การวิเคราะห์โปรติโอมและลักษณะสมบัติของโปรตีนที่ตอบสนองต่อ *Vibrio harveyi* ในกุ้งกุลาดำ *Penaeus monodon*

นายวรพนธ์ ชัยกีรติศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PROTEOMIC ANALYSIS AND CHARACTERIZATION OF *Vibrio harveyi* RESPONSIVE PROTEINS IN THE BLACK TIGER SHRIMP *Penaeus monodon*

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วรพนธ์ ชัยกีรติศักดิ์: การวิเคราะห์โปรติโอมและลักษณะสมบัติของโปรตีนที่ตอบสนองต่อ *Vibrio harveyi* ในกุ้งกุลาดำ *Penaeus monodon* (PROTEOMIC ANALYSIS AND CHARACTERIZATION OF *Vibrio harveyi* RESPONSIVE PROTEINS IN THE BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร. อัญชลี ทัศนาขจร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร.กุลยา สมบูรณ์วิวัฒน์, Prof. Peter B. Armstrong, Ph.D., 115 หน้า.

เชื้อแบคทีเรียวิบริโอฮาวิอายเป็นเชื้อแบคทีเรียก่อโรครุนแรงชนิดหนึ่งในกุ้งกุลาดำ (*Penaeus monodon*) และส่งผลกระทบต่อเศรษฐกิจการเพาะเลี้ยงกุ้ง ในงานวิจัยนี้ได้ใช้เทคนิคการศึกษาโปรตีโอมเพื่อระบุโปรตีนในอวัยวะน้ำเหลืองของกุ้งกุลาดำ ที่มีการแสดงออกเปลี่ยนแปลงไปภายหลังการติดเชื้อแบคทีเรียวิบริโอฮาวิอาย ในการระบุหาโปรตีนนี้ มีโปรตีนจำนวนมากที่มีการแสดงออกเพิ่มขึ้นและลดลง แสดงให้เห็นถึงความเกี่ยวข้องกับกลไกการต้านเชื้อแบคทีเรียและระบบภูมิคุ้มกันของกุ้งกุลาดำ โปรตีนATP synthase beta subunit และ Alpha-2-macroglobulin (A2M) ซึ่งเป็นโปรตีนที่แสดงออกเปลี่ยนแปลงไปอย่างมากภายหลังการติดเชื้อ ได้ถูกนำมาศึกษาต่อ พบว่าการยับยั้งการแสดงออกของยีนATP synthase beta subunit โดยใช้เทคนิค RNA interference (RNAi) ส่งผลทำให้กุ้งมีอัตราการตายสะสมสูงขึ้นและแตกต่างจากกลุ่มควบคุมอย่างเด่นชัด โดยในกลุ่มกุ้งที่ถูกลดการแสดงออกของยีน ATP synthase beta subunit มีปริมาณเซลล์เม็ดเลือดลดลงอย่างมาก การทดลองนี้แสดงให้เห็นว่า ATP synthase beta subunit อาจมีความสำคัญในระบบภูมิคุ้มกันของกุ้ง นอกเหนือจากนี้เราได้ใช้เทคนิค Yeast two-hybrid system ในการค้นหาโปรตีนที่ทำงานร่วมกับโปรตีนA2M ในการต่อต้านการติดเชื้อแบคทีเรียวิบริโอฮาวิอาย จากการทดลองพบว่า Receptor binding domain ของโปรตีน A2M สามารถจับกับปลาย Carboxyl ของเอนไซม์ Transglutaminase ชนิดที่ 2 ซึ่งเป็นเอนไซม์ที่เกี่ยวข้องกับกระบวนการแข็งตัวของเลือดได้ รวมทั้งยังตรวจพบการเรียงตัวของโปรตีน A2M ร่วมกับโปรตีน Clottable protein บนลิ่มเลือดอีกด้วย โดยผลการทดลองนี้แสดงให้เห็นถึงความเกี่ยวข้องของโปรตีน A2M ในกระบวนการแข็งตัวของเลือด เมื่อทดลองลดการแสดงออกของยีน A2M เราพบว่าระบบการแข็งตัวของเลือดสามารถดักจับเชื้อแบคทีเรียวิบริโอฮาวิอายได้ลดลง โดยจำนวนของเชื้อแบคทีเรียในระบบหมุนเวียนเลือดของกุ้งกลุ่มที่ถูกลดการแสดงออกของยีนA2M มีปริมาณสูงกว่ากุ้งกลุ่มควบคุมถึง 3.3 เท่า ภายหลังการฉีดเชื้อ5 นาที เมื่อใช้กล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราด ในการสังเกตลิ่มเลือดของกุ้งกลุ่มที่ถูกลดการแสดงออกของยีน A2M ในสภาวะติดเชื้อ พบว่ามีบริเวณย่อยสลายของลิ่มเลือดเกิดขึ้นรอบๆเซลล์ของแบคทีเรียอย่างชัดเจนผลการทดลองนี้บ่งชี้ว่าโปรตีน A2M ทำหน้าที่สำคัญในระบบการแข็งตัวของเลือดโดยอาจมีผลยับยั้งโปรตีเอสที่หลั่งจากเชื้อแบคทีเรีย อย่างไรก็ตามเชื้อแบคทีเรียสามารถย่อยสลายลิ่มเลือดของกุ้งปกติและเชื้อแบคทีเรียจำนวนหนึ่งสามารถหลบหนีได้ภายใน2 ชั่วโมง ถึงแม้ว่าเชื้อแบคทีเรียจะถูกจับอย่างเหนียวแน่นในระยะเวลาช่วงเริ่มต้นก็ตาม นอกเหนือจากนี้เมื่อศึกษาฤทธิ์ในการยับยั้งการย่อยสลายลิ่มเลือดของ Extracellular products ในน้ำเลี้ยงเชื้อแบคทีเรียวิบริโอฮาวิอาย และความสามารถในการหลบหนีของเชื้อแบคทีเรียจากลิ่มเลือด โดยใช้ตัวยับยั้งการทำงานของโปรตีเอสชนิดต่างๆ พบว่าโปรตีเอสที่ใช้ย่อยสลายลิ่มเลือดถูกจัดอยู่ในกลุ่ม Metallo protease และ Serine protease

สาขาวิชา ……..…..ชีวเคมี…… ลายมือชื่อนิสิต ……..

สาขาวิชา ……....ชีวเคมี…… ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…….....

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ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……

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VORRAPON CHAIKEERATISAK: PROTEOMIC ANALYSIS AND CHARACTERIZATION OF *Vibrio harveyi* RESPONSIVE PROTEINS IN THE BLACK TIGER SHRIMP *Penaeus monodon.* ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., CO-ADVISOR: ASST. PROF. KUNLAYA SOMBOONWIWAT, Ph.D., PROF. PETER B. ARMSTRONG, Ph.D., 115 pp.

Vibriosis, caused by bacteria from the genus *Vibrio*, is one of the major diseases in shrimp aquaculture resulting in a high mortality and so economic losses. Here, proteomic analysis of the lymphoid organ of *V. harveyi* challenged *Penaeus monodon* was performed in order to identify potential proteins responsible for the bacterial infection. A number of bacterial responsive proteins regarding antibacterial immunity and/or bacterial infection mechanism were discovered. Among the bacterial responsive proteins obtained from the proteomic screening, ATP synthase beta subunit and alpha-2-macroglobulin (A2M) exhibited considerably altered expression levels against *V. harveyi* infection. Hence, both of them were further characterized for their potentially necessary roles in the shrimp response to bacterial infection. Partial transcript knockdown of ATP synthase beta subunit showed a high cumulative mortality of the transcript silenced shrimp and a substantial decrease of the number of circulating hemocytes in the survival shrimp. We speculate that ATP synthase beta subunit might serve vital functions in the immune system of shrimp upon the infection. The yeast two-hybrid system revealed that *Pm*A2M's receptor binding domain interacted with the carboxyl-terminus of transglutaminase type II, a major enzyme related to the shrimp clotting system. In accord with this, *Pm*A2M was found to colocalize with coagulation associated proteins on the extracellular blood clots. RNA interference-mediated knockdown of A2M transcript levels impaired the bacterial seizing ability of the shrimp clotting system, resulting in an up to 3.3-fold higher number of *V. harveyi* that systemically spreaded into the blood circulation at 5 min post-infection before later elimination by the immune system. A characteristic of *Pm*A2M depleted clots in the presence of *V. harveyi* obviously illustrated fibrinolysis areas surrounding the bacteria. *In vitro* observation of *V. harveyi* entrapping normal shrimp clots; nevertheless, disclosed that the bacterial cells were initially immobilized firmly, but later, they can continue to proliferate and escape when the clots were digested by fibrinolytic enzymes. Additionally, *V. harveyi* secreted enzymes responsible for the clot lysis were further identified. The fibrinolytic activity of bacterial conditioned media was completely inhibited when adding 1,10 phenanthroline (metallo-protease inhibitor) and AEBSF (serine protease inhibitor) as well as the number of motile bacteria entrapped in the clots was significantly diminished when supplemented with AEBSF. This suggests that both metallo-proteases and serine proteases released from the bacteria are required for substantial fibrinolysis.

Department: …...Biochemistry..….. Student's Signature ……..

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LIST OF ABBREVIATIONS

2-DGE	Two-dimensional gel electrophoresis
A	Ampere
A2M	Alpha-2-macroglobulin
AbA	Aureobasidin A,
AD	Activating domain
Ade	Adenine
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
BD	Binding domain
bp	Base pair
BSA	Bovine serum albumin
CMB	Complete marine broth
CFU	Colony forming unit
Da	Dalton
DDO	Double dropout medium: SD/–Leu/–Trp
DDO/X/A	Double dropout medium: SD/–Leu/–Trp supplemented with X-α-Gal and Aureobasidin A
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
E64	[1-[N-[(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane]
EST	Expressed sequence tag
F	Forward
FBS	Fetal bovine serum
g	Gram
GFP	Green fluorescence protein
h	Hour, Hours
His	Histidine

HPI	Hour post infection
IEF	Isoelectric focusing
K	Kilo
l	Liter
LacZ	*LacZ* reporter gene (β-galactosidase activity.)
LB	Luria-Bertani
LC–ESI–MS/MS	Liquid chromatography-electrospray ionisation-mass spectrometry
Leu	Leucine
m	Milli
M	Molar
MB	Marine broth
Mel1	*MEL1* reporter gene (α-galactosidase activity)
min	Minute, Minutes
Ms	mouse
MW	Molecular weight
No.	Number
PAGE	polyacrylamide electrophoresis
PCR	Polymerase chain reaction
PBS	1x phosphate buffered saline
Pm	*Penaeus monodon*
QDO	Quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp
QDO/X/A	Quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp supplemented with X-a-Gal and Aureobasidin A
R	Reverse
Rb	Rabbit
RBD	Receptor binding domain
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcription

SD	Standard deviation
SDO	Single dropout medium
sec	Seconds
SPF	Specific pathogen free
SPI	Serine proteinase inhibitor
ssRNA	Single-stranded RNA
SSS	Shrimp saline solution
TCBS	thiosulfate–citrate–bile–sucrose
THC	Total hemocyte number
Trp	Tryptophan
μ	Micro
UV	Ultraviolet
V	Volt
Vh	Accumulated voltage (Volt x hours)
X	X-α-Gal
Y2H	Yeast two-hybrid
YPD	A blend of yeast extract, peptone, and dextrose in optimal proportions for growth of most strains of *S. cerevisiae*
YPDA	YPD medium supplemented with adenine hemisulfate

CHAPTER I
INTRODUCTION

1.1 History of the shrimp culture industry

Over the last few decades, aquafarming such as fish farming, oyster farming, shrimp farming, and algaculture has been greatly expanding to reach higher demands of the world consumption rate. Among those aquaculture, shrimp farming has been a multi-billion dollar industry and thereby has become one of the most economically significant farming in many countries in Asia, especially China and Thailand, which share up to 75% of the world shrimp trade (Source: FAO databases, 2007). Thailand has been the world leader in farmed shrimp exportation since 1990s, and used to be the biggest exporter of the black tiger shrimp, *Penaeus monodon*, of the world market in prawn and shrimp (Wyban, 2007). Dispersively located along the Southern coastal areas, the hatcheries and farms of the black tiger shrimp in Thailand are mostly founded in Surat Thani and Nakon Sri Thammarat provinces.

