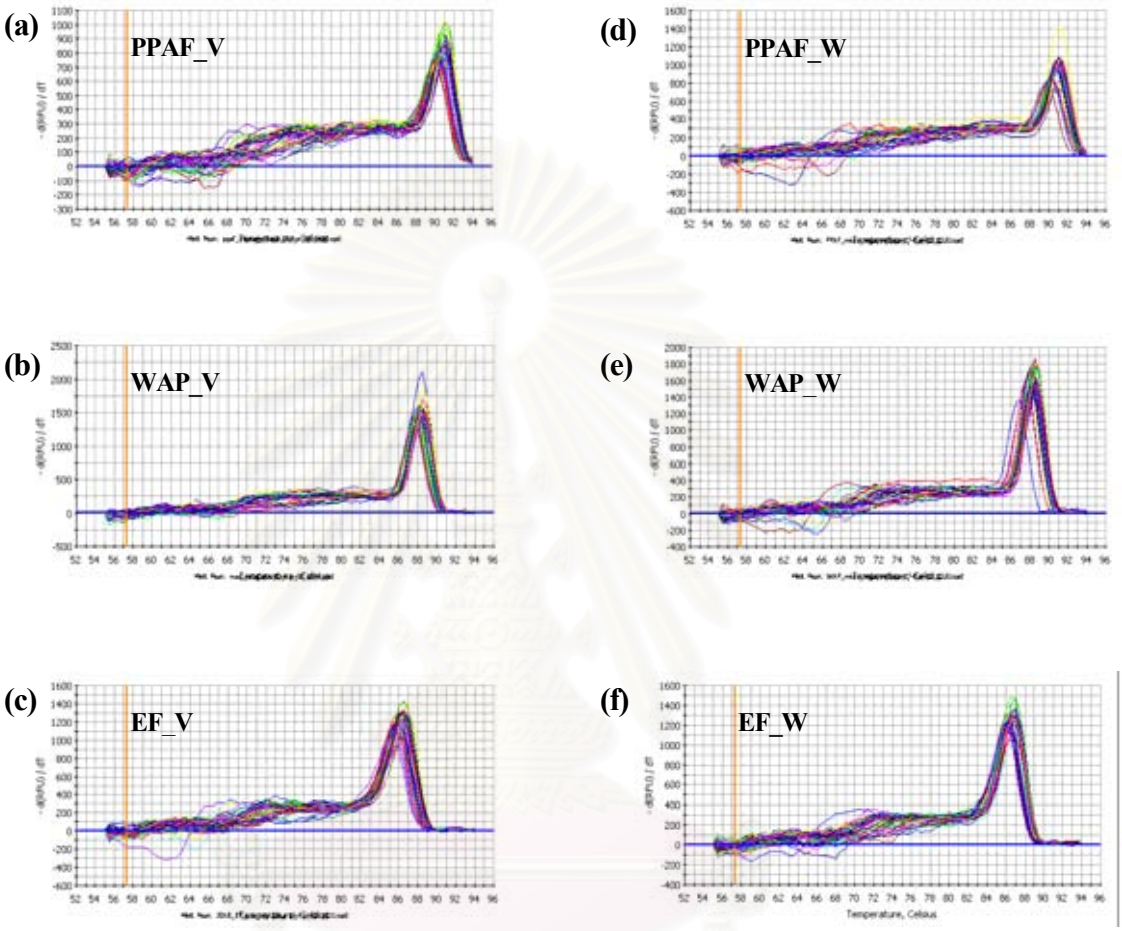


Figure 3.12 The dissociation curves of immune-related genes; PPAF (a, d) and WAP (b, e), and reference gene EF-1α (c, f) after injected with *V. harveyi* (a, b, c) or WSSV (d, e, f).

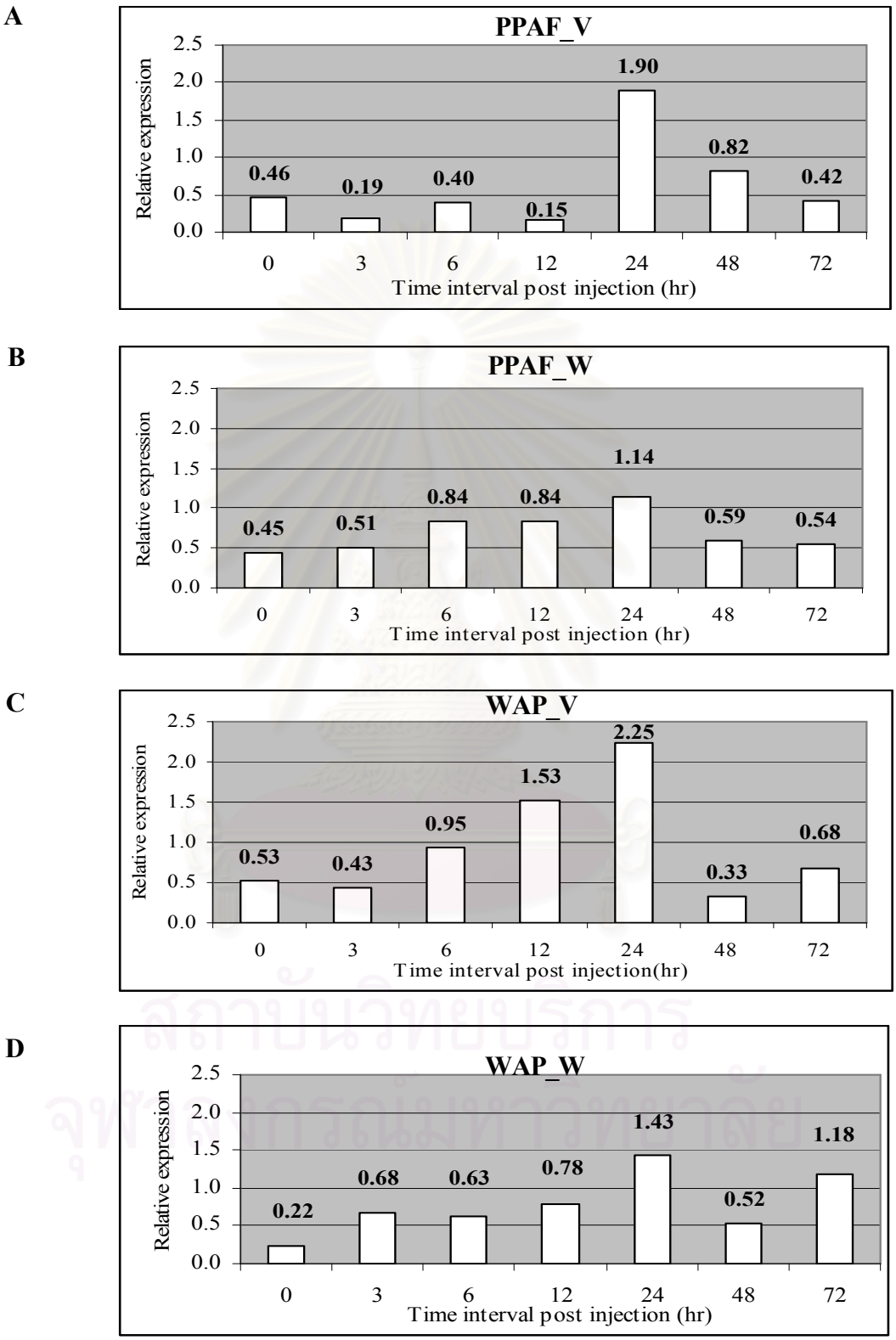


Figure 3.13 Time-course analysis of PPAF (A,B) and WAP (C,D) transcrips in *V. harveyi* (A,C)or WSSV (B,D) challenge shrimp using real time PCR.

Table 3.6 The PCR efficiency for each amplified gene and melting temperature of its product

Gene name	Melting temperature (°C)	Correlation Coefficient		Slope		PCR Efficiency*	
		V. harveyi injection	WSSV injection	*V. harveyi* injection	WSSV injection	*V. harveyi* injection	WSSV injection
PPAF	90.5	1.000	0.998	-3.729	-3.412	1.85	1.96
WAP	88.5	0.999	1.000	-3.682	-3.690	1.87	1.87
EF	86.0	1.000	0.999	-3.466	-3.645	1.94	1.88

PCR Efficiency* was $10^{-1/slope}$

Table 3.7 Expression levels of immune-related gene using real-time PCR in *V. harveyi* challenged shrimp.

Genes	Relative Expression						
	0 hr	**3 hr**	**6 hr**	**12 hr**	**24 hr**	**48 hr**	**72 hr**
PPAF	0.46 (±0.09)	0.19 (±0.15)	0.40 (±0.07)	0.15 (±0.02)	1.90 (±0.10)	0.82 (±0.01)	0.42 (±0.05)
WAP	0.53 (±0.03)	0.47 (±0.06)	0.95 (±0.08)	1.53 (±0.10)	2.25 (±0.07)	0.33 (±0.01)	0.68 (±0.04)

Table 3.8 Expression levels of immune-related gene using real-time PCR in WSSV challenged shrimp.

Genes	Relative Expression						
	0 hr	**3 hr**	**6 hr**	**12 hr**	**24 hr**	**48 hr**	**72 hr**
PPAF	0.45 (±0.03)	0.51 (±0.05)	0.84 (±0.21)	0.84 (±0.08)	1.14 (±0.16)	0.59 (±0.08)	0.54 (±0.00)
WAP	0.22 (±0.02)	0.68 (±0.02)	0.63 (±0.06)	0.78 (±0.02)	1.47 (±0.10)	0.52 (±0.02)	1.18 (±0.09)

3.4 Expression patterns of immune related genes in different developmental stages of *P. monodon* by a semi-quantitative RT-PCR

Genes showing up-regulation against the pathogens were subjected to further investigation for their expression at different developmental stages. Those genes were PPAF, GPx, WAP, TG, PPI and HSP 60. Shrimp samples were collected in early-larval (NaupliusIII (N3), Zoea II (Z2), Mysis II (M2)), post-larval (PL5 and 15) and juvenile 1 month (J1) stages. At each stage, the sample was collected in triplicates.

The cDNA was synthesized from total RNA of each developmental stage of *P. monodon*. The cDNA was used as template for PCR that amplification and the resulting amplicons were analyzed by electrophoresis. The EF was used as an internal control gene. The results are shown in Fig. 3.14. From the expression pattern, transcripts of PPAF, GPx, TG, PPI and HSP 60 were detected throughout *P. monodon* development. However, transcripts of WAP were not observed in N3, but they were appeared in the later developmental stages. The relative expression levels of PPAF, GPx, WAP, TG, PPI and HSP 60 in different developmental stages of *P. monodon* were shown in Table 3.9 and Fig3.15 and the data analysis were shown in Appendix E.

The expression levels of PPAF and TG were significantly increased ($p < 0.05$) in PL5 when compared with the relative expression in larval stage (N3, Z2 and M2) and decreased in the juvenile stages. Likewise, the expression levels of GPx were significantly increased ($p < 0.05$) in PL15 when compared with the relative expression in the larval stage (N3, Z2 and M2). In both PL5 and PL 15 stages, the expression levels of PPI were higher than those other stages. In contrast, the expression levels of HSP 60 were significantly decreased ($p < 0.05$) in M2 and 1 month old shrimp.

Interestingly, transcripts of WAP were not found in N3 stage, but they were shown to markedly significant increase ($p < 0.05$) during the development of Z2 to PL15 and slightly significant decrease ($p < 0.05$) of the juvenile stage. The results were show in Table 3.9

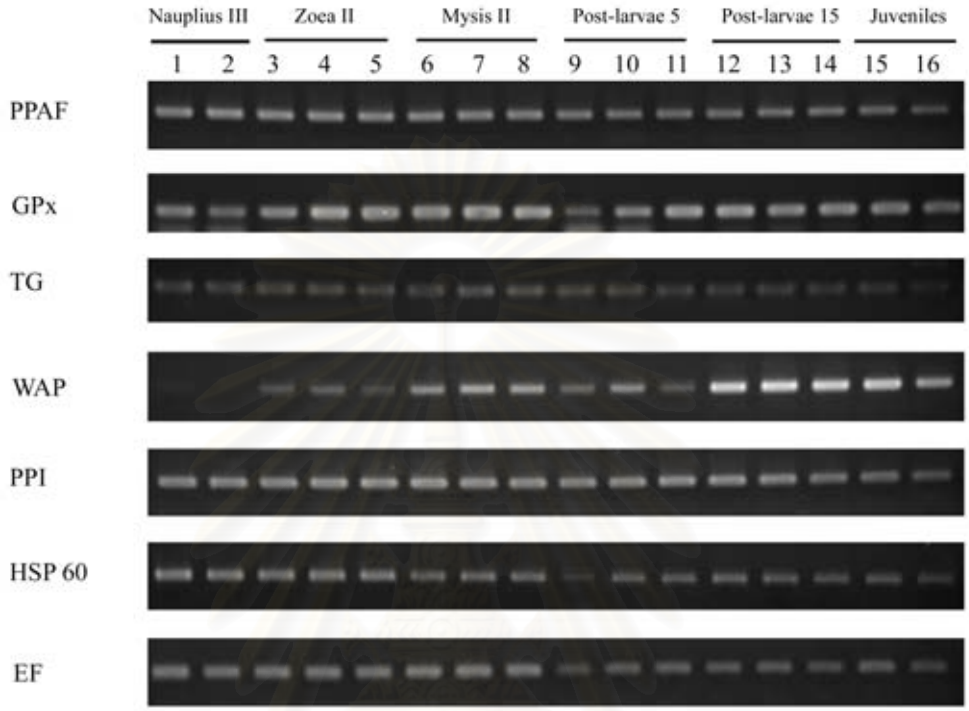


Figure 3.14 Reverse transcriptase-polymerase chain reaction (RT-PCR) of immune-related genes expression from total RNA at various developmental stages of the shrimp *P. monodon*; NaupliusIII (1-3), Zoea II (4-6), Mysis II (7-9), Post-larvae 5 (10-12), Post-larvae 15 (13-15) and Juvenile (16-18). EF was used as an internal control.