With the rapid expansion of the shrimp farming as well as the sudden improvement in aquaculture innovations and technologies, many problems in cultural management of the shrimp broodstocks and in the outbreaks of pandemic diseases caused by severe pathogens have been occurred (Mohan et al., 1998). The white spot syndrome (WSSV), taura syndrome (TSV) and yellow head (YHV) viruses and the luminescent *Vibrio harveyi* bacteria have been then recognized as deadly infectious pathogens causing main losses in majority of the shrimp production, perhaps resulting in economic collapses (Flegel, 2006). Consequently, the black tiger shrimp production in Thailand has been substantially dropped during the period from 2002 to 2009 (Figure 1.1). The white shrimp, *Litopenaeus vannamei*, has then become another alternative of the cultured shrimp species because the *L. vannamei*'s traits, which are availability of specific pathogen free stocks, specific pathogen resistance, high growth rate, and high survival rate, are more favorable than *P. monodon* does. However, some preferences of *P. monodon* are still more advantageous. They are, for instance, fast growing, they are tolerant to a broad range of salinity and, particularly they are a native shrimp species in Thailand. It is, therefore, important to maintain the farming industry of the black tiger shrimp, *P. monodon*, by using genetic tools to understand its immune system and to improve its pathogen resistance, develeping better cultural management, and inventiing the effective means to control these infectious diseases.

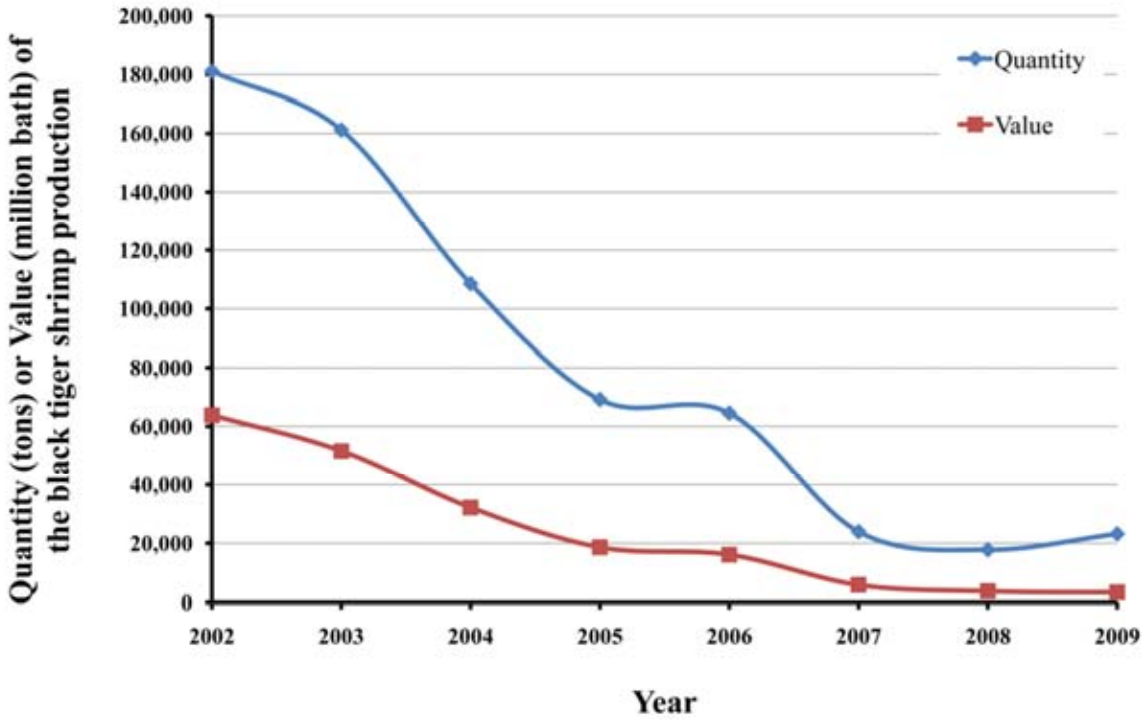

Figure 1.1 The production of the black tiger shrimp, *P.monodon*, of Thailand in the period from 2002 to 2009 (Source: www.oae.go.th/oae_report/export_import/expor t .php).

1.2 Taxonomy of the black tiger shrimp, *Penaeus monodon*

Penaeid shrimp are catergorized into the Arthropoda, which is the largest phylum in the Kingdom of Animalia. The presences of protective exoskeletons covering the whole body of animals and pairs of jointed appendages are obvious characteristics of the animals classified into this phylum. The Crustacea subphylum consists of approximately 42,000 aquatic species belonging to ten classes. Shrimp including crabs, lobsters, and crayfish belong to the Decapoda order which is under the Malacostraca class. The appearent aspect of the *Penaeus monodon* are the black colored stripes on their cuticles appearing like tiger traits and so the *P. monodon* is normally called the black tiger shrimp. The taxonomic meaning of the black tiger shrimp, *P. monodon*, is as following (Baily-Brock and Moss, 1992):

P**hylum** 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* (*P. monodon*)

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

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

1.3 Major bacterial diseases in shrimp

Although the shrimp diseases caused by severe viral pathogens such as white spot syndrome (WSSV), yellow head (YHV) and taura syndrome (TSV) viruses are much more pandemic than others, the shrimp diseases caused by bacteria belonging to the genus *Vibrio* also contribute to morbidity and even mass mortality in the shrimp. Caused by *Vibrio* spp., vibriosis is notably known as a lethal shrimp disease resulting in almost 100% cumulative mortality of the infected hosts (Alvarez et al., 1998; Flegel, 2006).

The apparent characteristics of the genus *Vibrio* are gram-negative, motile, and bioluminescent bacteria possessing a rod shape of around 0.5 μm in width and 1.4 μm in length. Normally found in seawater, *Vibrio* spp. are facultative anaerobes and most of them are mortal pathogenic strains in a wide spectrum of hosts such as *Vibrio parahaemolyticus*

and *Vibrio vulnificus* found in mammals, and *Vibrio fischeri* and *Vibrio harveyi* found in aquaculture (Rao, 2002).

1.3.1 *Vibrio harveyi*

Of the genus *Vibrio*, the luminescent *Vibrio harveyi* bacterium is recognized as one of the most devastating species of penaeid shrimp by causing many deadly diseases (Diggles BK et al., 2000; Thompson FL et al., 2004). The penaeid shrimp are typically infected with *V. harveyi* in all life cycle, but mostly in the larvae stage. During the infection, several pathogenic mechnisms of *V. harveyi*, such as quorum sensing factors and biofilm forming, are necessary for the bacteria to circumvent from host immune defenses. The evident symptoms caused by *V. harveyi* in the infected penaied shrimp are called luminous vibriosis (disorder development of basal tissues in the digestive system) and bolitas negricans (glowing in the dark, visible on the thoracic and head part). Extracellular products (ECPs) which consist of proteases, phospholipase and hemolysin, are regarded as the dominant virulent factors and these exotoxins exhibit brutally pathogenic effect to penaeid shrimp, especially the black tiger shrimp, *P. monodon* (Austin and Zhang, 2006; Harris and Owens., 1999.; Lavilla-Pitogo et al., 1998).

1.4 Invertebrate immunity

All creatures always encounter to various invading pathogenic organisms; thus they rely on efficient immune defense mechanisms in order to protect themselves against the pathogenic attack. Unlike vertebrate, invertebrates possess solely the innate immunity as a major immune defense. The innate immune system is considered as an evolutionarily conserved immune system. Upon the pathogen intrusion, this system non-specifically and immediately reacts against the pathogens by stimulation a massive array of associated and concerted host defense mechanisms (Figure 1.2).

This immune defense comprises of two major responses; cellular mediated and humoral immune responses (Jiravanichpaisal et al., 2006). The defense mechanism in which host hemocytes directly participate are catagorized in the cellular mediated reponse including phagocytosis, nodule formation and encapsulation. Otherwise, the humoral immune reponse defines as the defense mechanism without any direct participations of immune related cells, but immune effectors originally synthesized from those cells mainly involve in activation of

signal transduction to initiate immune cascades. These effectors are varied in biological functions and act as potential biomolecules, such as soluble substances, enzymes, and proteins, involved in prophenoloxidase system, hemolymph coagulation system, agglutination, proteases, proteinase inhibitors, and antimicrobial peptides (Hoffmann et al., 1999; Iwanaga and Lee, 2005; Söderhäll, 1999). There is, however, an overlap between both types of the immune responses because several humoral effectors involve the hemocytic responses as well as the blood cells are a principle source of a number of humoral molecules (Elrod-Erickson et al., 2000; Lavine and Strand, 2002).

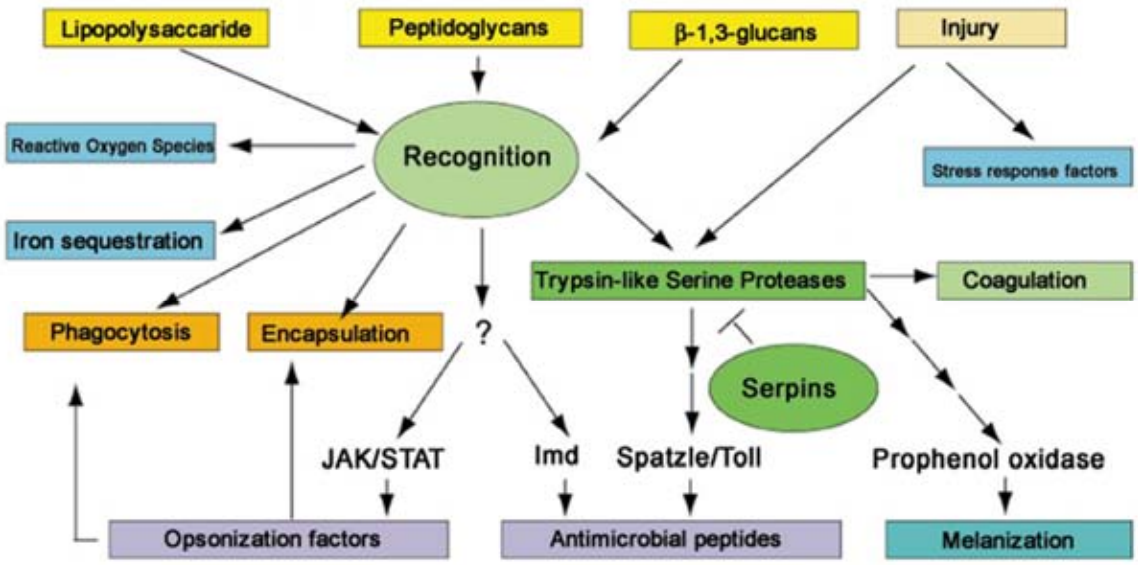

Figure 1.2 Schematic llustration of the innate immune defense mechanism of the *Drosophila melanogaster* (Source: CGM - Department Cellular Dynamics and Development)

1.5 The crustacean immune response

In crustaceans, in addition to the innate immune response as described above, they also have physical protections against the invading pathogen which are the rigid exoskeleton covering all body surfaces and the mucin barrier to trap pathogenic components.

The hemocytes in crustaceans are a major immune related cells because they can involve both cellular mediated immune responses and humoral immune responses. These immune responsive cells, hemocytes, are fundamentally classified into three groups; hyaline cell (agranular), semigranular cell (small granular) and granular cell (large granular)

(Bauchau, 1980; Tsing et al., 1989). The hyaline hemocytes serve potential roles in phagocytosis and in initiation of the blood clotting via cytolysis (Smith and Söderhäll, 1983; Vargas-Albores F. et al., 1998), while the granular hemocytes generally function in apoptosis, encapsulation, melanization and nodulation via exocytosis (Kobayashi et al., 1990; Pech and Strand, 2000; Sung et al., 1998).

In shrimp, another important immune responsive organ is the lymphoid organ which plays crucial roles in pathogen filtation from the blood circulation. The synergy between both immune associated tissues, the lymphoid organ and hemocytes, results in a significant immune defense response to various pathogenic microorganisms (Burgents JE et al., 2005).

1.5.1 Cellular-mediated defense mechanisms

The cell-mediated immune defense, one of the innate immunity, includes three main mechanisms which are phagocytosis, encapsulation, and nodule formation (Millar and Ratcliffe, 1994). Circulating blood cells in the blood stream play major roles in these mechanisms and the different cellular defense mechanisms depend on the type of the hemocytes (hyaline, semigranular, and granular cells) and the aspects of the pathogenic invasion.

Phagocytosis sharing the same ordinary processes in various organisms consists of 3 major steps; foreign particles attachment, internalization, and destruction. This reaction can remove the invading pathogens as well as the cell debris (Jeon et al., 2010). In case that the invaders are too huge to be phagocytosed, multiple layers of hemocytes are formed and adhered surrounding the foreign material to diminish its severity. This mechanism is called encapsulation (Gillespie et al., 1997). Nodule formation is responsible for entrapment of the high number of invading microbes. The entrapped particles are in the center of the forming nodules and are subsequently destroyed by nutrient deficiency and oxygen species produced by the prophenoloxidase activating system (Jiravanichpaisal et al., 2006).

1.5.2 Pattern recognition proteins

When the pathogenic microorganisms invade animals, cell surfaces components of the pathogens will be detected and recognized by the first line of the innate immune response of the hosts. These factors are referred to as Pathogen-associated molecular patterns (PAMPs) which vary among groups of the invading pathogens; for instance, lipopolysaccharides (LPS) of gram-negative bacteria, lipoteichoic acids (LTA) of gram-positive bacteria, peptidoglycan of of microbial cell wall, β-1,3-glucan of fungi, mannans of yeasts, glycolipids of

mycobacteria, and double-stranded RNA of viruses (Hoffmann et al., 1999). The recognition processes to these PAMPs are mediated by a variey of receptors or proteins of the host immune system, so-called Pattern Recognition Receptors or Proteins (PRRs or PRPs). The interactions between these recognition proteins and their partners are able to trigger a number of distinct immune casecades in response to the invading microorganisms (Figure 1.2).

In crustaceans, several pattern recognition proteins have been recently identified such as LPS binding proteins (LBPs), β-1,3-glucan-binding proteins (bGBPs), peptidoglycan recognition protein, lectins, and hemolin (Lee and Söderhäll, 2002). Additionally, many LPS or/and β-glucan binding proteins (bGBPs or/and LGBPs) has been found in penaied shrimp (Amparyup et al., 2012).

1.5.3 The prophenoloxidase (proPO) activating system

The prophenoloxidase (proPO) activating system is one of the most important innate immune sytems in crustaceans. This system comprises of several proteases participating in the immune cascade in order to generate cytotoxic products and melanin as final products, so-called melanization process. It is believed that the proPO acitivating system involves in phagocytosis, encapsulation and cell adhesion as well (Söderhäll and Cerenius, 1998).

Initially stimulated by pathogen-associated molecular patterns (PAMPs) such as microbial cell wall elements (LPS, LTA and peptidoglycan of bacteria) and β-1,3-glucan of fungi via pattern recognition proteins, prophenoloxidase-activating factor, enzyme or proteinases (ppA, PPAEs, PPAFs, PPAPs) which are stored as a zymogen, an inactive form, will be activated and transformed to phenoloxidase-activating factor, enzyme or proteinases (PAEs, PAFs, PAPs), an active form. This process lead to many active enzymes stepwise occured in the proPO cascade, which are controlled by serine proteases and their inhibitors, to eventually produce prophenoloxidase (PO) (Figure 1.3) (Ariki et al., 2004).

In crayfish, ppA, a trypin-like serine proteinase, and proPO are stored as an inactive form in granules of the hemocytes (Aspán, 1991; Wang et al., 2001a). Upon the elicitor stimulation, these enzymes are released together by exocytosis and then become activated. The active ppA converts the proPO to PO, an active form, a key enzyme of the melanin synthesis (Shiao et al., 2001; Söderhäll and Cerenius, 1998). The coversions of O-hydroxylation of monophenols to diphenols and oxideized diphenols to quinones are then catalyzed by PO. Quinones non-enzymatically polymerize themselves to form insoluble melanin, as can be seen by black spots at the wound sites or blackening of the microorganisms in the host plasma. In the melanization step, generated intermediates and insoluble melanin

can restrain the pathogen growth (Söderhäll and Ajaxon, 1982). Moreover, the production of melanin serves roles in wound healing, sclerotisation and encapsulation of foreign particales as well (Theopold, 2004).

Until now, four members of the proPO activating system designated as *Pm*proPO1, *Pm*proPO2 (proPO genes), and *Pm*PPAE1 and *Pm*PPAE2 (PPAE genes) have been found in the black tiger shrimp, *P. monodon*. The dsRNA mediated gene silencing (RNAi) of these genes result in significant reduction of total PO activity, causing the higher mortality rate of the *V. harveyi* challenged shrimp and increase in the number of bacteria counted in the challenged shrimp (Amparyup et al., 2009; Charoensapsri et al., 2009; Charoensapsri et al., 2011).

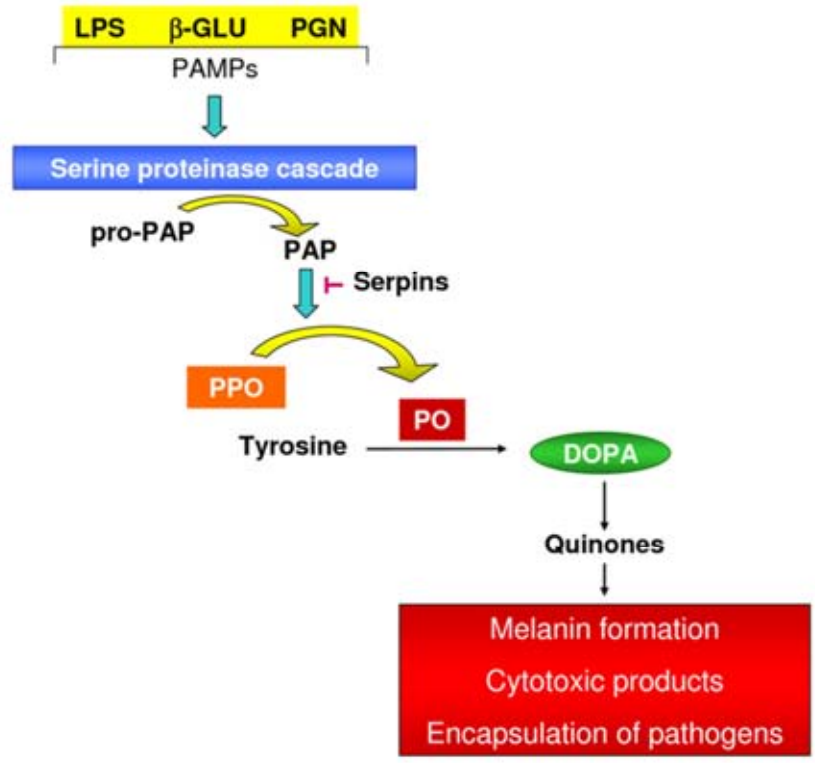

Figure 1.3 Serine proteinase cascade of the prophenoloxidase (proPO) activating system (Source: Garcia E S, Castro D P, Figueiredo M B, Genta F A, Azambuja P. (2009). "*Trypanosoma rangeli*: a new perspective for studying the modulation of immune reactions of *Rhodnius prolixus*". *Parasit Vectors* 2(1):33.)

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1.5.4 Antimicrobial peptides (AMPs)

Found in a variety of organisms including mammals and invertebrates, antimicrobial peptide (AMPs) are evolutionarily conserved elements of the innate immune defense. The AMPs are originated from many immune related tissues and various cell types. These peptides have a broad range of microorganism killing such as gram-negative and gram prositive bacteria, fungi, enveloped viruses, and even antibiotic resistant strains, so-called host defense peptide. They are generally small in size, approximately less than 200 amino acid residues, with total cationic charge and amphipathic structure; however, a few of anionic peptides are also found.

Due to these properties, not only the AMPs can be simply synthesized and rapidly translocated to the wound sites, but also they can attach and insert themselves into membrane bilayers to form pores. The pore forming on the membranes will destroy the membrane permeability barrier and completely lyse the cells resulting in the leakage of cellular components. To date, catagorized by the pore forming mechanisms, there have been 3 distinct models as follows; barrel-stave, carpet, and toroidal-pore models (Figure 1.4) (Brogden, 2005).