Table 3.9 Expression levels of some immune related genes in different developmental stages of *P. monodon* by a sequantitative RT-PCR.

Genes	Relative expression					
	N3	**Z2**	**M2**	**PL5**	**PL15**	**J1**
PPAF	109.79^{ab} (±5.18)	110.35^{ab} (±3.84)	93.40^{a} (±4.69)	140.15^{c} (±23.14)	135.26^{bc} (±13.11)	97.00^{a} (±1.41)
GPx	103.36^{a} (±3.92)	153.25^{ab} (±23.99)	158.73^{ab} (±8.8)	185.10^{bc} (±60.32)	243.35^{c} (37.12)	174.695^{bc} (±10.54)
TG	51.43^{ab} (±7.67)	50.29^{ab} (±2.90)	54.35^{ab} (±7.8)	93.88^{c} (±24.9)	74.12^{bc} (±1.68)	39.27^{a} (±8.08)
WAP	0.00^{a} (±0.00)	58.17^{b} (±11.73)	116.78^{c} (±13.52)	173.43^{d} (±26.08)	417.67^{e} (±43.68)	308.00^{f} (±6.15)
PPI	132.77^{a} (±15.75)	141.53^{a} (±7.67)	132.78^{a} (±2.75)	227.98^{b} (±24.26)	220.60^{b} (±23.11)	135.02^{a} (±15.20)
HSP60	116.48^{a} (±7.10)	110.43^{a} (±2.20)	74.71^{b} (±4.87)	89.87^{ab} (±31.04)	119.65^{a} (±7.06)	79.36^{b} (±2.71)

* Data in the same row having different letters are significantly different ($P<0.05$) in a mean expression level of the immune-related gene.

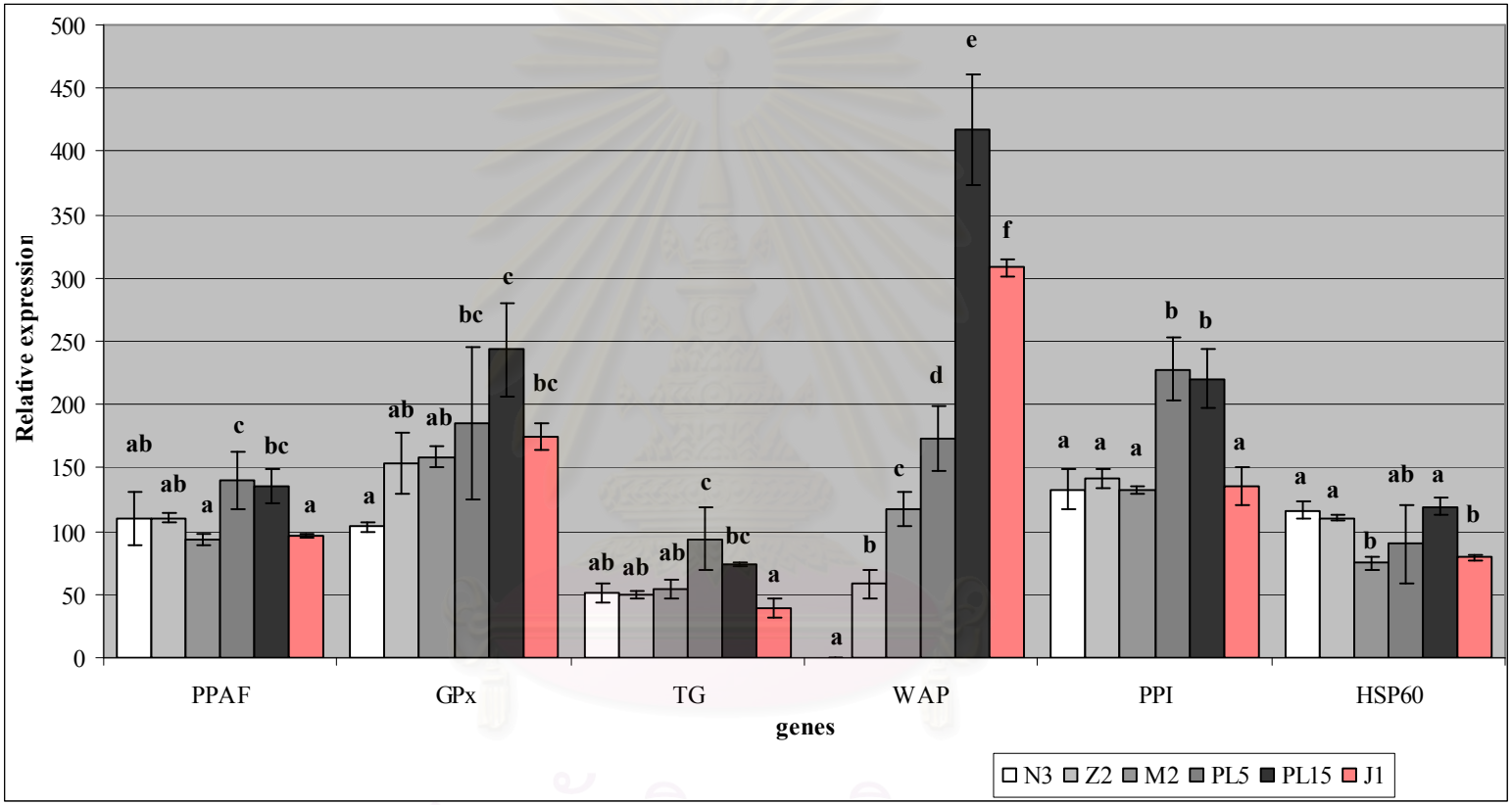


Figure 3.15 Expression levels of the 6 immune-related genes in NaupliusIII (N3), Zoea II (Z3), Mysis II (M3), post-larvae (PL5 and 15) and juvenile (J1) stages of *P. monodon* by a semiquantitative RT-PCR. Different letters indicate significant ($p < 0.05$) difference in a mean expression level of each the immune-related gene.

3.5 Gene expression analysis of *P. monodon* from different populations

Expression levels of PPAF, GPx, WAP, TG, PPI and HSP 60, were examined by a semiquantitative RT-PCR from hemocytes of juvenile *P. monodon* (3 months old) from 3 different shrimp farms. The samples (15 individuals) were collected from 2 commercial farms in Suphunburi, Chachoengsao and a selective domesticated *P. monodon* from Nakhonsithammarat (specific-pathogen-free (SPF) shrimp).

Total RNA was prepared from hemocytes of individual shrimp by using a Trizol reagent. After the first stranded cDNAs were synthesized, they were pooled into triplicate composing of 5 individual shrimps in each group. The pooled-cDNAs were used as the templates, in the semi-quantitative RT-PCR amplification.

The amplification products were run on a 2% of agarose gel and a ratio of band intensity of the target gene and the control gene (elongation factor 1 alpha, EF) was analyzed using Genetools analysis software (Syngene). The expression was determined as the signal ratio of the interested gene: elongation factor 1 alpha (EF) while the expression of EF was normalized to 100. The results are shown in Fig. 3.16. .

The transcription levels of PPAF, WAP, PPI and TG were significantly higher ($p < 0.05$) in the domesticated *P. monodon* than those from the two commercial farms. The GPx expression of the domesticated *P. monodon* was significantly higher ($p < 0.05$) than that of *P. monodon* from Supunburi but was not significantly different from those in Chachoengsao farm. No significant difference ($p > 0.05$) in the expression level of HSP60 was observed in *P. monodon* from the 3 farms. The results are shown in Fig. 3.17 and Table 3.10 and the data analysis were shown in Appendix F.

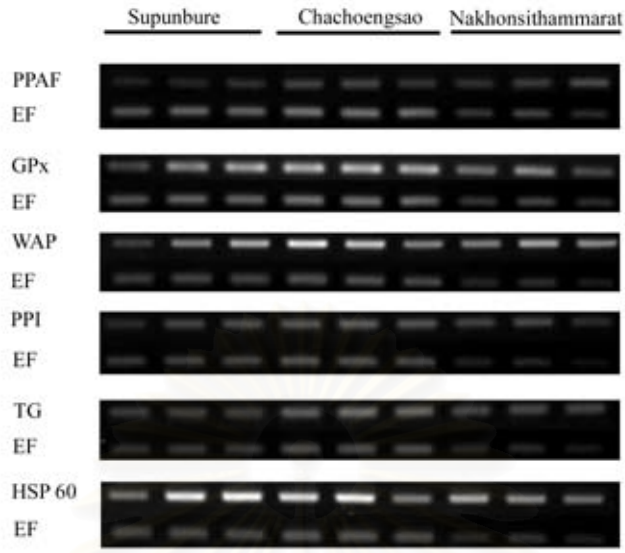


Figure 3.16 Analysis of expression levels of PPAF, WAP, PPI, TG and HSP 60 transcripts in hemocytes of *P. monodon* from three different farms. EF was used as an internal control.

Lanes 1-3 : *P. monodon* from the commercial farm in Suphunburi

Lanes 4-6 : *P. monodon* from the commercial farm in Chachoengsao

Lanes 7-9 : *P. monodon* from the domesticated farm in Nakhonsithammarat

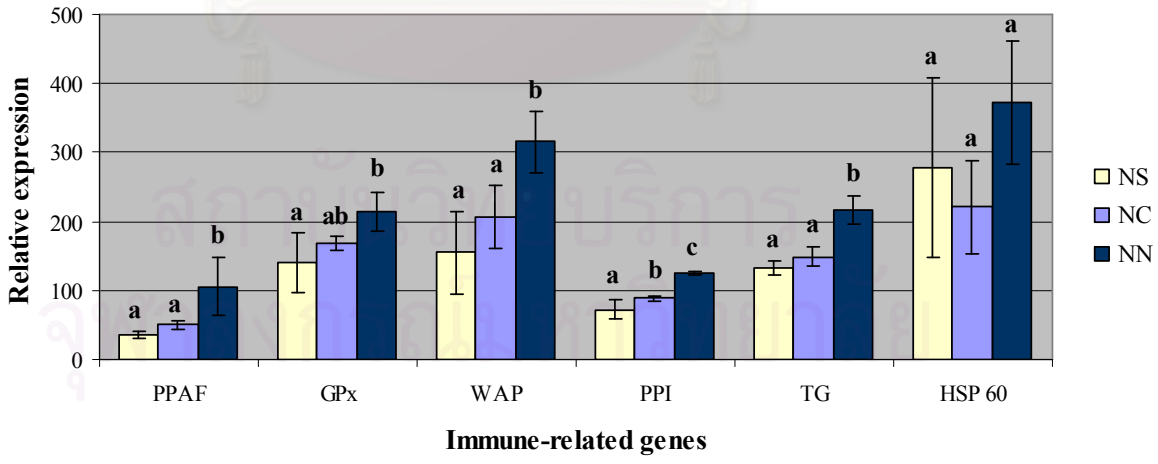


Figure 3.17 Expression level of immune-related genes of *P.monodon* from commercial farm in Suphunburi (NS) and Chachoengsao (NC) and a domesticated farm in Nakhonsithammarat (NN). Different letters indicate significant ($p < 0.05$) difference in a mean expression level of the immune-related gene.

Table 3.10 The expression level of the immune-related genes of *P. monodon* from the commercial and domesticated farms. The experiment were performed in triplicate (n=5).

Genes	Relative Expression		
	Commercial farms		domesticated farm
	Suphunburi	Chachoengsao	Nakhonsithammarat
PPAF	36.20^{a} (±5.18)	50.43^{a} (±5.87)	105.65^{b} (±42.26)
GPx	140.15^{a}(±44.15)	168.22^{ab}(±9.30)	214.21^{b}(±28.86)
WAP	155.09^{a} (±59.96)	206.88^{a} (±45.52)	316.07^{b} (±44.60)
PPI	72.03^{a} (±13.50)	88.39^{b} (±3.14)	125.37^{c} (±2.95)
TG	132.97^{a} (±10.03)	148.58^{a} (±14.23)	216.45^{b} (±19.83)
HSP 60	278.04^{a} (±128.86)	221.07^{a} (±67.08)	372.86^{a} (±89.77)

Data in the same row having different letters are significantly different ($P<0.05$) in a mean expression level of the immune-related gene.

CHAPTER IV
DISCUSSIONS

Tremendous economic losses are realized in the culture of crustaceans due to viral and bacterial epizootics. At present, the problem of infectious disease outbreaks in cultured shrimp is still uncontrollable. Therefore, the genetic selection for healthy or disease resistant broodstock might solve the problem. To select the healthy or disease resistant broodstock, the understanding of shrimp immunity is thus important.

The shrimp defense mechanisms are based mainly on innate immune responses. Innate immunity comprises both cellular and humoral reaction components of the circulatory system, which interplay for detecting and for eliminating foreign and potentially harmful microorganisms and parasites. The first immune process is the recognition of invading microorganisms, which is mediated by the haemocytes and by plasmatic proteins (Vargas-Albores et al., 1993). Distinct pattern recognition proteins (PRPs) recognize and bind to molecules present on the surface of microorganisms but absent in multicellular organisms such as bacterial lipopolysaccharides (LPS), peptidoglycans (PG), and fungal beta-1,3- glucans, and thus trigger responses such as phagocytosis, nodule formation, encapsulation, activation of proteinase cascades, and synthesis of antimicrobial peptides (Cerenius et al., 1994; Kim et al., 2000; Tho¨rnqvist et al., 1994; Yu et al., 2002). Several PRPs like LPS- and glucan-binding protein (LGBP) genes involve in the activation of pro-PO are identified in *Penaeus stylirostris* (Roux et al., 2002). Furthermore, He et al. (2004) identified genes in hemocyte of *P. japonicus* following microbial challenge found that Kunitz-type protease inhibitor, chaperonin (chaperonin containing TCP1 (CCT)), and antioxidant (cytochrome *c* oxidase and NADH dehydrogenase) were expressed at a higher level after the challenge. For viral stimulation, WSSV caused up-regulation of lipopolysaccharide and beta-1, 3-glucan binding protein (LGBP), protease inhibitors, apoptotic peptides and tumor-related proteins in Penaeus shrimp (Roux et al., 2001; Rojtinnakorn et al., 2002). On the other hand, the haemolymph, total haemocyte count, phagocytosis, phenoloxidase (PO), superoxide anion (O_2^-) and superoxide

dismutase (SOD) production are significantly reduced, which occurred in the first 1–4 days. The immune system of shrimp consequently crashed and high mortality occurred (Chang et al., 2003). Besides, Song et al. (2003) has reported that in Taura syndrome virus-infected shrimp, plasma phenoloxidase (PO) activity was significantly increased.

In the present study, we examined the expression of some immune related genes in order to identify candidate genes that can be use as biomarkers for health monitoring and have potential used for broodstock selection. At present, a variety of methods are used to quantify mRNA expression such as northern blotting and *in situ* hybridisation (Parker and Barnes, 1999), RNAse protection assays (Hod, 1992; Saccomanno et al., 1992), the reverse transcription polymerase chain reaction (RT-PCR) (Weis et al., 1992) and the cDNA arrays (Bucher, 1999). Northern analysis is the only method providing information about mRNA size, alternative splicing and the integrity of RNA samples. The RNAse protection assay is most useful for mapping transcript initiation and termination sites and intron/exon boundaries, and for discriminating between related mRNAs of similar size, which would migrate at similar positions on a northern blot. *In situ* hybridisation is the most complex method of all, but is the only one that allows localisation of transcripts to specific cells within a tissue. The main limitation of Northern analysis, *in situ* hybridisation, RNAse protection assays techniques is their comparatively low sensitivity (Melton et al., 1984). The cDNA arrays, is still limited in its use by cost considerations. Quantitative RT-PCR is the method of choice used to quantify the mRNA expression which is useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts (Carding and Bottomly, 1992). It is the most sensitive and accurate of the quantification methods (Wang and Brown, 1999). Since the discovery of PCR numerous applications have been described to quantify the results, such as semi-quantitative and quantitative competitive RT-PCR and its latest innovation quantitative “real-time” RT-PCR.

Competitive PCR is carried out based on the competition of the target RNA and known amount of a synthetic homologous competitor template, usually engineered to share the primer recognition site with the target sequence but to differ either in length or by a short heterologous sequence stretch and the overall PCR

efficiency is not affected by the modification (Orlando et al., 1998; Goerke et al., 2001). This technique is an "end-point" measuring method occurs when the PCR reaction is completed. An amplification curve with the internal competitor is then built for each sample. Quantification is rather accurate, but this technique requires a relatively large amount of cDNA and a large number of amplification reactions per sample. Moreover, intensive initial works are required when different RNAs have to be analyzed and hence different competitors need to be constructed.

Semi-quantitative RT-PCR for evaluation transcriptional levels of immune-related genes found in this study was developed. This method simultaneously amplifies interesting genes and the internal control (a housekeeping gene). The PCR product is quantitatively analyzed at the exponential phase of amplification. As a result, the PCR product at this point is surely related to the amount of mRNA at the initiation. This method is a highly sensitive, specific and easy to set up (Marone et al., 2001). Semi-quantitative RT-PCR assumes that the experimental conditions do not alter the expression levels of the selected control RNA. The PCR product of the interesting genes are analyzed relatively to the internal control. However, Semi-quantitative RT-PCR is only able to tell that one transcript is expressed at a higher level than the other.

Real-time reverse transcription (Real-time RT-PCR) based on fluorescence-kinetic RT-PCR enables quantification of the PCR product in "real-time.". This sensitive and accurate technique measures PCR product accumulation during exponential phase of the reaction (Bustin, 2002; Klein, 2002). The technique much faster than the previous endpoint RT-PCR as it is designed to provide information as rapidly as amplification process itself, thus requiring no post-PCR amplification. Real-time RT-PCR can be used to compare and quantitate expression of selected genes in different biological samples. Real-time RT-PCR data analysis methods may be broadly classified as absolute quantitation, based either on standard curve method or relative based on the comparative threshold method. The comparative threshold method was used to report the changes in expression of interested genes relative to reference gene in a given treatment. Expression of target genes was normalized with reference genes of housekeeping genes. In this study, elongation factor-1 alpha (EF-1 alpha was used as reference gene.

Housekeeping genes are present in all nucleated cell types since they are necessary for basis cell survival, like glyceraldehydes-3-phosphate dehydrogenase (G3PDH or GAPDH), actins, cyclophilin, 18S rRNA, 28S rRNA or EF were applicable.

The glycolytic tetramer glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional enzyme involved in cellular metabolism. Although it was initially believed that GAPDH is expressed as a single-copy nuclear gene (Bhatia, et al., 1994), genome sequencing data have identified at least two functionally independent GAPDH genes in Caenorhabditis elegans, Drosophila melanogaster and humans. Predictably this gene also has limitations too. For example it has been reported that its use should be avoided in experimental hypoxia (Zhong and Simons, 1999), cancer cell lines (Rondinelli et al., 1997), during ontogeny and acute pancreatitis (Calvo et al., 1997), cell proliferation and carcinogenisis (McNutty and Toscano, 1995).

Actin is one of the major components of cytoplasmic microfilaments in eukaryotic cells. It plays an important role in diverse cellular functions, such as cyctoplasmic streaming, changes in cell shape, cell motility, phagocytosis, cell division, the distribution of plasma membrane proteins and the generation of contractile force in both muscle and non-muscle cells (Romans et al., 1995; Kusakabe, 1997). As a member of a multigene family, every organism typically possesses three to four highly homologous actin isoforms. Although actin is one of the most widely used internal control genes, there is an increasing volume of evidence suggesting that the relative amounts of each isoform expressed and the total actin content can vary with muscle type, development, cell culture conditions, pathologically and potentially between cells within tissues (Drew and Murphy, 1997)

Cyclophilins, or peptidyl-prolyl cis-trans isomerases, are enzymes belonging to the superfamily of immunophilins. Their biological significance is manifested by the catalysis of protein folding via peptide bond rotation on the amino side of proline residues (Fischer et al., 1989; Takahashi et al., 1989), the action as a chaperone for protein trafficking as well as the nucleolytic degradation of the genome (Montague et al., 1997). Numerous reports have observed a differential induction of CYP, in general, mainly during development or exposure to certain stressors. It has been

shown for example, that mercuric chloride (Martinez-Gonzalez and Hegardt, 1995), development (Marivet et al., 1992), heat shock, virus infection, ethephon and salicylic acid exposure (Marivet et al., 1994) significantly induce CYP expression in plants. Similarly, differential regulation was observed in hypoxia and heat stressed myogenic cells (Andreeva et al., 1997).

The 18S and 28S ribosomal subunits are a further example of commonly used internal controls. A literature review of its uses revealed a reoccurring theme: when directly compared to other housekeeping genes they compare extremely favourably in terms of steady-state expression levels. The ribosomal subunits are not polyadenylated they cannot be exploited when dealing with poly(A)+RNA or cDNA derived from total RNA utilising poly T-primers in the RT reaction. It is precisely for this reason that the ribosomal subunits have failed to replace the use of other housekeeping genes. Although it possesses very promising invariant characteristics, its uses are limited, especially in the light of the fact that an increasing number of laboratories prefer the use of mRNA or polyT-primers in the RT step.

The elongation factor-1 alpha (EF-1 alpha) is an ubiquitous protein that binds aminoacyl-transfer RNA to ribosomes during protein synthesis. It has been stipulated to be a good invariant control to adjust for differences in tube-to-tube loading and/or degradation (Dostal et al., 1994). In shrimp, elongation factor 1-alpha gene (EF-1α) was identified as constitutively expressed gene and was used as reference gene for real-time RT-PCR analysis (Astrofsky et al., 2002). Furthermore, Dhar et al. (2004) suggest that EF1-alpha will be a good internal control for moderate and highly expressed genes, whereas GAPDH will be a better control for low expressed genes for measuring gene expression in WSSV shrimp by real-time RT-PCR.

To study expression of some immune related genes in *P. monodon*, selected immune gene from ESTs in different tissues and conditions. The EST information could be accessed from the *P. monodon* EST project (http://pmodon.biotec.or.th).

A total of 2,312 ESTs obtained from the *P. monodon*'s hepatopancreas, *Vibrio harveyi*-challenged lymphoid organ, healthy and *V. harveyi*-challenged hemocytes were analyzed the putative gene identification. One hundred and eighty clones representing 6.3% of the total clone sequences were identified as putative immune-

related genes and categorized into 7 different subgroups consisting of antimicrobial molecules, ProPO systems, oxidative enzymes, proteinases and inhibitors, heat shock proteins, apoptotic- and tumor-related proteins and other immune-related molecules. In this study, genes involving immune responses of *Peneaus monodon* were selected from the EST database and analyzed the expression level in order to identify candidate markers for the application in broodstock selection.

The total of fourteen putative immune genes were isolated from the ESTs of *P. monodon* based on functional categories consisting of the prophenoloxidase activating factor (PPAF), transglutaminase (TG), WAP four-disulfide core domain protein 5 precursor (WAP), glutathione peroxidase (GPx), cytosolic manganese superoxide dismutase precursor (SOD), chaperonin containing TCP1 subunit 4 (CCTP), heat shock protein 60 (HSP60), apoptosis inhibitor survivin (survivin), P 109 protein, Peptidyl-prolyl cis-trans isomerase 5 precursor (PPI), cyclophilin 18, thrombospondin 4, techylectin-5B and lectin C-type as previously illustrated to homologous with other species.

The immune reaction of shrimp and crustaceans is mainly accomplished through circulating hemocytes and many immune factors located in hemocytes or released into plasma from hemocytes (Bachère, 2000). Therefore, 14 immune-related genes from the ESTs of the black tiger shrimp were determined the expression pattern in hemocytes. From the results, PPAF, TG, WAP, GPx, SOD, CCTP, HSP 60, Survivin, P109, PPI and Cyclophilin genes were found significantly expressed in shrimp hemocytes

Expression levels of eleven putative genes including PPAF, TG, WAP, GPx, SOD, CCTP, HSP 60, survivin, P109, PPI and cyclophilin in hemocytes of *P. monodon* were analyzed at different treatment periods after shrimps were challenged with the appropriate amount of *V. harveyi* or WSSV using semi-quantitative RT-PCR and the expression levels of PPAF and WAP were quantitatively determined by Real-time RT-PCR. The results showed that both pathogens caused up-regulation of PPAF mRNA. The higher expression of PPAF at 24 hours (4.13 and 2.53 fold difference comparing with relative expression at 0 hpi) after injected with *V. haeveyi* and WSSV, respectively. Microbial cell wall (β-1,3-glucan, peptidoglycan and lipopolysaccharide

(LPS)) and viral components (probably capsid proteins) might induce the activation of the pro-PO-activating enzyme (Aspán and Söderhäll, 1991; Duvic and Söderhäll, 1992; Yoshida et al., 1994; Song et al., 2003). It has also been the hypothesis of the pattern recognition molecules (binding protein), a complex with the proPO-activating enzyme(s) and microbial cell wall components, to activate proPO activating enzyme(s) and converts the proPO to an active phenoloxidase (PO) by limited proteolysis (Söderhäll and Cerenius, 1998; Ashida and Brey, 1998; Gillespie et al., 1997).

Likewise, change in expression of WAP protein were significant increase with the maximum at 24 h (4.23 and 6.68 fold difference compared with relative expression at o hpi, respectively) after *V. harveyi* and WSSV stimulation. A similar expression profile was observed in *L. vannamei* where the WAP domain (SWD) mRNA levels increased at 3 and 6 h and returned slowly to non-stimulated levels from about 12 to 24 h after injection of *V. alginolyticus* (Jim-Vega et al., 2004). In vertebrates, WAP domain can also be stimulated as found in mouse, by the SLPI synthesis was observed from 60 min, obtaining the maximum expression in 24-36 h (Jin et al., 1998). It's indicated that the probable role of this protein in the immune system may be necessary to protect host from microbial proteases or virus and to regulate the proteases cascades (the proPO and coagulation system) (Laskowski and Kato, 1980; Aspan et al., 1990).

Cytosolic manganese superoxide dismutase (SOD) is an important enzyme in the antioxidant defense pathway responses to oxidative stress produced by activated phagocytes (Fridovich, 1995). The mRNA level of SOD in *P. monodon* was significant decrease after *V. harveyi* and WSSV stimulation, similar to the enzymatic activity reported in *Palaemonetes argentinus* after infected with *Probopyrus ringueleti* (Neves et al., 2000). Besides, these results are supported by the previous evidence in ileum of rabbit that significant decrease in the levels of SOD enzyme in the enterocytes isolated from *V. cholerae* 0139 treated ileum (Gorowara et al., 1998). 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 (Munoza et al., 2000; Thörnqvist and Söderhäll. 1997).