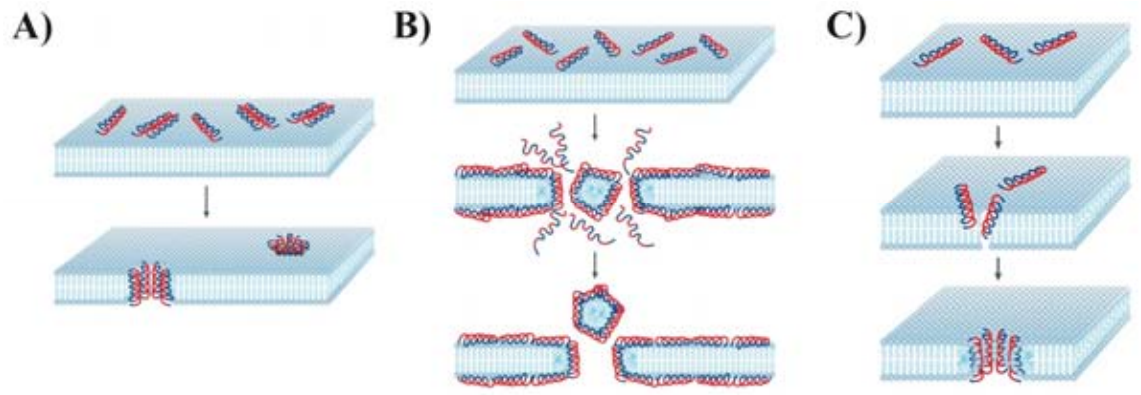


Figure 1.4 Overview of pore forming mechanisms of antimicrobial peptides (AMPs). A) The barrel-stave model of AMP-induced killing. B) The carpet model of AMP-induced killing. C) The toroidal model of AMP-induced killing. (Source: Brogden K.A. (2005). "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?". *Nature Reviews Microbiology* 3: 238-250)

In penaeid shrimp, several AMPs have been discovered and their biological functions have been studied (For review: (Tassanakajon et al., 2010). Penaeidins, one of the AMPs

family, exhibit antimicrobial and antifungal activities reported in the white shrimp, *L. vannamei*; additionally, penaeidin5 that have recently been found in the black tiger shrimp, *P. monodon*, serves immune functions agaist viral infection (Destoumieux-Garzon, 1997; Woramongkolchai et al., 2011). Besides, referred to as the crustacean antimicrobial pepteides, crustins such as crustin*Ls* crustin*Lv*, and crustin*Pm* have been identified in the penaeid shrimp, *L. setiferus, L. vannamei* and *P. monodon*. (Bartlett et al., 2002; Tassanakajon et al., 2010). They displayed no homology with other recognized AMPs in databases, but occupied sequence identity to the whey acidic protein (WAP), a member of proteinase inhibitory proteins. Antil-ipopolysaccharide factors (ALFs), a majority of AMPs identified in the *P. mondon* EST database, also show a wide range of antimicrobial activities to bacteria, viruses and filamentous fungi (Li et al., 2008; Liu et al., 2006; Somboonwiwat et al., 2005; Tassanakajon et al., 2006). Thus, it is evidenced that the AMPs play a major and effective role in host immune defense against diverse pathogens.

1.5.5 Blood coagulation system

Due to the fact that invertebrates have an open circulatory system, hemolymph coagulation system, one of the most funadamental innate immune defense, therefore, becomes necessary. The blood clotting serves important roles in preventing hemolymph bleeding at the wound sites as well as obstructing pathogen dissemination throughout the body (Martin et al., 1991).

In many organisms including crustaceans, plasma clotting occurs in hemolymph without direct involvement of circulating hemocytes. During an acitivation of the clotting, the reaction of cross-linking of coagulation-associated proteins in plasma is catalyzed by hemocyte-derived factors (Cerenius and Söderhäll, 2010).

In invertebrates, two distinct blood clotting reactions of horseshoe crab and crayfish have been most extensively studied (Figure 1.5). The plasma coagulation process of horseshoe crab is much more complicated than that of the crayfish. Upon stimulation of pathogen-associated molecular patterns (PAMPs) through pattern recognition proteins, hemocyte derived factors involved in the clotting are released via exocytosis and they are then coverted to proteolytically active forms by the PAMPs, especially LPS and β-1,3-glucan. These enzymes are responsible for activation of proclotting enzyme to clotting enzyme which can transform coagulogen to coagulin. The coagulin, coagulation-associated proteins, can non-covalently polymerize itself to form a large insoluble gel. Even though tranglutaminase (TGase) is not involved in the coagulin formation, it is yet necessay in cross-linking proxin

and stablin onto the coagulin gel for more productive bacterial immobilization (Cerenius and Söderhäll, 2010; Kawabata S et al., 2009).

Unlike horseshoe crab, blood clotting in crayfish and shrimp results from the polymerization between free glutamine and lysine residues of clottable proteins (CPs) which is catalyzed by a calcium ion-dependent TGase (Chen et al., 2005; Hall et al., 1999; Wang et al., 2001b). The CP in the black tiger shrimp, *P.monodon*, is a 380 kDa homodimeric glycoprotein linked by a disulfide bond and is mainly produced in stromal cells of the lymphoid organ, but not in hemocytes (Yeh et al., 2007; Yeh et al., 1999; Yeh et al., 1998). So far, there have been three isoforms of TGase found in the black tiger shrimp, *P.monodon*, which are *Pm*STG I (Huang et al., 2004) and two members of *Pm*STG II (Chen et al., 2005; Yeh et al., 2006). These *Pm*STG IIs exert the polymerization activity using CPs as a substrate; whilst *Pm*STG I lacks the blood coagulation activity. In addition, alpha-2-macroglobulin (A2M), a broad range proteinase inhibitor, was previously found to be decorated on the blood clots in both horseshoe crab and crayfish for some extra functions of the clotting system (Armstrong and Armstrong, 2003; Hall and Söderhäll, 1994).

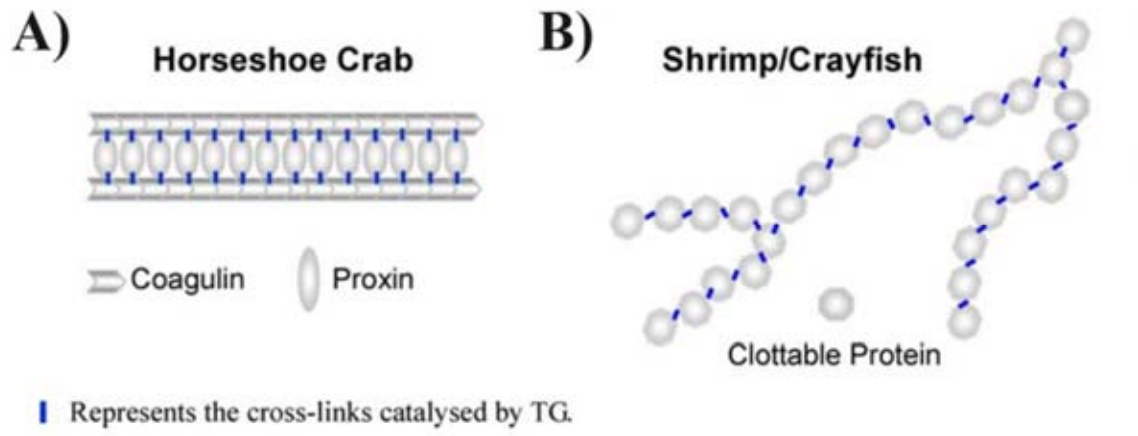


Figure 1.5 A comparative illustration of blood coagulation products between (A) horseshoe crab and (B) shrimp and crayfish. (Source: Chen M.Y., Hu K.Y., Huang C.C. and Song Y.L. (2005). "More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation.". *Developmental and Comparative Immunology* 29: 1003-1016.)

1.5.6 Apoptosis

Apoptosis is a process of programmed cell death which mainly involves in cellular development, differentiatiopn and cell-mediated immune defense including pathogen invasions. The apoptosis may result from biochemical changes such as cellular blebbing and shrinkage, DNA fragmentation, and chromatin condensation leading to cell morphology disorder, and finally cell death (Kerr et al., 1977; Thompson, 1995). Upon the pathogen infection, the apoptosis in the host cell is regulated by complicated interations between host and parasite proteins. Nevertheless, the programmed cell death is also implicated to the host immune defense against the pathogens (Hasnain et al., 2003).

In crustacean, apoptosis serves a crucial function in the antiviral immunity (Liu et al., 2009). It is evidenced that DNA fragmentation and chromatin condensation were observed in tissues of the black tiger shrimp, *P. monodon*, infected with either WSSV or YHV (Khanobdee et al., 2002; Sahtout et al., 2001). In addition, a number of apoptosis regulators such as caspase and apoptosis 1 inhibitor have been revealed in the *Vibrio spp.* challenged penaeid shrimp (de Lorgeril et al., 2005; Zhang et al., 2010).

1.5.7 Other immune-related molecules

1.5.7.1 ATP synthase beta subunit

Found in the chloroplast thylakoid membranes and mitochondrial inner membranes of various organisms, ATP synthase is a key enzyme complex involved in the energy production that controls the flow of the proton gradient across the respective membrane. The ATP synthases, also called F_1F_0-ATP synthases, are comprised of two main parts; a soluble part (the F_1 sector) and a membrane bound part (the F_0 sector). The F_1 sector functions in ATP formation and hydrolysis utilizing its subunits, such as the α, β and the central stalk subunit (γ, δ and ε), while the F_0 sector is a proton channel made up from several subunits, such as the C ring, subunit a and peripheral stalk subunit (b, d, F_6 and OSCP) (Devenish et al., 2008). Nevertheless, the roles of the F_1F_0-ATP synthase as a cell surface receptor with diverse cellular activities in various mammalian cell types, such as adipocytes, endothelium, hepatocytes and tumor cells, have been reported (Champagne et al., 2006; Chi and Pizzo, 2006). In crustaceans, the ATP synthase beta subunit located on the membranes of the hematopoietic tissue of the crayfish, *P. leniusculus*, was identified to function as the receptor of the crayfish cytokine like hormones, prokineticin and astakine, which are essential for hemocyte differentiation and secretion (Lin et al., 2009). The ATP synthase beta subunit- in

the Pacific white shrimp, *L. vannamei*, was also found to be involved in WSSV infection (Liang et al., 2010).

1.6 Proteinases and their inhibitors in the immune system

Classified by the basis of their active-site catalytic residue, proteinases can be divided into main proteinase families as follows; i) metalloproteinase, ii) serine proteinase , iii) cysteine proteinase , iv) threonine proteinase , and v) aspartic proteinase, and vi) unidentified proteinase families. Although a variety of proteinases, such as serine proteinases, cysteine proteinases, and aspartic proteinases, serves major functions in the immunity in vertebrates, serine proteinases are supposed to be one of the most vital elements in the immune system of insects and arthropods due to the relatively high concentration of their inhibitors in these organisms' hemolymph (Kanost, 1999; Tyndall et al., 2005; van Eijk et al., 2003). Otherwise, proteinases produced by pathogenic parasites are virulence factors playing various roles in pathogenesis. It has been reported that almost all types of classified proteinases (serine, cysteine, metallo, and aspartyl proteinases) were found to associate in the pathogenic evasions of the host immune system (Armstrong, 2006; Wladyka and Pustelny, 2008).

Expectedly, protease inhibitors are hence ubiquitously found in various creatures and consequentially become essential for controlling both exogenous and endogenous proteases. These proteinase inhibitors can be categorized by their inhibitory mechanisms into 2 major groups; active site inhibitors and alpha-2-macroglobulin (A2M). The inhibitory mechanism of the active site inhibitors is that the inhibitors directly interact with proteinases resulting in activity losses, while A2M possesses its distinctly unique mechanism to inhibit the proteinase activity (Armstrong, 2001; Laskowski and Kato, 1980). Since serine proteases and their inhibitors are a major component in arthropod immunity, a specific classification of the serine proteinase inhibitors has also been reported. Based on their functions and three dimensional structures, the serine proteinase inhibitors in arthopods can be mainly divided to Serpins, Alpha-2-macroglobulin (A2M), Kunitz, Kazal, and Pacifastin (Kanost, 1999).