After that, SOD level returns to normal level suggested that host cell might protect themselves from oxidative damage.

Survivin and P 109 protein are an inhibitor of apoptosis (IAP) protein regulating apoptosis at cell division (Liston et al., 1996; Uren et al., 1996). The decreasing level of survivin transcript in *P. monodon*, after *V. haeveyi* and WSSV challenged, appeared earlier in hemocytes (3 h). In addition, this study found that survivin gene increasingly expressed after 48-72 h after *V. harveyi* stimulation whereas returned to normal level in WSSV stimulation. The lower expression of survivin at early injection might promote the apoptosis in infected-cells due to avoid virus spreading by eliminating microbial and virus-infected cell (Anggraeni and Owens, 2000). After that the upregulation of survivin may suppress apoptosis in shrimp hemocyte (Sahtout et al., 2001; Khanobdee et al., 2002). Furthermore, Wongprasert et al. (2003) reported that apoptosis in various tissues of the black tiger shrimp occurred following WSSV injection. On the other hand, no significant differences in the level of P109 mRNA in *P. monodon* were observed after challenged with *V. harveyi* or WSSV. From those results, it is suggested that survivin involves in inhibition of apoptosis in hemocyte of *P. monodon*, whereas P109 is still unknown properties in immune system of *P. monodon*.

For the RT-PCR results showed only a significant increase expression of TG in hemocyte of *V. harveyi*-injected *P. monodon* as well as the previous evidence in horseshoe crab that the components of hemolymph coagulation were released and the system was initiated upon stimulation by microbial polysaccharides (Iwanaga and Kawabata et al., 1998). Although, the expression levels of TG in WSSV-injected *P. monodon* were not changed, Song et al. (2003) observed that the coagulation system of *L. vannamei* seemed likely to be a target in virus attacks which hemocytic TG activity was declined concomitantly after the Taura Syndrome Virus (TSV) infection. Differential immune response may be according to host factors, for example species, age, physiological state and pathogens species.

The glutathione peroxidase (GPx) has been reported as an important extracellular antioxidant, to remove the hydrogen peroxide and various hydroperoxides from cells. These are mechanisms to protect host cell from free-

radical damage (Ho et al., 1998; Rich-Evans et al., 1993). Mates et al. (1999) reported that gene expression of glutathione peroxidase was up-regulated by H_2O_2 and other ROS. The mRNA level of GPx in *P. monodon* was significantly increased after *V. harveyi* stimulation. The upregulation of GPx is suggested that *V. harveyi* may be over induced reactive oxygen species (ROS) generation in hemocyte of *P. monodon* (Song and Hsieh, 1994) or *V. harveyi* may have some mechanism that induces GPx transcription in shrimp in order to protect them from free-radical damage or it may employ a novel way(s) to activate GPx transcription to evade the host defense. In contrast, the mRNA level of GPx was significantly decreased after WSSV stimulation. With a significant decrease of GPx mRNA, it might accumulate H_2O_2 which H_2O_2 are potentially deleterious to microorganism (Munoza et al., 2000; Thörnqvist and Söderhäll, 1997). The expression profiles of GPx in response to exterior pathogen and the difference of expression profiles between *Vibrio* and WSSV infection provides some clues to further understanding the complex innate immune mechanism in shrimp.

Mammalian Hsp60 is the assistance to correct the folding protein and may be the potential activators of the innate immune system (Wallin et al., 2002; Tsan and Gao, 2004. However, no significant differences in the transcription level of HSP 60 in *P. monodon* were observed after *V. harveyi* stimulation. Deane et al. (2004) observed that the transcription levels of HSP60 remained unchanged after sea bream fish infected by *V. alginolyticus*. On the other hand, the transcript levels of HSP60 were observed significant increase in WSSV-injected *P. monodon*. Interestingly, Hsp60 was found to involve in the activation of apoptosis that the Hsp60 cooperates to induce apoptosis by acting as a chaperone to procaspase-3 and aiding in its maturation into active caspase-3 (Lim et al., 2001; Samali et al., 1999; Xanthoudakis et al., 1999). HSP60 may be important in the stimulation of the innate immune system against invaded of WSSV in *P. monodon*.

Cyclophilin, a peptidylprolyl isomerase is known in mammals to accelerate the folding of proteins and to mediate signalling events leading to T-cell activation (Shida et al., 2003). Recently, a fish cyclophilin was found from head kidney cells of carp stimulated with peptidoglycan that associated with the increasing protection against infectious disease (Konoa et al., 2004). In *P. monodon*, the mRNA level of

Peptidyl-prolyl cis-trans isomerase 5 (PPI) was significantly increased after *V. harveyi* stimulation, whereas, no significant differences after WSSV stimulation. Besides, PPI-5 was reported that involving in activation of coagulation system, degradation of chromatin during apoptosis and cell signaling after *V. harveyi* infection (Mattila et al., 1990; Montague et al., 1997; Allain et al., 1999). On the contrary, no significant differences in the mRNA level of cyclophilin 18 after *V. harveyi* or WSSV injection. The function cyclophilin in immune system of shrimp have not been previously studied.

Until today, little is known about chaperonin containing t-complex polypeptide-1 (CCTP) and the immune system of shrimp. From suppression subtractive hybridization (SSH) of *P. japonicus* following microbial challenge found that CCTP was also expressed at a higher level after the challenge (He et al., 2004). RT-PCR results showed that the transcription levels of CCTP in *P. monodon* after *V. haeveyi* or WSSV challenged were no significant difference.

In microbial-challenged shrimp as observed in other crustacean species (Smith and Ratcliffe, 1980; Martin et al., 1993; Liu et al., 2005; Somboonwiwat et al., 2005), the total number of circulating hemocytes dramatically drop in the early hours post-injection (3-12 hours), followed by a recovery (with a return to initial level) at about 24-48 hours. During the very low number of total circulating hemocytes in the first phase of pathogen response, PPAF, WAP, GPx, PPI, TG and HSP60 transcripts were found highly expressed and up-regulated. This implies that PPAF, WAP, GPx, PPI, TG and HSP60 genes involve by up-expression in order to function against pathogens and presumably to be the innate immune responses in *P. monodon*.

In general, the early developmental stages of animal are sensitive to diseases than adults. Some immune genes are differentially expressed between larval and adult stages of shrimp. However, until now, little is known about the immune system and the larval and post-larval stages of shrimp development and ontogeny of the immune system in invertebrates as their small size precludes direct study. This study analyzed the expression level of PPAF, GPx, WAP, TG, PPI and HSP 60 in different developmental stages (NaupliusIII (NIII), Zoea II (ZII), Mysis II (MII), post-larvae (PL5 and 15) and juvenile (1 month)) of *P. monodon*. The results showed that the

transcripts of PPAF, GPx, TG, PPI and HSP 60 were detected throughout *P. monodon* development. Those genes might express during embryogenesis or important in stabilize cells under mechanical stress or participate in morphogenesis in some other ways (Chasan and Anderson, 1989; Murugasu-Oei et al., 1995; Singer et al., 1992; Sugino et al., 2002). Likewise, the transcripts of penaeidin are already present in the early stage of nauplius V by RT-PCR analyses (Munoz et al., 2003). However, the transcripts of WAP were not found in NaupliusIII stage, but they were observed in the later developmental stages tested.

In *Drosophila melanogaster*, PPAF is essential for pattern formation during normal embryonic development and expressed during embryogenesis, larval, and pupal developments (Chasan and Anderson., 1989; Murugasu-Oei et al 1995). Likewise, PPI-5 in *Caenorhabditis elegans* was relatively constant throughout the larval and adult stages. However the embryonic expression level was two-fold higher than that observed in larval and adult stages (Picken et al., 2002). Saito et al. (1999) proposed that Cyp5 played a role in postembryonic development rather than acting as a stress-responsive chaperone. In addition, TG mRNA was found during the early blastula stage and increases thereafter in starfish and grasshoppers (Singer et al., 1992; Sugino et al., 2002). Also in vertebrates, the expression of HSP60 was demonstrated in early mouse embryos (Bensaude and Morange, 1983).

Aguirre-Guzmán et al. (2002) demonstrated that the Nauplii to protozoea III stages showed greater susceptibility to the pathogens, compared to mysis I to postlarvae 1. For *P. monodon*, larval substages from N3 to M2 infected with *V. campbellii* showed significantly (p<0.001) lower survival rates than PL stage (Hameed, 1995). From this study, the expression levels of PPAF, GPx, TG, WAP, PPI in post-larval stages (PL5 and PL15) were observed as higher than larval stages (N3, Z2 and M2). This indicates that the expression levels of immune genes simultaneously increase during larval development as reported the increasing resistance of larvae development to the *Vibrio* isolated (Hameed et al., 1996). However, the expression of these genes showed decreasing in juvenile stage, it's possibly due to the fluctuations in oxygen, temperature and salinity in pond that cause the increasing of physiological stresses, a result of the immune response is lowering (Le Moullac and Haffner, 2000).

Expression levels of PPAF, GPx, WAP, TG, PPI and HSP 60, were examined by semi-quantitative RT-PCR from hemocytes of juvenile *P. monodon* from 3 different shrimp farms: commercial farms (Suphunburi and Chachoengsao) and domesticated farm (Nakhonsithammarat). In experiment, shrimp from Nakhonsithammarat is specific-pathogen-free (SPF) shrimp (Free from WSSV and YHV). From the results, the transcription levels of PPAF, WAP, PPI and TG were significantly higher ($p < 0.05$) in the domesticated *P. monodon* than those from the commercial farms. The GPx expression of the domesticated *P. monodon* was significantly higher ($p < 0.05$) than that of *P. monodon* from Supunburi but no significant difference from those in Chachoengsao farm. No significant difference ($p > 0.05$) in the expression level of HSP60 was observed in *P. monodon* from the 3 farms. The different expression levels might be due to genetics, environment, nutrition and farm practices of shrimp. For example geography, genetics, sensitivity to pathogen, size, age, breeding stage, health status of the broodstock, temperature, salinity, pH, dissolved oxygen, feeding and nutrition status, have been reported to effect the immune response in many species of decapod crustaceans (Le Moullac and Haffner, 2000). Furthermore, dietary administration of β-Glucan or peptidoglycan has been used to enhance the immune system in shrimp (Chang et al., 2003; Rattanachai et al., 2005).

Consequently, it was suggested that, the domesticated *P. monodon* might be more healthy than those commercial shrimp (wild animal) based on the level of immune-related genes. This should be confirmed by testing the shrimp from different origins and determine their survival rate after challenged with pathogens in order to select for healthy or disease resistant broodstock.

At present, it has a little biomarkers for immune of shrimp, Song et al. (2003) reported that immunological parameters (haemocyte counts, agglutinin, bacterial growth inhibitory activity, clotting response (transglutaminase activity), phagocytosis-related reactive oxygen intermediates (production of superoxide anion) and phenoloxidase activity) can be used as biomarkers for evaluate shrimp health. In this research, genetic parameters were used as biomarker to accurately assess shrimp health status.

These present results indicate that the expression level of PPAF, WAP, GPx and PPI can be expected to assess shrimp health for selection of healthy shrimp that potential used as broodstock. The additional information from the EST data would further be selected for analyzing the expression levels in order to fine suitable use as biomarker for monitoring shrimp health.

CHAPTER V
CONCLUSIONS

1. A total of fourteen immune-related genes were selected from EST libraries of the black tiger shrimp genome project. The expression of Thrombospondin, Techylectin and Lectin,C-type could not be detected by RT-PCR in hemocyte of *P.monodon*. Therefore, the expression levels of 11 genes including PPAF, TG, WAP, GPx, SOD, CCTP, HSP 60, survivin, PPI and cyclophilin gene were successfully detected in *P .monodon* hemocytes.
2. The determination of expression level of 11 immune-related genes after challenged with *Vibrio harveyi* by semi-quantitative RT-PCR showed that the pathogens caused up-regulation of PPAF, GPx, TG, WAP and PPI genes whereas the expression levels of SOD and survivin were significantly decreased. No significant difference in the expression levels of P109 protein, cyclophilin, CCTP and HSP 60 was observed.
3. The examination of expression levels of 11 immune-related genes after challenged with WSSV by semi-quantitative RT-PCR showed that the expression levels of PPAF, WAP and HSP60 were significantly increased whereas the expression levels of SOD, GPx and survivin were significantly decreased. No significant difference in the expression levels of TG, P109 protein, PPI, cyclophilin and CCTP was observed.
4. By real-time PCR analysis, it was shown that PPAF and WAP transcripts were up-regulated upon *V.harveyi* and WSSV infections. The higher expression levels of PPAF were at 24 hours (4.13 and 2.53 fold difference compared with relative expression at 0 hpi, respectively) after injected with *V. haeveyi* and WSSV, respectively. Likewise, the maximum expression levels of WAP were at 24 h (4.23 and 6.68 fold after compared with relative expression at 0 hpi, respectively) after *V. harveyi* and WSSV stimulation

5. The semi-quantitative RT-PCR was used to determine the expression patterns of 6 immune-related genes in early-larval (NaupliusIII, Zoea II, Mysis II), post-larval (PL5 and 15) and juvenile (1 month) stages of *P.monodon*. The transcripts of PPAF, GPx, TG, PPI and HSP 60 were detected throughout *P.monodon* development, whereas the transcript of WAP could not be found in the NaupliusIII stage, but it was observed in the later developmental stages. Furthermore, the expression levels of PPAF, GPx, TG, WAP and PPI in the post-larval stages were higher than the larval stages.
6. The transcription levels of PPAF, WAP, PPI and TG in hemocytes of juvenile shrimps from domesticated farm were significantly higher ($p < 0.05$) than shrimps from the commercial farms, whereas no significant difference ($p > 0.05$) in the expression level of HSP60 was observed in *P. monodon* from different farms.
7. From expression analysis some immune related genes such as PPAF, WAP, PPI and TG can be candidate biomarkers for health monitoring and have potential used for broodstock selection.

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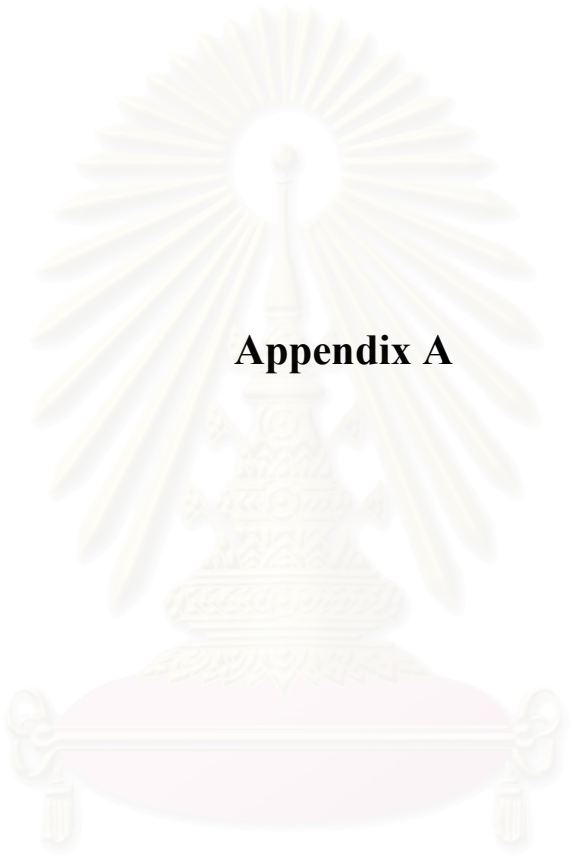
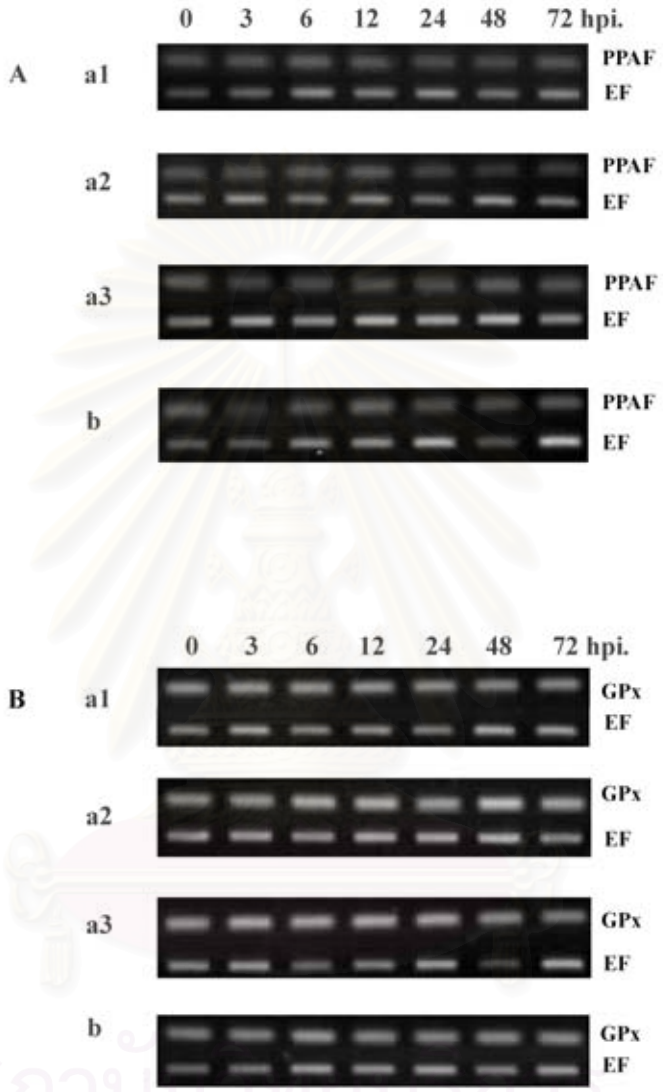
APPENDICES

Appendix A





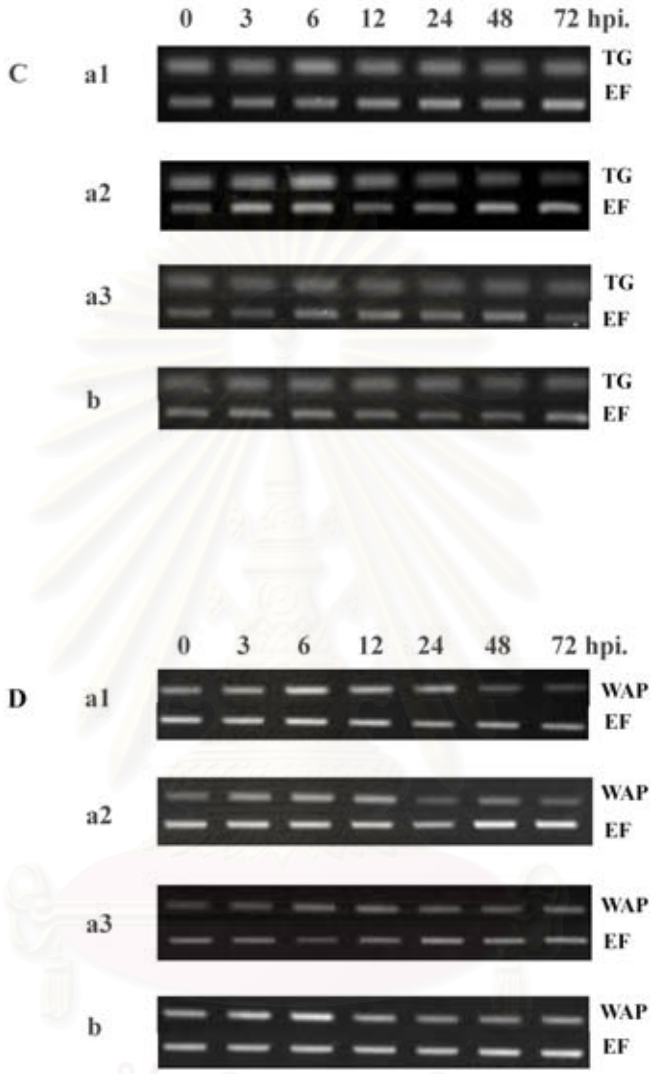
I. Analysis of expression levels of PPAF (A) and GPx (B) transcripts after injection with *V. harveyi* for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : *V. harveyi* injected shrimps, replication 1

Panel a2 : *V. harveyi* injected shrimps, replication 2

Panel a3 : *V. harveyi* injected shrimps, replication 3

Panel b : saline injected shrimps



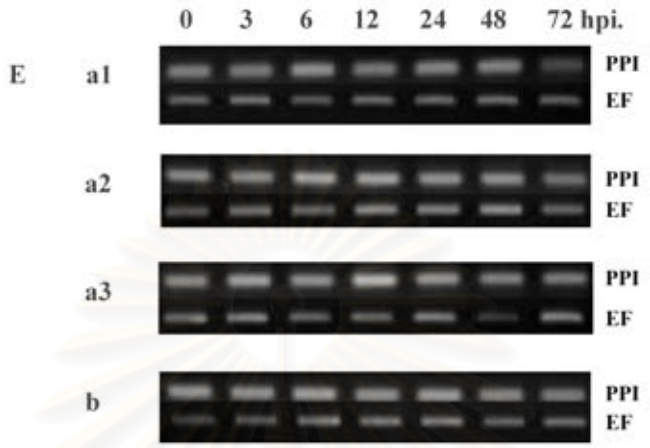
I. (continued) Analysis of expression levels of TG (C) and WAP (D) transcripts after injection with *V. harveyi* for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : *V. harveyi* injected shrimps, replication 1

Panel a2 : *V. harveyi* injected shrimps, replication 2

Panel a3 : *V. harveyi* injected shrimps, replication 3

Panel b : saline injected shrimps



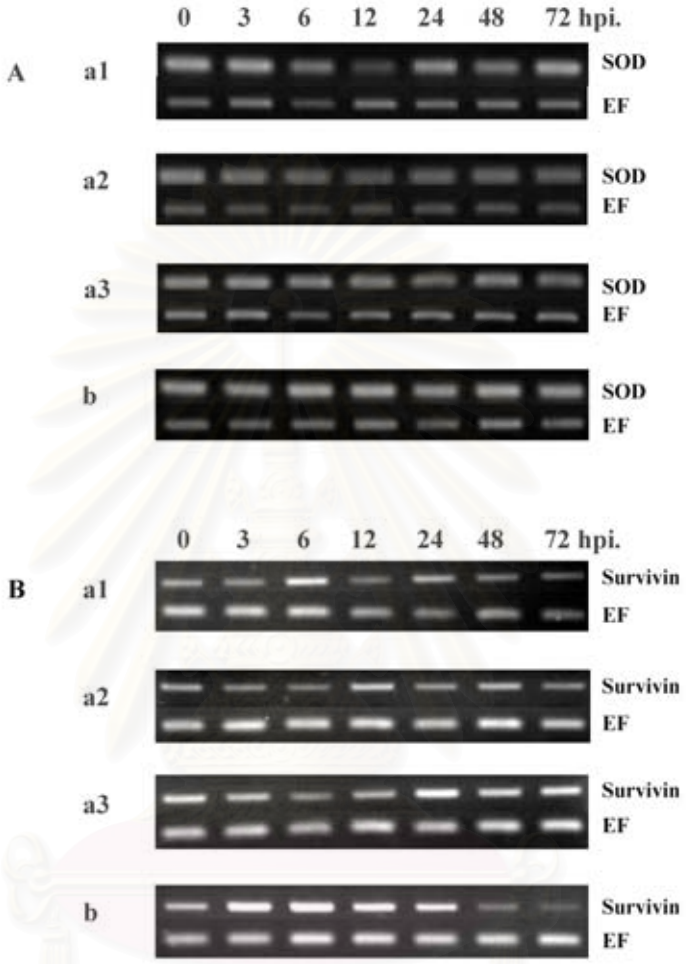
I. (continued) Analysis of expression levels of PPI (E) transcripts after injection with *V. harveyi* for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : *V. harveyi* injected shrimps, replication 1

Panel a2 : *V. harveyi* injected shrimps, replication 2

Panel a3 : *V. harveyi* injected shrimps, replication 3

Panel b : saline injected shrimps



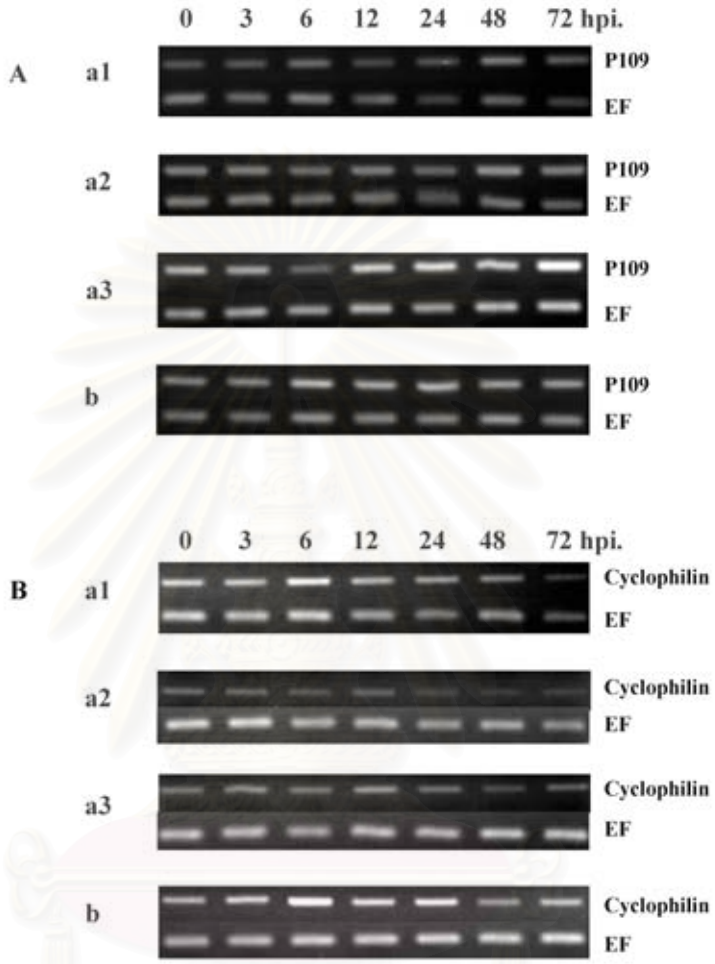
II. Analysis of expression levels of SOD (A) and survivin (B) transcripts after injection with *V. harveyi* for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : *V. harveyi* injected shrimps, replication 1