1.7 Alpha-2-macroglobulin (A2M)

Alpha-2-macroglobulin (A2M) is a broad range-protease inhibitory molecule and is an evolutionarily conserved component in the innate immunity. A2M possesses a unique mechanism to entrap a targeted proteinase inside its molecule without active site inactivation, so-called the physical entrapment mechanism (Figure 1.6). This molecular compaction of the A2M hinders an access of macromolecular substrates to the entrapped proteinase.

Nevertheless, the proteinase activity of the targeted proteinase is almost completely inhibited; unless small substrates can diffuse through this molecular cage into the active site of the entrapped proteinse. In many organisms including mammals and invertebrates, A2M is recognized as a highly abundant plasma protein whose well-characterized function is to eliminate active proteases from the circulatory system (Armstrong, 2010).

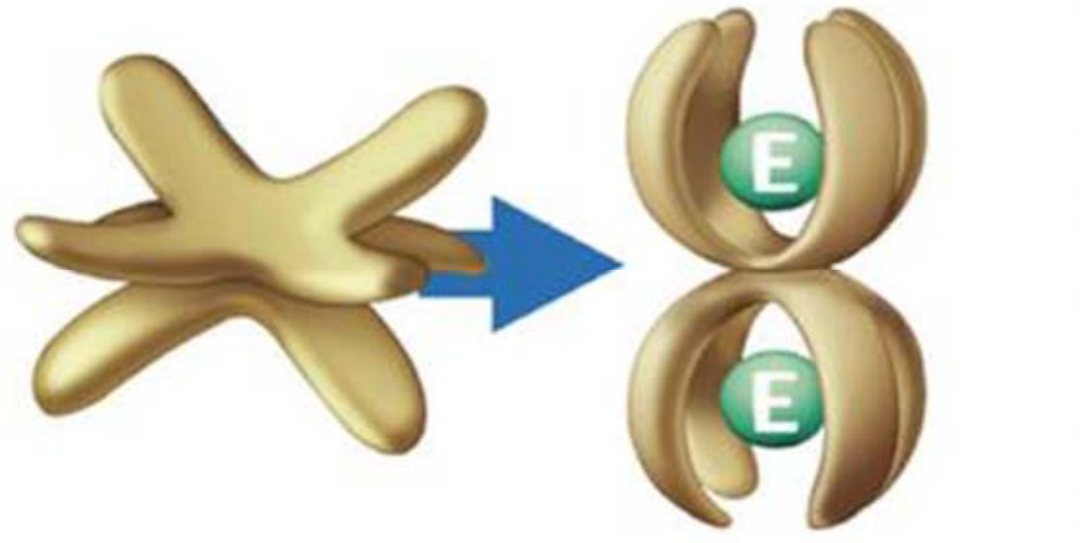


Figure 1.6 Brief overview of the physical entrapment mechanism of alpha-2-macroglobulin. "E" is representative of a protease (Source: http://www.systemicenzyme support.org/alpha-2-macroglobulin/ index.htm).

A2M molecule consists of 3 important regions as follows; bait region, thiol ester, and receptor binding domain (RBD). The bait region, a solvent exposed stretch, has approximately 50 amino acid residues in length where contains one or more proteolytic attack sites. Proteolysis of this region causes the molecular compaction of A2M to entrap the reacting protease. This conformation change initiates the cleavage of the thiol ester bond, therefore a highly reactive group that is the carbonyl of glutamic acid residue is exposed for subsequent crosslinking to either the reactive protease or another A2M subunit to stablize its structure. The RBD is then disclosed following this physical entrapment process and this domain will be recognized by cell surface receptors for later clearance of the protease-reacted A2M (For reviews: (Armstrong and Quigley, 1999)).

There have been a number of studies reporting that A2M serves crucial roles in the innate immune response in arthropods such as phagocytosis in the mosquito, *Anopheles gambiae* (Levashina et al., 2001) and the hard tick, *Ixodes ricinus* (Buresova et al., 2009), the blood clotting system in crayfish, *Pacifastacus leniusculus* (Hall and Söderhäll, 1994) and the horseshoe crab, *Limulus polyphemus* (Armstrong et al., 1984), and the proPO activating system in the crayfish, *P. leniusculus* (Aspán et al., 1990). In penaied shrimp, A2Ms has been cloned and characterized including the white shrimp, *L. vannamei* (Gollas-Galván et al., 2003), the kuruma shrimp, *Marsupenaeus japonicus* (Rattanachai et al., 2004), the black tiger shrimp, *P. monodon* (Lin et al., 2007), and the chinese white shrimp, *Fenneropenaeus chinesis* (Ma et al., 2010). Altered A2M expression levels upon endotoxin and pathogen challenges as well as A2M binding partners, syntenin and phagocytosis activating protein, have been also reported (Chotigeat et al., 2007; Ho et al., 2009; Lin et al., 2007; Rattanachai et al., 2004; Tonganunt et al., 2005). Most recently, proteomic analysis revealed that A2M was the most strongly altered protein in hemocytes of the black tiger shrimp, *P. monodon*, upon *V. harveyi* infection (Somboonwiwat et al., 2010).

1.8 Purposes of the thesis

In order to better understand the shrimp innate immunity agaist bacterial infection and/or antibacterial mechanisms, protein expression profiles of the lymphoid organ of *V. harveyi*-challenged *P. mondon*, the black tiger shrimp, were initially examined using two-dimensional electrophoresis (2-DGE) and mass spectrometry (MS). ATP synthase beta subunit, the highest up-regulated protein, and alpha-2-macroglobulin (A2M), a highly down-regulated protein, appeared to be the most interesting proteins among other immune responsive proteins, thereby they were selected for further characterization. It was evidenced that ATP synthase beta subunit involves in hemocyte production as well as shrimp survival. Moreover, A2M was found to serve essential roles in the blood clotting system by protecting the shrimp clots against protease-mediated fibrinolysis and bacterial escape.

CHAPTER II
MATERIALS AND METHODS

2.1 Equipments and Chemicals

2.1.1 Equipments

- 20 °C Freezer (Whirlpool)
- 80 °C Freezer (ThermoForma)

0.45 mm glassbeads

Amicon Ultra concentrators (Millipore)

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

Automatic micropipettes P2, P10, P100, P200 and P1000 (LioPette / Select BioProduct / Gilson Medical Electrical S.A.)

Balance Satorius 1702 (Satorius)

Centrifuge 5804R (Eppendorf), Centrifuge Avanti^{TM} J-301 (Beckman Coulter)

Confocal Laser Scanning Microscopy (OLYMPUS FV1000)

Critical Point Dryer (Balzers, model CPD 020)

Ettan IPGphor 3 IEF system (GE healthcare)

Gel documentation system (GeneCam FLEX1,SYNGENE)

Gene Pulser (Bio-RAD)

Hematocytometer (Cole-Parmer)

Hybridization oven (Hybrid)

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

Ion sputter (Balzers, model SCD 040)

IPG strips (nonlinear pH 4 - 7; 13 cm long) (GE healthcare)

Incubator (Memmert)

Innova 4080 incubator shaker (New Brunswick Scientific)

Imagescanner III (GE healthcare)

JEOL scanning electron microscope, model JSM-6510 (JEOL Ltd.)

LABO Autoclave (SANYO)

Laminar Airflow Biological Safety Cabinets (NuAire, Inc.)

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

Minicentrifuge (Costar, USA)

Nipro disposable syringes (Nissho)

Nano-LC–ESI–MS/MS

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)

PD-10 column (GE Healthcare)

pH meter Model # SA720 (Orion)

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

Polysine slides (Thermo Scientific)

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

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

Touch mixer Model # 232 (Fisher Scientific)

TCBS (thiosulfate–citrate–bile–sucrose) (Becton, Dickinson)

Sterring hot plate (Fisher Scientific)

Trans-Blot® SD (Bio-RAD Laboratories)

Ultra Sonicator (SONICS Vibracell)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

Whatman® 3 MM Chromatography paper (Whatman International Ltd., England)

2.1.2 Chemicals, Reagents and Biological substance

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

1,10-Phenanthroline (Sigma)

2-Mercaptoethanol, C_2H_6OS (Fluka)

3-(N-morpholino) propanesulfonic acid (MOPS) (USB)

5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (Fermentas)

5-bromo-4-chloro-indolyl phosphate (BCIP) (Fermentas)

Absolute ethanol, C_2H_5OH (BDH)

Absolute methanol, CH_3OH (Scharlau)

Acetic acid glacial, CH_3COOH (BDH)

Acetic anhydride (Sigma)

Acrylamide (Plus one)

AEBSF (Sigma)

Agarose (Sekem)

Alexa Fluor® 488 goat anti-rabbit IgG antibody (Invitrogen)

Alexa Fluor® 568 goat anti-mouse IgG antibody (Invitrogen)

Alexa Fluor® 647 goat anti-rabbit IgG antibody (Invitrogen)

Alkaline phosphatase-conjugated goat anti-mouse IgG (Millipore)

Alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.)

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

Amplicillin (BioBasic)

Anti-His antiserum (GE Healthcare)

Aureobasidin A (Clontech)

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (MERCK)

Bovine serum albumin (Fluka)

Bromophenol blue (MERCK)

Calcium chloride (MERCK)

CHAPS (Biorad)

Chloramphenicol (Sigma)

Chloroform, $CHCl_3$ (MERCK)

Coomassie brilliant blue G-250 (Fluka)

Coomassie brilliant blue R-250 (Sigma)

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

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

Dithiothreitol (Pharmacia)

E64 (Sigma)

Ethanol (MERCK)

Ethidium bromide (Sigma)

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

Formaldehyde (BDH)

Formamide deionized (Sigma)

GeneRuler™ 100bp DNA ladder (Fermentas)

GeneRuler™ 1kb DNA ladder (Fermentas)

GelCode Blue Stain Reagent (Pierce)

Glacial acetic acid (J.T. Baker)

Glucose (Ajax chemicals)

Glycerol, $C_3H_8O_3$ (Scharlau)

Glycine, NH_2CH_2COOH (Scharlau)

HALT (protease inhibitor mix) (Pierce)

Hydrochloric acid (HCl) (MERCK)

Imidazole (Fluka)

Iodoacetamide (GE healthcare)

Isopropanol, C_3H_7OH (MERCK)

Isopropyl-β-D-thiogalactoside (IPTG) , $C_9H_{18}O_5S$ (USBiological)

Kanamycin (BIO BASIC Inc.)