Panel a2 : *V. harveyi* injected shrimps, replication 2

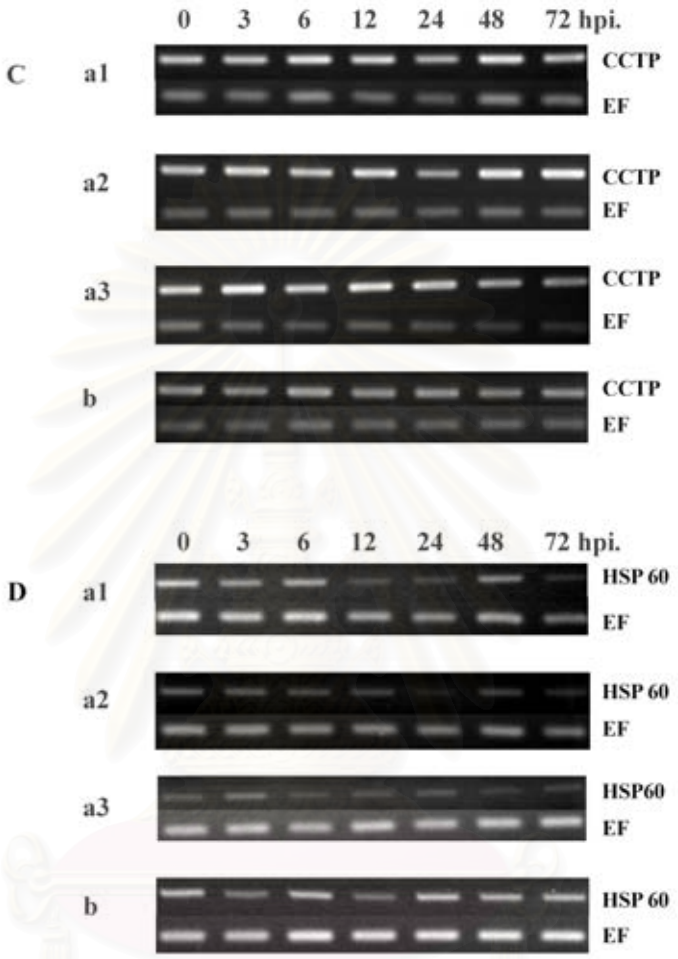
Panel a3 : *V. harveyi* injected shrimps, replication 3

Panel b : saline injected shrimps



III. Analysis of expression levels of P109 (A), Cyclophilin (B) transcripts after injection with *V. harveyi* for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : *V. harveyi* injected shrimps, replication 1
Panel a2 : *V. harveyi* injected shrimps, replication 2
Panel a3 : *V. harveyi* injected shrimps, replication 3
Panel b : saline injected shrimps



III. (continued) Analysis of expression levels of CCTP (C) and HSP 60 (D) transcripts after injection with *V. harveyi* for 0, 3, 6, 12, 24, 48 and 72 hrs.

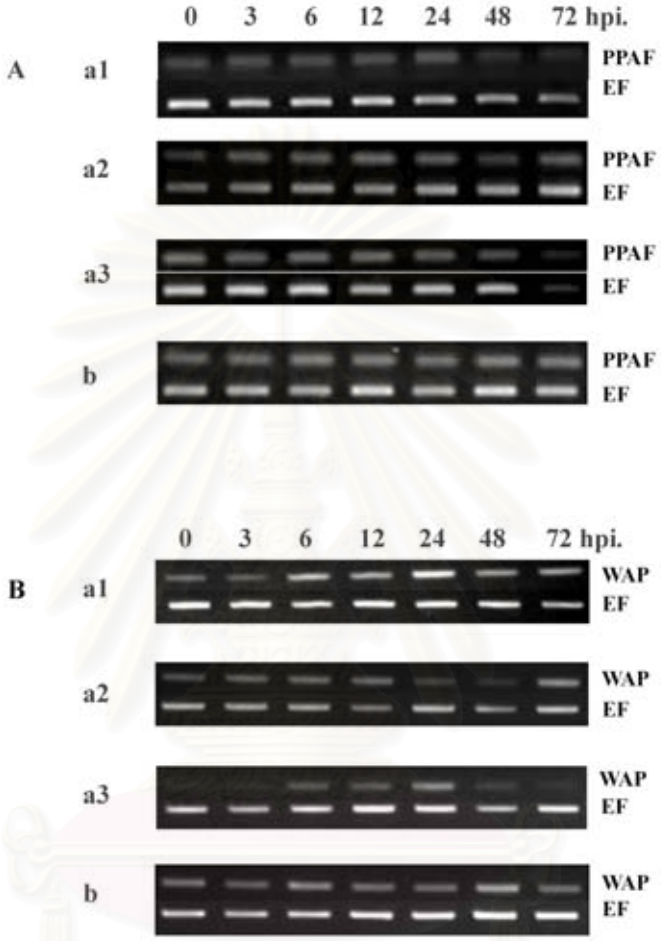
Panel a1 : *V. harveyi* injected shrimps, replication 1

Panel a2 : *V. harveyi* injected shrimps, replication 2

Panel a3 : *V. harveyi* injected shrimps, replication 3

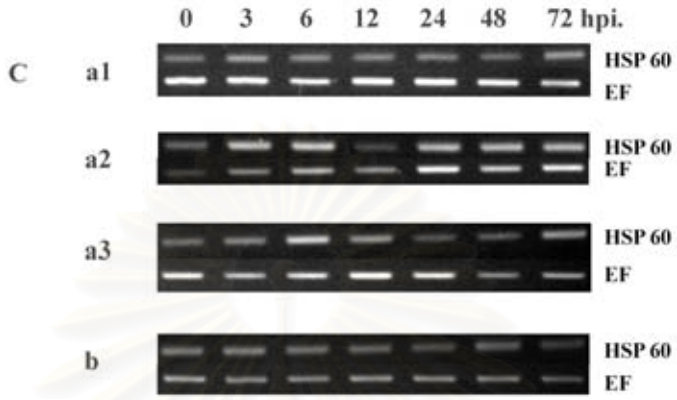
Panel b : saline injected shrimps

Appendix B



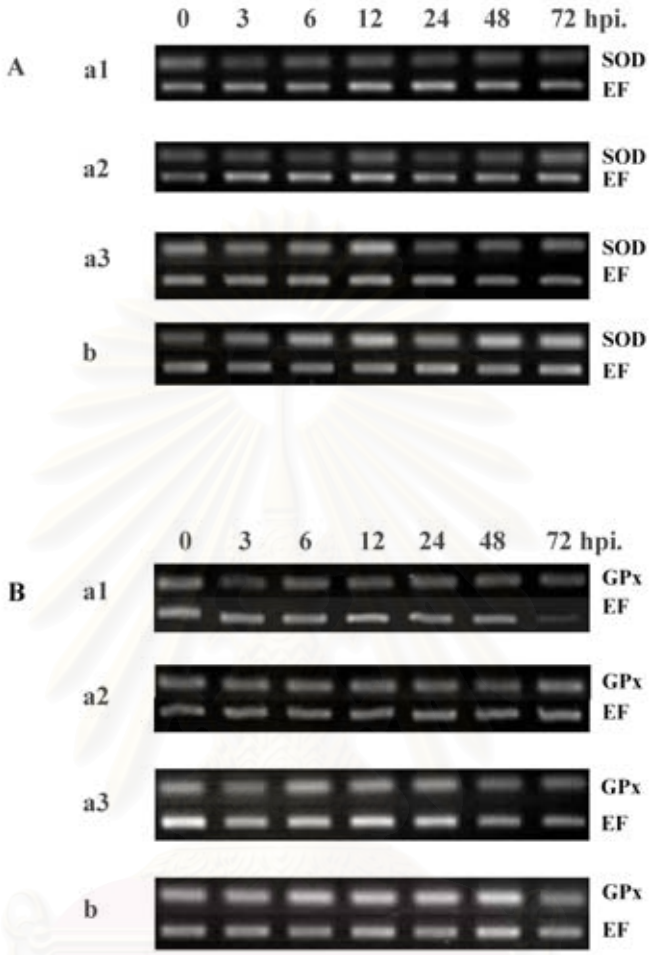
I. Analysis of expression levels of PPAF (A) and WAP (B) transcripts after injection with WSSV for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : WSSV injected shrimps, replication 1
Panel a2 : WSSV injected shrimps, replication 2
Panel a3 : WSSV injected shrimps, replication 3
Panel b : LHM injected shrimps



I. (continued) Analysis of expression levels of HSP 60 (D) transcripts after injection with WSSV for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : WSSV injected shrimps, replication 1
Panel a2 : WSSV injected shrimps, replication 2
Panel a3 : WSSV injected shrimps, replication 3
Panel b : LHM injected shrimps



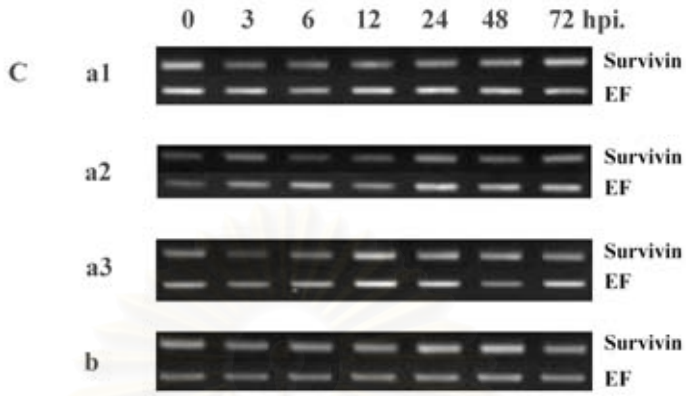
II. Analysis of expression levels of SOD (A) and GPx (B) transcripts after injection with WSSV for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : WSSV injected shrimps, replication 1

Panel a2 : WSSV injected shrimps, replication 2

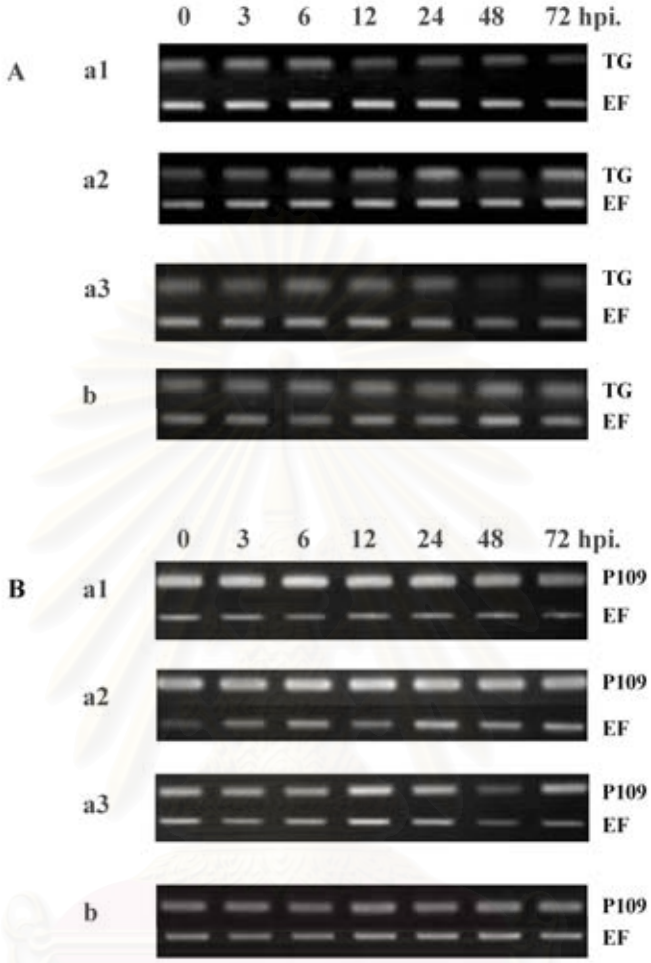
Panel a3 : WSSV injected shrimps, replication 3

Panel b : LHM injected shrimps



II. (continued) Analysis of expression levels of Survivin (C) transcripts after injection with WSSV for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : WSSV injected shrimps, replication 1
Panel a2 : WSSV injected shrimps, replication 2
Panel a3 : WSSV injected shrimps, replication 3
Panel b : LHM injected shrimps



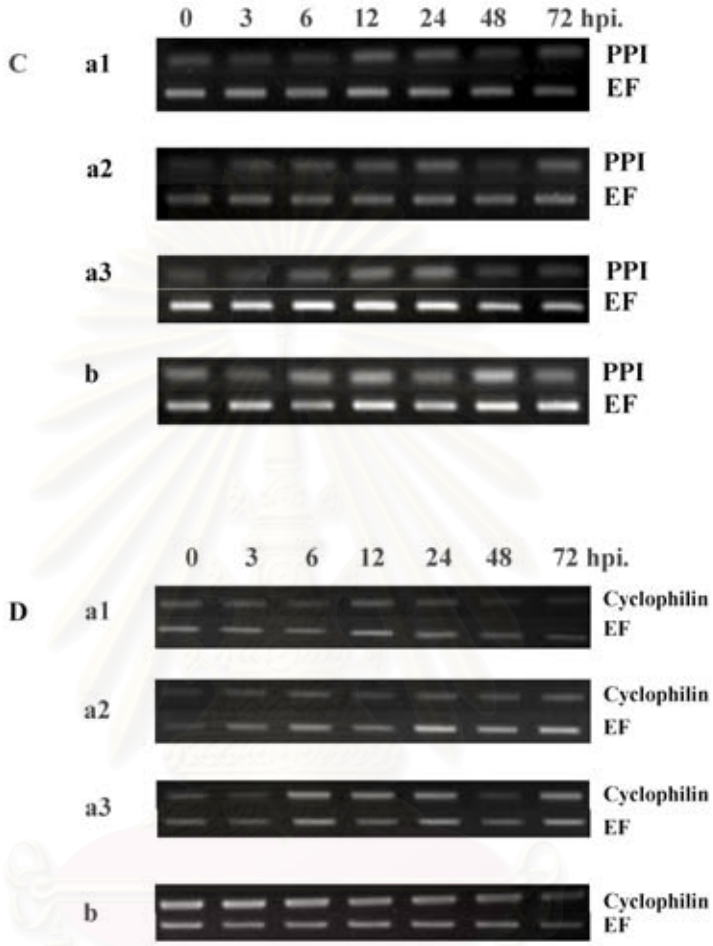
III. Analysis of expression levels of TG (A) and P109 (B) transcripts after injection with WSSV for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : WSSV injected shrimps, replication 1

Panel a2 : WSSV injected shrimps, replication 2

Panel a3 : WSSV injected shrimps, replication 3

Panel b : LHM injected shrimps



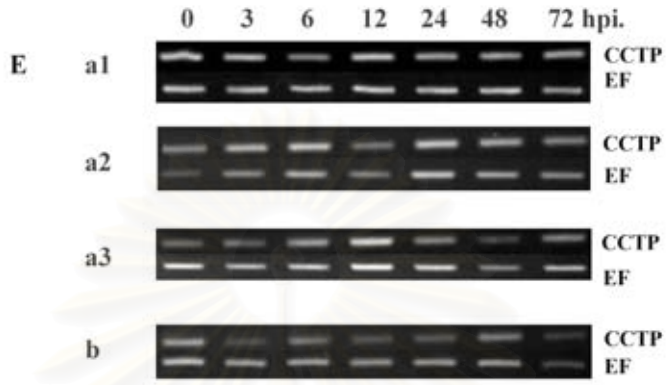
III. (continued) Analysis of expression levels of PPI(C) and Cyclophilin (D) transcripts after injection with WSSV for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : WSSV injected shrimps, replication 1

Panel a2 : WSSV injected shrimps, replication 2

Panel a3 : WSSV injected shrimps, replication 3

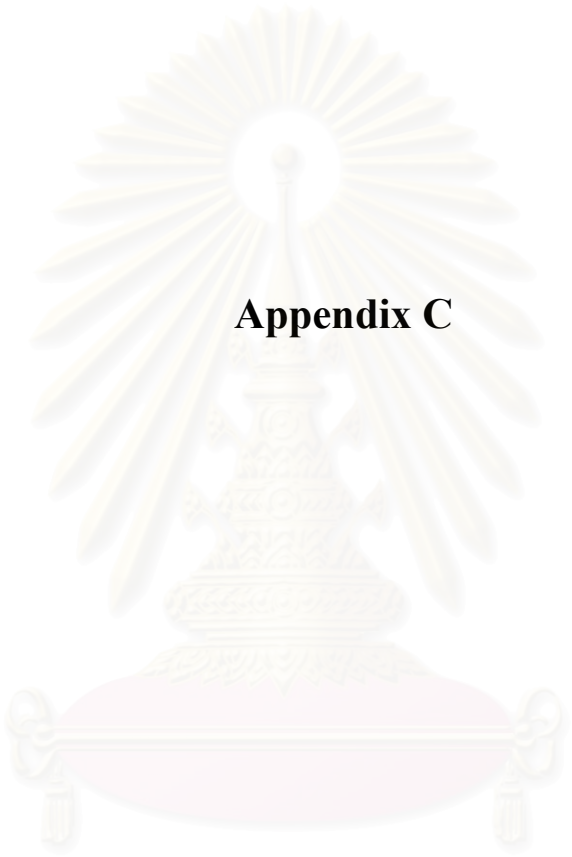
Panel b : LHM injected shrimps



III. (continued) Analysis of expression levels of CCTP (E) transcripts after injection with WSSV for 0, 3, 6, 12, 24, 48 and 72 hrs.

Panel a1 : WSSV injected shrimps, replication 1
Panel a2 : WSSV injected shrimps, replication 2
Panel a3 : WSSV injected shrimps, replication 3
Panel b : LHM injected shrimps

Appendix C



I. Anova test of PPAF expression in *V. harveyi*-challenged *P. monodon*.

ANOVA

PPAF_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.093	6	.182	5.102	.006
Within Groups	.500	14	3.571E-02		
Total	1.593	20			

II. Dancan test of PPAF expression in *V. harveyi*-challenged *P. monodon*.

PPAF_V

Duncan[a]

		Subset for alpha = .05	
HOURS	N	1	2
3.00	3	.6622	
72.00	3	.7349	
.00	3	.7971	
6.00	3	.8785	
12.00	3	.9139	
48.00	3	1.0059	
24.00	3		1.4095
Sig.		.065	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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

ANOVA

GPX_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.039E-02	6	1.507E-02	4.275	.012
Within Groups	4.933E-02	14	3.524E-03		
Total	.140	20			

IV. Dancan test of GPx expression in *V. harveyi*-challenged *P. monodon*.

GPX_V

Duncan[a]

		Subset for alpha = .05		
HOURS	N	1	2	3
.00	3	.9067		
3.00	3	.9133		
72.00	3	.9633	.9633	
48.00	3	.9933	.9933	.9933
24.00	3		1.0400	1.0400
6.00	3		1.0600	1.0600
12.00	3			1.0867
Sig.		.120	.086	.096

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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

ANOVA

TG_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.227	6	3.784E-02	12.594	.000
Within Groups	4.207E-02	14	3.005E-03		
Total	.269	20			

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

TG_V

Duncan[a]

		Subset for alpha = .05		
HOURS	N	1	2	3
.00	3	.9733		
3.00	3	1.0033	1.0033	
6.00	3	1.0533	1.0533	
72.00	3	1.0567	1.0567	
48.00	3		1.0867	
24.00	3			1.2067
12.00	3			1.2867
Sig.		.107	.107	.096

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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

ANOVA

WAP_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.605	6	.101	3.201	.034
Within Groups	.441	14	3.151E-02		
Total	1.046	20			

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

WAP_V

Duncan[a]

		Subset for alpha = .05		
HOURS	N	1	2	3
.00	3	.7296		
3.00	3	.8297	.8297	
6.00	3	.9332	.9332	
72.00	3	.9464	.9464	.9464
48.00	3	.9647	.9647	.9647
24.00	3		1.1338	1.1338
12.00	3			1.2792
Sig.		.163	.077	.051

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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

ANOVA

PPI_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.964	6	.161	11.845	.000
Within Groups	.190	14	1.357E-02		
Total	1.154	20			

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

PPI_V

Duncan[a]

		Subset for alpha = .05			
HOURS	N	1	2	3	4
.00	3	.7100			
3.00	3	.8567	.8567		
72.00	3		.9433		
48.00	3		1.0667	1.0667	
24.00	3			1.1900	1.1900
12.00	3				1.2967
6.00	3				1.3267
Sig.		.145	.054	.216	.194

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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

ANOVA

SOD_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.575	6	9.576E-02	5.117	.006
Within Groups	.262	14	1.871E-02		
Total	.837	20			

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

SOD_V

Duncan[a]

		Subset for alpha = .05		
HOURS	N	1	2	3
12.00	3	.8367		
3.00	3	1.0067	1.0067	
6.00	3	1.0200	1.0200	
24.00	3	1.0400	1.0400	
48.00	3	1.0933	1.0933	
72.00	3		1.2600	1.2600
.00	3			1.3800
Sig.		.055	.058	.301

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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

ANOVA

SURVI_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.098	6	.683	32.468	.000
Within Groups	.295	14	2.104E-02		
Total	4.393	20			

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

SURVI_V

Duncan[a]

		Subset for alpha = .05			
HOURS	N	1	2	3	4
3.00	3	.3900			
6.00	3	.4633			
12.00	3	.6400			
24.00	3		.9067		
.00	3		.9233		
48.00	3			1.2367	
72.00	3				1.7567
Sig.		.064	.890	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XV. Anova test of P109 expression in *V. harveyi*-challenged *P. monodon*.

ANOVA

P109_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.385E-02	6	1.231E-02	1.236	.346
Within Groups	.139	14	9.962E-03		
Total	.213	20			

XVI. Dancan test of P109 expression in *V. harveyi*-challenged *P. monodon*.

P109_V

Duncan[a]

HOURS	N	Subset for alpha = .05
		1
6.00	3	.6267
3.00	3	.6433
.00	3	.6567
12.00	3	.6967
48.00	3	.7667
72.00	3	.7667
24.00	3	.7733
Sig.		.131

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XVII. Anova test of cyclophilin expression in *V. harveyi*-challenged *P. monodon*.

ANOVA

CYP18_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.135	6	2.247E-02	.352	.897
Within Groups	.895	14	6.392E-02		
Total	1.030	20			

XVIIII. Dancan test of cyclophilin expression in *V. harveyi*-challenged *P. monodon*.

CYP18_V

Duncan[a]

HOURS	N	Subset for alpha = .05
		1
48.00	3	.5640
3.00	3	.6200
.00	3	.6533
72.00	3	.6867
24.00	3	.6933
6.00	3	.6967
12.00	3	.8433
Sig.		.246

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XIX. Anova test of CCTP expression in *V. harveyi*-challenged *P. monodon*.

ANOVA

CCTP_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.162E-03	6	5.270E-04	.098	.995
Within Groups	7.567E-02	14	5.405E-03		
Total	7.883E-02	20			

XX. Dancan test of CCTP expression in *V. harveyi*-challenged *P. monodon*.

CCTP_V

Duncan[a]

HOURS	N	Subset for alpha = .05
		1
6.00	3	1.0167
12.00	3	1.0400
.00	3	1.0433
48.00	3	1.0433
24.00	3	1.0467
3.00	3	1.0500
72.00	3	1.0600
Sig.		.527

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XXI. Anova test of HSP 60 expression in *V. harveyi*-challenged *P. monodon*.

ANOVA

HSP60_V

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.146	6	2.437E-02	3.092	.038
Within Groups	.110	14	7.881E-03		
Total	.257	20			

XXII. Dancan test of HSP 60 expression in *V. harveyi*-challenged *P. monodon*.

HSP60_V

Duncan[a]

		Subset for alpha = .05		
HOURS	N	1	2	3
72.00	3	.8833		
24.00	3	.9433	.9433	
48.00	3	.9567	.9567	.9567
.00	3	1.0267	1.0267	1.0267
12.00	3		1.0667	1.0667
3.00	3		1.1100	1.1100
6.00	3			1.1200
Sig.		.088	.055	.059

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix D

I. Anova test of PPAF expression in WSSV-challenged *P. monodon*.

ANOVA

PPAF_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.957	6	.160	5.106	.006
Within Groups	.437	14	3.124E-02		
Total	1.394	20			

II. Dancan test of PPAF expression in WSSV-challenged *P. monodon*.

PPAF_W

Duncan[a]

		Subset for alpha = .05		
HOURS	N	1	2	3
72.00	3	1.0028		
48.00	3	1.0647		
.00	3	1.1800		
3.00	3	1.2899	1.2899	
6.00	3	1.3045	1.3045	
24.00	3		1.5287	1.5287
12.00	3			1.6314
Sig.		.078	.138	.489

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

III. Anova test of WAP expression in WSSV-challenged *P. monodon*.

ANOVA

WAP_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.956	6	.493	2.992	.043
Within Groups	2.305	14	.165		
Total	5.262	20			

IV. Dancan test of WAP expression in WSSV-challenged *P. monodon*.

WAP_W

Duncan[a]

		Subset for alpha = .05	
HOURS	N	1	2
.00	3	.5127	
3.00	3	.6986	
48.00	3	.7133	
6.00	3	.7563	
72.00	3	.8936	
12.00	3	.9116	
24.00	3		1.7600
Sig.		.297	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

V. Anova test of HSP 60 expression in WSSV-challenged *P. monodon*.

ANOVA

HSP60_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.282	6	4.698E-02	6.369	.002
Within Groups	.103	14	7.376E-03		
Total	.385	20			

VI. Dancan test of HSP60 expression in WSSV-challenged *P. monodon*.

HSP60_W

Duncan[a]

		Subset for alpha = .05	
HOURS	N	1	2
12.00	3	.6167	
24.00	3	.6200	
48.00	3	.6567	
.00	3	.7367	
72.00	3	.7700	.7700
6.00	3		.9067
3.00	3		.9100
Sig.		.066	.078

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

VII. Anova test of SOD expression in WSSV-challenged *P. monodon*.

ANOVA

SOD_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.753	6	.292	6.973	.001
Within Groups	.587	14	4.190E-02		
Total	2.340	20			

VIII. Dancan test of SOD expression in WSSV-challenged *P. monodon*.

SOD_W

Duncan[a]

		Subset for alpha = .05	
HOURS	N	1	2
24.00	3	.5333	
12.00	3	.6333	
6.00	3	.6667	
48.00	3	.6667	
72.00	3	.7667	
3.00	3	.8667	
.00	3		1.4667
Sig.		.095	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

IX. Anova test of GPx expression in WSSV-challenged *P. monodon*.

ANOVA

GPX

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.287	6	4.782E-02	5.703	.003
Within Groups	.117	14	8.386E-03		
Total	.404	20			

X. Dancan test of GPx expression in WSSV-challenged *P. monodon*.

GPX

Duncan[a]

		Subset for alpha = .05			
HOURS	N	1	2	3	4
12.00	3	.7333			
24.00	3	.8067	.8067		
6.00	3	.8100	.8100		
3.00	3	.8267	.8267	.8267	
.00	3		.9333	.9333	.9333
48.00	3			.9933	.9933
72.00	3				1.0933
Sig.		.268	.139	.052	.061

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XI. Anova test of survivin expression in WSSV-challenged *P. monodon*.