Magnesium chloride ($MgCl_2$) (MERCK)

Methanol, CH_3OH (MERCK)

Minimal Media Single Dropouts (SDO) (Clontech)

SD/-Trp

SD/-Leu

Minimal Media Double Dropouts (DDO) (Clontech)

SD/-Leu/-Trp

Minimal Media Quadruple Dropouts (QDO) (Clontech)

SD/-Ade/-His/-Leu/-Trp

Monoclonal antibody to ATP synthase subunit beta (Invitrogen)

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

N, N', methylenebisacrylamide (Fluka)

Ni Sepharose 6 Fast Flow (GE Healthcare)

Nitroblue tetrazolium (NBT) (Fermentas)

Nytrans® super charge nylon membrane (Schleicher&Schuell)

Paraformaldehyde (Sigma)

Pepstatin (Sigma)

Phenol, saturated (MERCK)

Potassium chloride, KCl (Ajax)

Prestained protein molecular weight marker (Fermentas)

Prolong Gold Antifade Reagent (Invitrogen)

PMSF (phenylmethanesulfonylfluoride) (Sigma)

RNase A (Sigma)

RNA markers (Promega)

Skim milk powder (Mission)

Sodium acetate, CH_3COONa (Carlo Erba)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium chloride, NaCl (BDH)

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

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

Sodium hydroxide, NaOH (Eka Nobel)

Sucrose, $C_{12}H_{22}O_{11}$ (Sigma)

Thiourea (GE healthcare)

TO-PRO-3 iodide (Invitrogen)

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

TRI Reagent® (Molecular Research Center)

Triton® X-100 (MERCK)

Tween™-20 (Flula)

Unstained protein molecular weight marker (Fermentas)

Urea (Fluka)

Xylene cyanol FF, C25H27N2O6S2Na (Sigma)

X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) (Clontech)

YPDA Broth/Agar (Clontech)

2.1.3 Enzymes and Kits

2-D Quant kit (GE healthcare)

Advantage®2 Polymerase Mix (Clontech)

*Bam*HI (Biolabs)

ImProm-II™ Reverse Transcription system kit (Promega)

Matchmaker™ Gold Y2H System (Clontech)

MakeYour Own "Mate & Plate™" Library system (Clontech)

Maxima™ SYBR Green qPCR Master Mix (Fermentas)

*Nde*I (Biolabs)

NucleoSpin® Extract II Kits (MACHEREY-NAGEL)

QIAprep® Miniprep kits (Qiagen)

RevertAID™ first strand cDNA synthesis kit (Fermentas)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase (Promega)

T7 RiboMAX™ Express Large Scale RNA Production System (Promega)

T&A cloning vector kit (RBC Bioscience)

Taq DNA polymerase (Fermentas)

*Xho*I (Biolabs)

Yeastmaker™ Yeast Transformation System 2 (Clontech)

2.1.4 Microorganisms

Escherichia coli strain BL21 (DE3)

Escherichia coli strain XL-1-Blue

Vibrio harveyi strain 639

Saccharomyces cerevisiae strain Y2HGold (Clontech)

Saccharomyces cerevisiae strain Y187 (Clontech)

2.1.5 Software

BlastN and BlastX (http://www.ncbi.nlm.nih.gov/BLAST/)

ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

ClustalX (Thompson, 1997)

ExPASy ProtParam (http://au.expasy.org/tools/protparam.html)

EMBOSS Pairwise Alignment (http://www.ebi.ac.uk/Tools/emboss/align/)

GENETYX (Software Development Inc.)

Image J program (The National Institutes of Health)

ImageMaster 2D Platinum 6.0 software (GE healthcare)

ImageQuant™ TL (GE Healthcare)

MASCOT MS/MS ion search tool (http://www.matrixscience.com/cgi/search_form.pl ?FORMVER=2&SEARCH=MIS)

Penaeus monodon EST database (http://pmonodon.biotec.or.th/home.jsp)

PHYLIP (Felsenstein, 1993)

SECentral (Scientific & Educational Software)

SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/)

SPSS statistics 17.0 (Chicago, USA)

2.1.6 Vectors

pET-22b(+) (Novagen)

pGADT7 (Clontech)

pGADT7-T Control Vector (Clontech)

pGBKT7 (Clontech)

pGBKT7-53 Control Vector (Clontech)

pGBKT7-Lam Control Vector (Clontech)

T&A cloning vector (RBC Bioscience)

2.2 Animal cultivation

Specific pathogen free (SPF)-healthy juvenile black tiger shrimp, *Penaeus monodon*, which size around 3 or 20 grams body weight used in the RNA interference experiment and the general purposes, respectively, were obtained from the Broodstock and Larval Development Research Center at Walailuk University, Nakornsrithammarat Province, Thailand. Prior to use, shrimp were acclimatized in artificial seawater with salinity of 15 ppt, 28 ± 4°C and continuously aerated for at least a week.

2.3 *V. harveyi* challenged *P. monodon*

Experimental shrimp were divided into 2 groups, which were challenged and non-challenged groups. Each solution were introduced to the experimental shrimp by injection at the fourth abdominal segment. Three experiments were individually performed with at least 3 individuals in each experiment. Determination of *V. harveyi* infection in the experimental shrimp was verified before and after the bacterial challenge.

2.3.1 Preparation of *V. harveyi* strain 639

V. harveyi strain 639 which is a severe pathogen of the black tiger shrimp, *P. monodon*, was grown on a tryptic soy agar (TSA) plate supplemented with 2% (w/v) NaCl. The plate was incubated upside down at 30 °C for overnight. A single colony was then inoculated in a tryptic soy broth (TSB) supplemented with 2% (w/v) NaCl and cultured at 30 °C, with shaking at 250 rpm for 12-16 h. The culture was then transferred into sterile TSB supplemented with 2% (w/v) NaCl to make a dilution of 1/100 and later inoculated at 30 °C, with orbitally shaking at 250 rpm until A_{600} reached 0.6 where bacterial cell densities was 10^8 CFU/ml monitored by the plate count method. The culture was later diluted 1/100 in 0.85% (w/v) saline solution, to make cell suspension of 10^6 CFU/ml.

2.3.2 *V. harveyi* challenge

The shrimp in the control group were injected with 100 µl of 0.85% (w/v) sterile saline solution while those in the experimental group were intramuscularly injected with 100 µl of the *V. harveyi* diluted with 0.85% (w/v) sterile saline solution (10^5 CFU, a lethal dose).

2.3.3 Diagnosis of *V. harveyi* infection

PCR technique was performd in order to detect the *V. harveyi* infection in the experimental shrimp. A template for PCR amplification was prepared as following. The gills of individual *V. harveyi* infected shrimp were collected, and then homogenized in 200 µl of lysis buffer (2.5 N NaOH and 10% (w/v) SDS) to extract total DNA. The homogenized samples were boiled for 10 min, chilled on ice for 3 min, and then centrifuged for 10 min at 8,000 x g at 4 °C. The collected supernatant was diluted 1:100 in sterile DI H_2O used as a template.

The PCR amplification of a product fragment of 363 bp using the gyrB primer pair (forward primer FA2: 5' TCTAACTATCCACCGCGG 3' and reverse primer RB3: 5' AGCAATGCCATCTTCACGTTC 3') was carried on. The PCR reactions consisted of 2 µl of diluted DNA template, 5 µl of each 2 µM primer, 1.6 µl of 2.5 mM dNTPs, 2 µl of 10X PCR buffer, 2 µl of 2.5 mM MgCl2, and 0.1 µl of 5 U/µl Taq polymerase (Fermentas). The reaction volume was then adjusted to 20 µl by PCR-grade H_2O. The PCR condition comprised of 30 cycles of denaturation step at 94 ^oC for 1 min, annealing step at 63 ^oC for 1 min, and extension step at 72 ^oC for 2 min, followed by final extension step at 72 ^oC for 7 min. The positive and negative controls were amplified from the PCR samples with and without *V. harveyi*, respectively. The PCR products were analyzed by 1% TBE-agarose gel electrophoresis and ethidium bromide staining. The band of DNA was visualized under a UV transilluminator.

2.4 Collection of shrimp immune related tissues

The lymphoid organ was collected for the examination of differentially expressed proteins against *V. harveyi* infection using two-dimensional gel electrophoresis (2-DGE) and mass spectrometry while hemocytes were harvested for general experiments. Other important tissues including the lymphoid organ and hemocytes were collected for the determination of a transcript distribution in various tissues.

2.4.1 The lymphoid organ

The lymphoid organ was individually dissected from the experimental shrimp; *V. harveyi*-challenged and non-challenged shrimp. The collected organ was immediately frozen in liquid nitrogen (N_2) until use.

2.4.2 Hemocytes

Shrimp hemolymph was aspirated from the ventral cavity using 24 G / 1 inch needle fitted onto a 1.0 ml sterile syringe pre-filling with 100 µl of ice-cold 10% (w/v) trisodium citrate, an anticoagulant. The hemocytes were seperated by centrifugation at 800 x g for 15 min at 4 ^{o}C. The hemocyte pellets were immediately frozen in liquid nitrogen (N_2) until use.

2.4.3 Other tissues

Shrimp tissues such as the antennal gland, gill, epipodite, eye stalk, hemocytes, heart, hepatopancreas, hematopoietic tissue, intestine, stomach, and the lymphoid organ, were seperately dissected from normal shrimp and immediately frozen in liquid nitrogen (N_2) until use.

2.5 Protein sample preparation from the lymphoid organ

2.5.1 Total protein preparation

The lymphoid organ from each individual collected as described above was thoroughly homogenized in the lysis buffer (8 M urea, 2 M thiourea, 50 mM DTT, 0.2% (v/v) Triton X-100 and 1 mM PMSF). The cell debris was centrifuged at 12,000 x g, 4 °C for 20 min and the supernatant was collected. The total protein was precipitated with ice-cold acetone:methanol (3:1 (v/v)) at -20 °C overnight, followed by centrifugation at 12,000 x g, 4 °C for 10 min to collect the protein. Before the protein pellet was solubilized in the rehydration buffer (8 M urea, 2 M thiourea, 4% (w/v) CHAPS and 1 mM PMSF), the pellet was washed with cold acetone. The protein samples from 7 individuals were equally pooled and kept at -80 °C until use.

2.5.2 Protein quantification using 2-D Quant kit

The protein concentration of the prepared samples was assessed using a 2-D Quant Kit (GE healthcare). The protein samples were diluted by distilled-H_2O to an appropriate concentration, and then the sample was aliquoted into 2 centrifuge tubes with the volume of 20 and 40 µl, respectively. 500 µl of the precipitant solution was added into either protein

samples or BSA standard solution at the different concentrations. The samples were then mixed and incubated at ambient temperature for 3 min. Next, 500 µl of the co-precipitant solution were added and then mixed by inversion. Centrifugation at 12,000 x g for 10 min was performed to collect the pellets which were later resuspended in 100 µl copper solution and 400 µl of distilled-H_2O. After dissolving until the pellets totally disappeared, 1 ml of working solution (1:1 volume of solution A:solution B) was added and the samples were immediately mixed votexing. The samples were incubated at ambient temperature for 15 min and then measured the absorbance at 480 nm.

2.6 Two-dimensional gel electrophoresis (2-DGE)

To identify the altered proteins in expression level, the protein expression profiles of the lymphoid organ of *V. harveyi*-challenged shrimp were compared to that of the control shrimp. Each pooled protein sample was separated through the 2-DGE which composed of 1) the 13-cm long nonlinear pH 4–7 IPG strips (GE Healthcare) used for the protein separation in the first dimension based on isoelectric focusing (IEF) and 2) the reducing SDS-PAGE (12.5% (w/v) acrylamide resolving gel) used for the protein separation in the second dimension based on molecular weight of the seperated proteins.

2.6.1 First dimension (Isoelectric focusing, IEF)

In this experiment, 3 pooled protein samples, derived from a sum of 21 experimental shrimp at each time point, were analysed. The rehydration of IPG strip by a pooled protein sample dissolved in the rehydration buffer containing 1% (w/v) bromophenol blue, 50 mM DTT and 0.5% (v/v) IPG buffer pH 4–7 (PlusOne) at room temperature overnight was conducted. The rehydrated strips were then isoelectrical focused by the Ettan IPGphor 3 IEF system (GE healthcare) at 20 °C using a voltage focusing protocol as following: 300 V for 2 h, 500 V for 2 h, 1,000 V for 2 h, 4,000 V at 2 h and eventually at 8,000 V to reach 80,000 Vh.