ANOVA

SURVI_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.118	6	1.966E-02	4.216	.012
Within Groups	6.527E-02	14	4.662E-03		
Total	.183	20			

XII. Dancan test of survivin expression in WSSV-challenged *P. monodon*.

SURVI_W

Duncan[a]

		Subset for alpha = .05	
HOURS	N	1	2
12.00	3	.4933	
24.00	3	.5000	
6.00	3	.5100	
3.00	3	.5367	
48.00	3	.5967	.5967
72.00	3		.6633
.00	3		.6900
Sig.		.114	.134

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XIII. Anova test of TG expression in WSSV-challenged *P. monodon*.

ANOVA

TG

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.145	6	2.409E-02	2.801	.053
Within Groups	.120	14	8.600E-03		
Total	.265	20			

XIV. Dancan test of TG expression in WSSV-challenged *P. monodon*.

TG

Duncan[a]

		Subset for alpha = .05	
HOURS	N	1	2
6.00	3	.6067	
3.00	3	.6267	
12.00	3	.6300	
48.00	3	.7633	.7633
.00	3	.7667	.7667
24.00	3	.7833	.7833
72.00	3		.8233
Sig.		.054	.477

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XV. Anova test of P109 expression in WSSV-challenged *P. monodon*.

ANOVA

P109_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.029E-03	6	5.048E-04	.070	.998
Within Groups	.101	14	7.233E-03		
Total	.104	20			

XVI. Dancan test of P109 expression in WSSV-challenged *P. monodon*.

P109_W

Duncan[a]

HOURS	N	Subset for alpha = .05
		1
24.00	3	1.1000
12.00	3	1.1033
48.00	3	1.1033
3.00	3	1.1100
.00	3	1.1200
6.00	3	1.1267
72.00	3	1.1333
Sig.		.672

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XVII. Anova test of PPI expression in WSSV-challenged *P. monodon*.

ANOVA

PPI_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.087E-02	6	1.181E-02	1.128	.396
Within Groups	.147	14	1.047E-02		
Total	.217	20			

XVIII. Dancan test of PPI expression in WSSV-challenged *P. monodon*.

PPI_W

Duncan[a]

HOURS	N	Subset for alpha = .05
		1
.00	3	.6367
3.00	3	.6667
6.00	3	.7433
48.00	3	.7433
72.00	3	.7733
12.00	3	.7900
24.00	3	.8033
Sig.		.097

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XIX. Anova test of cyclophilin expression in WSSV-challenged *P. monodon*.

ANOVA

CYCLO_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.162E-03	6	8.603E-04	2.377	.085
Within Groups	5.067E-03	14	3.619E-04		
Total	1.023E-02	20			

XX. Dancan test of cyclophilin expression in WSSV-challenged *P. monodon*.

CYCLO_W

Duncan[a]

		Subset for alpha = .05	
HOURS	N	1	2
48.00	3	.6067	
3.00	3	.6133	
6.00	3	.6167	
.00	3	.6233	.6233
72.00	3	.6333	.6333
24.00	3	.6433	.6433
12.00	3		.6533
Sig.		.052	.095

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

XXI. Anova test of CCTP expression in WSSV-challenged *P. monodon*.

ANOVA

CCTP_W

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.448E-03	6	1.075E-03	1.085	.417
Within Groups	1.387E-02	14	9.905E-04		
Total	2.031E-02	20			

XXII. Dancan test of CCTP expression in WSSV-challenged *P. monodon*.

CCTP_W

Duncan[a]

HOURS	N	Subset for alpha = .05
		1
24.00	3	1.3833
48.00	3	1.3833
72.00	3	1.3967
.00	3	1.4067
12.00	3	1.4067
3.00	3	1.4167
6.00	3	1.4367
Sig.		.086

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix E

I. Anova test of PPAF expression in different developmental stages of *P. monodon*

ANOVA

PPAF

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5288.511	5	1057.702	5.405	.012
Within Groups	1957.049	10	195.705		
Total	7245.560	15			

II. Dancan test of PPAF expression in different developmental stages of *P. monodon*

PPAF

Duncan[a,b]

		Subset for alpha = .05		
STAGES	N	1	2	3
3.00	3	93.3967		
6.00	2	97.0000		
1.00	2	109.7850	109.7850	
2.00	3	110.3567	110.3567	
5.00	3		135.2600	135.2600
4.00	3			140.1500
Sig.		.229	.077	.700

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

* The 1-6 stages were NaupliusIII , Zoea II, Mysis II, PL5, PL15 and juvenile stage of *P. monodon*, respectively.

III. Anova test of GPx expression in different developmental stages of *P. monodon*

ANOVA

GPX

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26764.34	5	5352.867	4.668	.019
Within Groups	11467.91	10	1146.791		
Total	38232.25	15			

IV. Dancan test of GPx expression in different developmental stages of *P. monodon*

GPX

Duncan[a,b]

		Subset for alpha = .05		
STAGES	N	1	2	3
1.00	2	103.3650		
2.00	3	153.2500	153.2500	
3.00	3	158.7300	158.7300	
6.00	2		174.6950	174.6950
4.00	3		185.0967	185.0967
5.00	3			243.3467
Sig.		.107	.343	.052

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

* The 1-6 stages were NaupliusIII , Zoea II, Mysis II, PL5, PL15 and juvenile stage of *P. monodon*, respectively.

V. Anova test of TG expression in different developmental stages of *P. monodon*

ANOVA

TG

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5328.302	5	1065.660	7.061	.005
Within Groups	1509.323	10	150.932		
Total	6837.625	15			

VI. Dancan test of TG expression in different developmental stages of *P. monodon*

TG

Duncan[a,b]

		Subset for alpha = .05		
STAGES	N	1	2	3
6.00	2	39.2750		
2.00	3	50.2933	50.2933	
1.00	2	51.4300	51.4300	
3.00	3	54.3533	54.3533	
5.00	3		74.1167	74.1167
4.00	3			93.8767
Sig.		.223	.067	.098

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

* The 1-6 stages were NaupliusIII , Zoea II, Mysis II, PL5, PL15 and juvenile stage of *P. monodon*, respectively.

VII. Anova test of WAP expression in different developmental stages of *P. monodon*

ANOVA

WAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	323610.5	5	64722.110	110.507	.000
Within Groups	5856.820	10	585.682		
Total	329467.4	15			

VIII. Dancan test of WAP expression in different developmental stages of *P. monodon*

WAP

Duncan[a,b]

		Subset for alpha = .05					
STAGES	N	1	2	3	4	5	6
1.00	2	.0000					
2.00	3		58.1700				
3.00	3			116.7800			
4.00	3				173.4300		
6.00	2					307.9950	
5.00	3						417.6767
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

* The 1-6 stages were NaupliusIII , Zoea II, Mysis II, PL5, PL15 and juvenile stage of *P. monodon*, respectively.

IX. Anova test of PPI expression in different developmental stages of *P. monodon*

ANOVA

PPI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	29558.05	5	5911.611	20.706	.000
Within Groups	2855.077	10	285.508		
Total	32413.13	15			

X. Dancan test of PPI expression in different developmental stages of *P. monodon*

PPI

Duncan[a,b]

		Subset for alpha = .05	
STAGES	N	1	2
1.00	2	132.7700	
3.00	3	132.7800	
6.00	2	135.0200	
2.00	3	141.5333	
5.00	3		220.6033
4.00	3		227.9800
Sig.		.595	.631

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

* The 1-6 stages were NaupliusIII , Zoea II, Mysis II, PL5, PL15 and juvenile stage of *P. monodon*, respectively

XI. Anova test of HSP60 expression in different developmental stages of *P. monodon*

ANOVA

HSP60

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5070.506	5	1014.101	4.734	.018
Within Groups	2142.185	10	214.219		
Total	7212.691	15			

XII. Dancan test of HSP60 expression in different developmental stages of *P. monodon*

HSP60

Duncan[a,b]

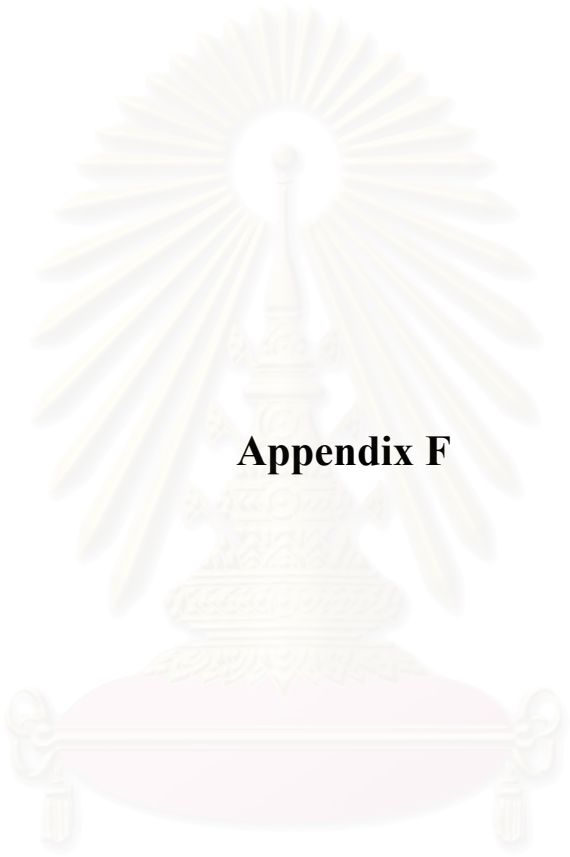
		Subset for alpha = .05	
STAGES	N	1	2
3.00	3	74.7067	
6.00	2	79.3600	
4.00	3	89.8667	89.8667
2.00	3		110.4300
1.00	2		116.4850
5.00	3		119.6500
Sig.		.288	.057

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.571.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

* The 1-6 stages were NaupliusIII , Zoea II, Mysis II, PL5, PL15 and juvenile stage of *P. monodon*, respectively



Appendix F

I. Anova test of PPAF expression.

ANOVA

PPAF

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8073.928	2	4036.964	6.555	.031
Within Groups	3695.051	6	615.842		
Total	11768.98	8			

II. Dancan test of PPAF expression.

PPAF

Duncan[a]

		Subset for alpha = .05	
SOURE	N	1	2
1.00	3	36.2033	
2.00	3	50.4267	
3.00	3		105.6467
Sig.		.509	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Indication Soure 1 : Suphunburi ;

Soure 2 : Chachoengsao;

Soure 3 : Nakhonsithammarat.

III. Anova test of GPx expression.

ANOVA

GPX

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8387.889	2	4193.944	4.389	.067
Within Groups	5733.873	6	955.645		
Total	14121.76	8			

IV. Dancan test of GPx expression.

GPX

Duncan[a]

		Subset for alpha = .05	
SOURE	N	1	2
1.00	3	140.1533	
2.00	3	168.2233	168.2233
3.00	3		214.2133
Sig.		.309	.118

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Indication Soure 1 : Suphunburi ;

Soure 2 : Chachoengsao;

Soure 3 : Nakhonsithammarat.

V. Anova test of WAP expression.

ANOVA

WAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40519.99	2	20259.993	7.938	.021
Within Groups	15314.38	6	2552.396		
Total	55834.36	8			

VI. Dancan test of WAP expression.

WAP

Duncan[a]

		Subset for alpha = .05	
SOURE	N	1	2
1.00	3	155.0933	
2.00	3	206.8767	
3.00	3		316.0733
Sig.		.256	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Indication Soure 1 : Suphunburi ;

Soure 2 : Chachoengsao;

Soure 3 : Nakhonsithammarat.

VII. Anova test of PPI expression.

ANOVA

PPI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4480.188	2	2240.094	33.466	.001
Within Groups	401.621	6	66.937		
Total	4881.809	8			

VIII. Dancan test of PPI expression.

PPI

Duncan[a]

		Subset for alpha = .05		
SOURE	N	1	2	3
1.00	3	72.0333		
2.00	3		88.3967	
3.00	3			125.3733
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Indication Soure 1 : Suphunburi ;

Soure 2 : Chachoengsao;

Soure 3 : Nakhonsithammarat.

IX. Anova test of TG expression.

ANOVA

TG

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11820.94	2	5910.469	25.456	.001
Within Groups	1393.077	6	232.180		
Total	13214.01	8			

X. Dancan test of TG expression.

TG

Duncan[a]

		Subset for alpha = .05	
SOURE	N	1	2
1.00	3	132.9667	
2.00	3	148.5767	
3.00	3		216.4533
Sig.		.256	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Indication Soure 1 : Suphunburi ;

Soure 2 : Chachoengsao;

Soure 3 : Nakhonsithammarat.

XI. Anova test of HSP 60 expression.

ANOVA

HSP60

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35273.83	2	17636.917	1.814	.242
Within Groups	58325.17	6	9720.861		
Total	93599.00	8			

XII. Dancan test of HSP 60 expression.

HSP60

Duncan[a]

SOURE	N	Subset for alpha = .05
		1
2.00	3	221.0767
1.00	3	278.0400
3.00	3	372.8600
Sig.		.118

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Indication Soure 1 : Suphunburi ;

Soure 2 : Chachoengsao;

Soure 3 : Nakhonsithammarat.

BIOGRAPHY

Miss Naritsara Pulsook was born on October 29, 1978. She graduated with the Bachelor of Science from the Department of Biochemistry at Chulalongkorn University in 2001. She has studied for the degree of Master of Science at the department of Biotechnology, Chulalongkorn University since 2002.