2.6.2 Second dimension (12.5% (w/v) reducing SDS-PAGE)

Afterward, the focused IEF strip was incubated in the SDS equilibration buffer (6 M urea, 2% (w/v) SDS, 50 mM Tris–HCl (pH 8.8), 30% (v/v) glycerol and 1% (w/v) bromophenol blue) containing 10 mg/ml DTT with gently shaking for 15 min at room temperature and subsequently incubated in the SDS equilibration buffer supplemented with 2.5 mg/ml IAA for 15 min at room temperature. The reducing SDS-PAGE was performed at

constant 20 mA until the front dye reached the bottom of the gel. The gel was finally stained with colloidal Coomassie Brilliant Blue G250.

2.6.3 Identification of differentially expressed protein spots

Imagescanner III (GE Healthcare) was used to scan the CCB G250-stained gel images. The protein spots were then detected and analyzed using the ImageMaster 2D Platinum 6.0 software (GE Healthcare). Before matching protein spots across all gels, the percentage of intensity volume (%vol) of each spot was normalized to the total intensity volume of all spots in each gel. The differences in the %vol of each spot between the control and *V. harveyi* challenged groups were then compared with acceptable statistical criteria of the Student's t test ($p < 0.05$). Only the eligible protein spots with at least a 3-fold alteration in the protein expression level in all gels were later subjected to a nano-LC–ESI–MS/MS for protein annotation.

2.7 Mass spectrometry based protein annotation

In-gel tryptin digestion was conducted to extract the proteins excised from the gels and then the peptides were determined by a LC–ESI–MS/MS. MS results of these proteins were identified and subsequently subjected to the MASCOT search program (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS) using the NCBI and EST databases. A peptide mass tolerance of 0.25 Da and up to one missed cleavage allowed were parameters used in the MS/MS ion search while methionine oxidation and cysteine carbamidomethylation were selected as the variable modification parameters. Based on significant hits upon searching, the protein was then annotated. The MW and pI of predicted proteins were confirmed in order to check the correction of identified protein spots.

2.8 Confirmation of protein alteration in response to *V. harveyi* infection by Western blot analysis

The total protein was extracted from the lymphoid organ of three individuals ,which were either saline- and *V. harveyi*-injected shrimp at 6 or 72 hpi, and then was quantitated for the concentration by the 2-D Quant Kit (GE Healthcare), as describe above. Fifty micrograms of the pooled proteins were run in a 10% (w/v) SDS-PAGE followed by transferred onto the nitrocellulose membranes, later analyzed by Western blot analysis. A dilution of 1:5,000 of ATP synthase subunit beta-monoclonal antibody (Invitrogen) was applied for the membrane incubation at room temperature for 1 h. The membrane was later incubated in AP-conjugated

goat anti-mouse IgG (Millipore), a secondary antibody, diluted 1:5,000 for additional 2 h at room temperature. Next, the bands were visualized by incubating in NBT/BCIP substrate solution. The intensity of positive bands was evaluated by ImageQuantTM TL (GE Healthcare). The band intensity of the ATP synthase beta subunit protein expressed in *V. harveyi*-injected shrimp was normalized to that of the normal saline-injected shrimp at each time point.

2.9 Quantitative analysis of gene transcript expression using real-time RT-PCR

To study the mRNA expression, the lymphoid organs were dissected from *V. harveyi*-challenged shrimp at 0, 6, 24 and 48 hpi. The organs were thoroughly homogenized in 1 ml of TRI Reagent® (Molecular Research Center) followed by adding 200 µl of chloroform and vigorous shaking for 15 sec. The reaction was incubated at ambient temperature and later centrifuged at 12,000 x g for 15 min at 4 °C. Upper phase was transferred to a new eppendrof and 500 µl of absolute isopropanol was added to precipitate the RNA. After incubation at - 20 °C for 15 min, the RNA pellet was centrifuged at 12,000 x g for 15 min at 4 °C and later washed with 1 ml of 70% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated H_2O. Prior to use, the RNA pellet was completely air-died and then dissolved in 20 µl of DEPC-treated H_2O. To remove contaminants such as chromosomal DNA, RNA was subsequently treated with RQ1 RNase-free DNase I (Promega) at 37 °C for 30 min and the total RNA was re-purified using protocol as described above. The amount of total RNA from three individual shrimp was measured by a spectrometer and later equally pooled for further cDNA synthesis using a RevertAID™ First Strand cDNA Synthesis Kit (Fermentas).

Using the iCycler iQTM Real-Time Detection System (Bio-Rad), quantitative real-time RT-PCR (qRT-PCR) was conducted. The target genes, which were ATP synthase beta subunit, 14-3-3 like protein, and Alpha-2-macroglobulin (A2M), were amplified by final concentrations of gene specific primers, listed in Table 2.1. The final volume of reaction was adjusted to 20 µl by Maxima™ SYBR Green qPCR Master Mix (Fermentas). The PCR profile was as following; the denaturation step for 10 sec at 95 °C, the annealing step for 15 sec at 60 °C (for ATP synthase beta subunit), at 55 °C (for 14-3-3 like protein), at 60 °C (for A2M), and at 58 °C (for EF-1a, the negative control). The expression of each target gene was firstly normalized to that of the internal control gene, elongation factor-1 alpha (EF-1a), at each time point. The PCR amplification of each was done in three trials. The relative expression ratios of each target gene the studied time points; 6, 24 and 48 hpi, were next

compared to that of the zero time point in order to calculate the fold changes of target gene expression. The significant changes in the data set were statistically analyzed by One way analysis of variance (ANOVA) and post hoc test (Duncan's new multiple range test), with criteria for eligible significance at p value < 0.05.

Table 2.1 List of the gene specific primers and final concentration of the primers used for quantitative real-time RT-PCR (qRT-PCR)

Primer name	Sequence (5'-3')	Final concentration (µM)
A2M_F	CCTCATATCCGGCTTCATCC	200
A2M_R	CCGTGAACTCCTCGATGTAG	200
14-3-3_F	ATCGCCAAGGCAGAGATGCAG	150
14-3-3_R	CCAACTCCGCAATAGCGTCGT	150
ATP_F	AGGCTCACGGTGGTTACTCT	300
ATP_R	CCTTGGAGGTGTCATCCTTCAG	300
EF1-α _F	GGTGCTGGACAAGCTGAAGGC	500
EF1-α _R	CGTTCCGGTGATCATGTTCTTGATG	500

2.10 Identification of protein partners using yeast two-hybrid (Y2H)

2.10.1 Competent cell preparation and transformation

2.10.1.1 Competent bacterial cells and electro-transformation

A colony of *E. coli* strain XL-1-Blue or BL21(DE3) was inoculated into LB media and incubated at 37 °C with shaking at 250 rpm to make an overnight culture. The overnight culture was then transferred into LB media containing an appropriate antibiotic and cultured until OD_{600} reached about 0.5 to produce a cell starter. The cell suspension was then chilled on ice for 30 min. The cell pellet was collected by centrifugation at 4,000 x g for 15 min and later washed twice using 1 and 0.5 volume, respectively, of sterile pre-cold DI water. After that, the cell pellet was dispersed in iced-cold 10% (v/v) glycerol and 40 µl of the cell suspension were then aliquoted, followed by immediately frozen at -80 °C until use.

Two microliters of plasmid solution was added to 40 µl of the competent cell. After incubateing on ice for 1 min, the suspension was transferred into pre-cold 0.2 cm electrode

gap cuvette for electroporation (Biorad). Minipulser electroporation system (Biorad) at constant 2.5 kV were then pulsed and 1 ml of fresh LB media was immediately added to the mixture. The solution containing transformants was next cultured at 37 °C with shaking at 250 rpm for 1 h. Afterward, the transformant was plated onto appropriate selective LB media.

2.10.1.2 Competent yeast cells and lithium acetate transformation

The competent yeast was prepared using the Yeastmaker™ Yeast Transformation System 2 (Clontech). *Saccharomyces cerevisiae* strain Y2HGold (Clontech) was firstly streaked on YPDA agar plate and the plate was incubated upside down in an oven at 30 °C for 3 days. A yeast colony with 3-5 mm in size was picked and transferred to 3 ml of YPDA medium followed by incubation at 30 °C with shaking at 250 rpm for 8 h. One microliter of the culture was later transferred to 10 ml of YPDA broth. The broth was cultured shaking until the OD_{600} reached 0.15-0.3. The yeast cells were harvested by centrifugation at 700 x g for 5 min at room temperature. After discarding supernatant, the cell pellet was resuspended by 20 ml of fresh YPDA and the culture was further incubated with shaking until the OD_{600} reached 0.4-0.5. The yeast cells were centrifuged twice at 700 x g for 5 min at room temperature. The pellet was firstly resuspended with 12 ml of DI-H_2O and secondly dispersed with 600 µl of TE buffer supplemented with 0.1 M Lithium Acetate. High speed centrifugation was then performed to sediment the cell pellet which was finally resuspended in 240 µl of TE buffer supplemented with 0.1 M Lithium Acetate. The competent cell of *S. cerevisiae* strain Y2HGold was ready to be transformed.

For lithium acetate transformation, a reaction consisted of 50 µl of the competent yeast cell, 5 µl of 95 °C pre-heated Yeastmaker carrier DNA and 100 ng of plasmid DNA. After mixing, 500 µl of polyethylene glycol 3350/lithium acetate (PEG/LiAc) was added to the reaction. The reaction was then incubated in a water bath at 30 °C and gently mixed every 10 min. Prior to incubate the reaction at 42 °C for 15 min, 20 µl of DMSO was added and the reaction was mixed. The competent cells were centrifuged with high speed for 15 sec and then resuspended with 1 ml of YPD plus medium followed by incubation with shaking at 30 °C for 90 min. After pelleting the transformant, the supernatant was discarded and the cells were finally resuspended in 0.9% (w/v) NaCl solution. Afterward, the transformant was plated onto appropriate selective media.

2.10.2 Preparation of the recombinant bait

2.10.2.1 Construction of the bait-recombinant pGBKT7

The receptor binding domain (RBD) of *Pm*A2M which was interesting to be further studied was amplified from the cDNA library of normal shrimp hemocyte using the gene specific primers (forward primer; 5'-GCCGATCATATGCTTGTCCACGAATTCACC-3' and reverse primer; 5'-ATCGAGGATCCTTTTGCTGCCGTCCAC-3'). The Advantage®2 Polymerase Mix (Clontech) was used to amplify the PCR product with the PCR profile as following; the pre-denaturation step at 94 °C for 5 min, 30 cycles of 1) the denaturation step at 94 °C for 30 sec, 2) the annealing step at 70 °C for 30 sec and 3) the elongation step at 72 °C for 30 sec, and then a final extension at 72 °C for 10 min. The amplified PCR product flanking *Nde*I and *Bam*HI containing sequences was then in frame-cloned into pGBKT7, a bait vector (Clontech). After that, the recombinant plamid (pGBKT7-RBD-A2M) was electroporated into *E. coli* strain XL-1-Blue in order to produce sufficient amount of the pGBKT7-RBD-A2M plasmid, as described above.

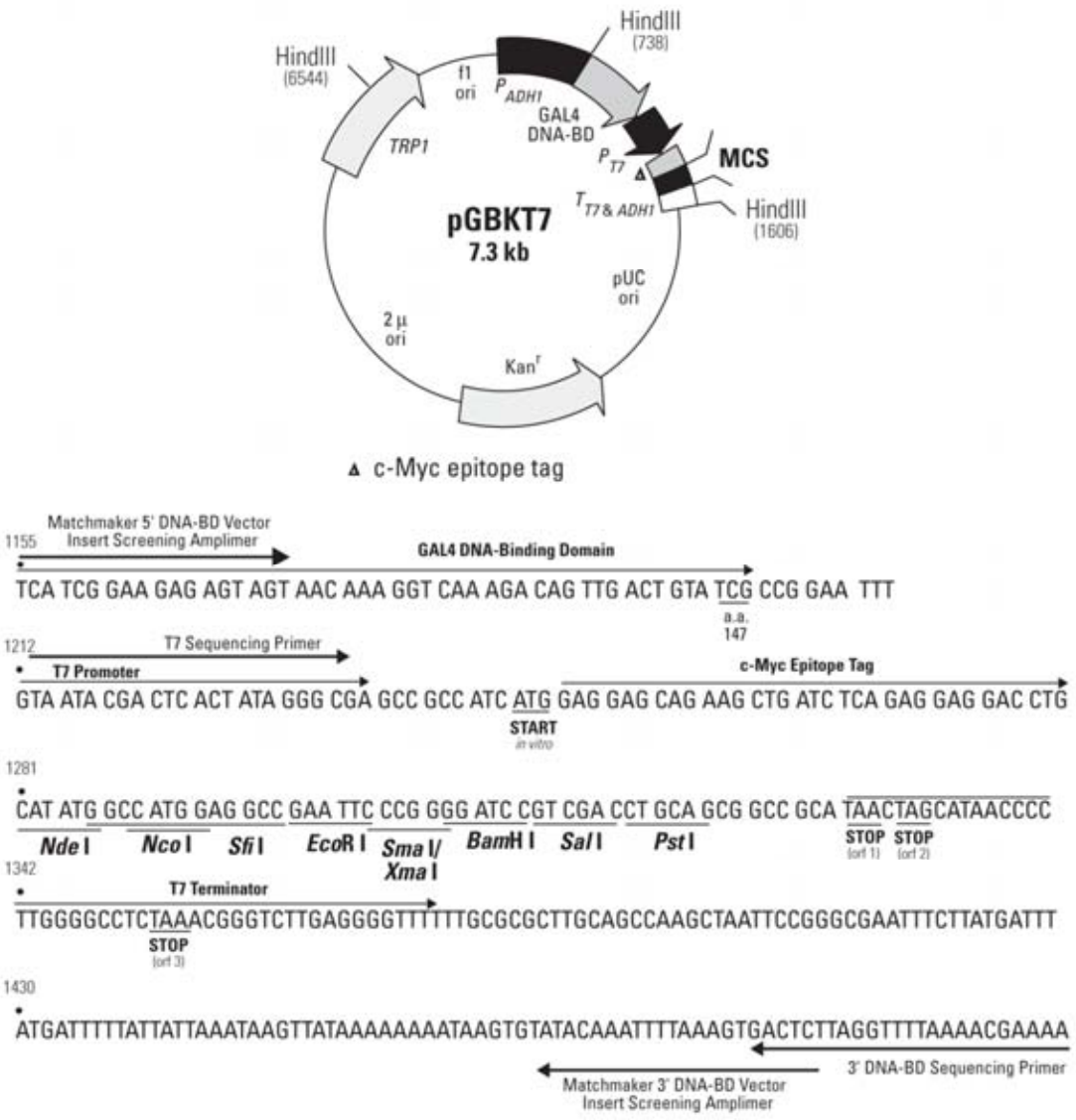


Figure 2.1 Map of the pGBKT7 vector (Clontech)

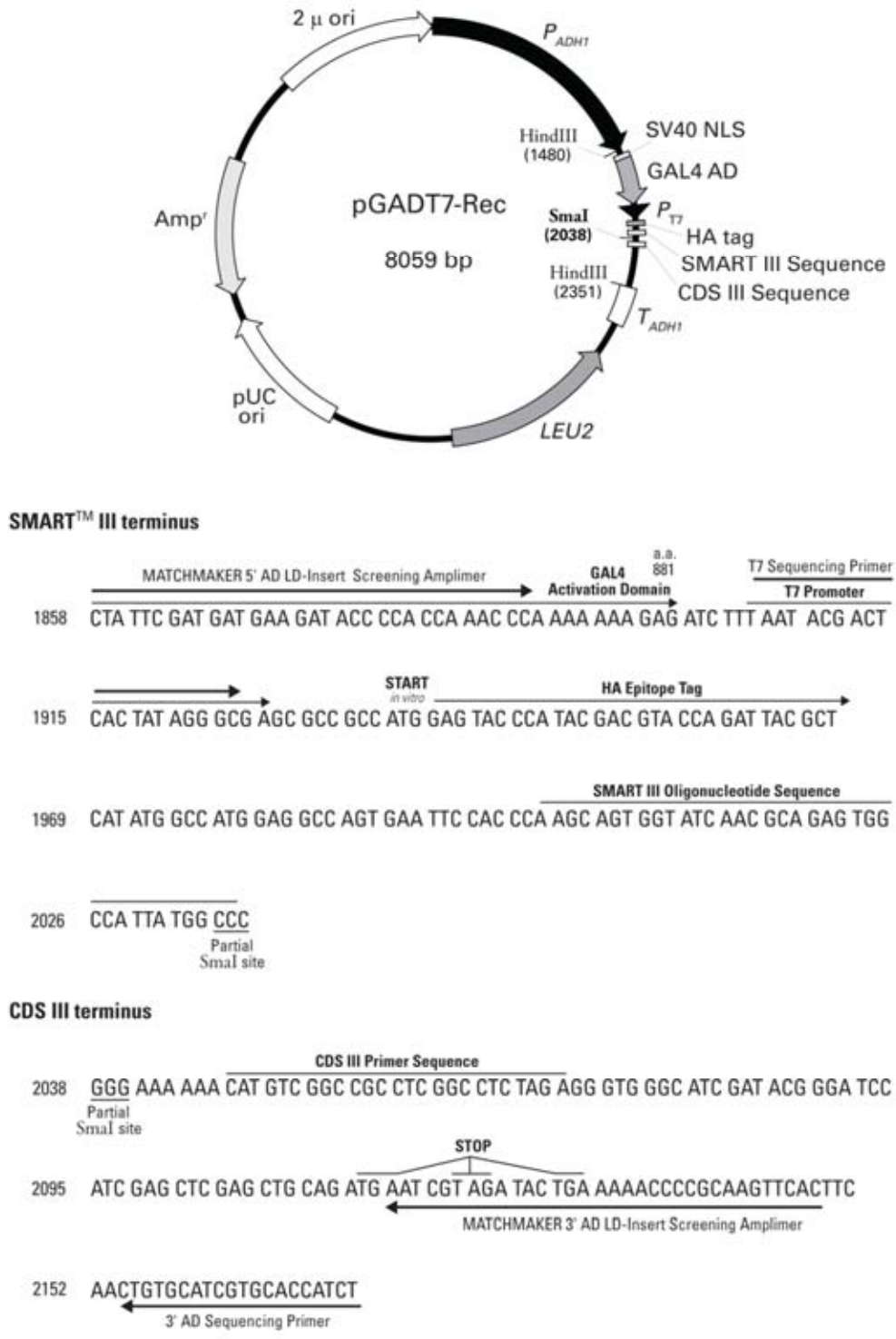


Figure 2.2 Map of the pGADT7 vector (Clontech)

2.10.2.2 Autoactivation and toxicity test

Autoactivation of the reporter genes by the plasmid and toxicity of the plasmid to the host cells were later tested. One hundred nanograms of the pGBKT7-RBD-A2M plasmid was transformed into the Y2HGold (Clontech) using the Yeastmaker™ Yeast Transformation System 2 (Clontech), as mentioned in section 2.10.1.2.

To test the bait for autoactivation, 100 µl of the transformants containing pGBKT7-RBD-A2M plasmid with a 1/10 dilution and a 1/100 dilution was spreaded onto SD/–Trp, SD/–Trp/X-α-Gal, and SD/–Trp/X-α-Gal/AbA. The plates were incubated upside down for 3-5 days at 30 °C.

To test the bait for toxicity, 100 ng of the empty pGBKT7 was also introduced to the Y2HGold (Clontech) used as the negative control. One hundred microliters of the transformants with the 1/10 and 1/100 dilutions were plated onto SD/–Trp. The plates were later incubated upside down for 3-5 days at 30 °C.

2.10.3 Control experiments

To indicate whether the protein-protein interaction discovered from the Y2H assay was authentic, these control experiments; negative control and positive control, were performed. Briefly, the Yeastmaker™ Yeast Transformation System 2 (Clontech) was used to produce transformants, which were Y2HGold containing either pGBKT7-Lam or pGBKT7-53 and Y187 containing pGADT7-T. These tranformants containing pGBKT7 and pGADT7 were then selected by SD/-Trp and SD/-Leu plating media, respectively, and then the plates were incubated at 30 °C for 3 days. Small-scale yeast mating of the negative and positive controls was conducted. Transformants containing different plasmids were matched as described below and transferred in 500 µl of 2X YPDA in an eppendrof tube followed by vortexing. After culturing with shaking at 200 rpm at 30 °C overnight, 100 µl of the mated cultures of 1/10, 1/100, and 1/1000 dilutions were plated on selective media; SD/-Trp, SD/-Leu, SD/-Leu/-Trp, and SD/-Leu/-Trp/X-α-Gal/AbA. Afterward, the plates were inbubated upside down in at 30 °C for 3-5 days until the colony sizes were 2-3 mm.

2.10.3.1 Negative control

The negative control was derived from a control mating between Y2HGold containg pGBKT7-Lam and Y187 containing pGADT7-T. The pGBKT7-Lam encodes the Gal4 DNA-binding domain fused with lamin while the pGADT7-T encodes the Gal4 DNA-activating

domain fused with SV40 large T-antigen. Since the lamin is not protein interacting partner of SV40 large T-antigen, this control mating will result in no activation of reporter genes in the host cell. The mating transformants grow on SD/-Trp, SD/-Leu, and SD/-Leu/-Trp minimal media because it contains pGBKT7 and pGADT7 but none grows on SD/-Leu/-Trp/X-α-Gal/AbA.

2.10.3.2 Positive control

The positive control was derived from a control mating between Y2H stain Gold containg pGBKT7-53 and Y187 containing pGADT7-T. The pGBKT7-53 encodes the Gal4 DNA-binding domain fused with murine p53 while the pGADT7-T encodes the Gal4 DNA-activating domain fused with SV40 large T-antigen. Since the murine p53 and SV40 large T-antigen are found to specifically interact in the Y2H assay, this control mating will result in the activation of reporter genes in the host cell. The mating transformants grow on all minimal media; SD/-Trp, SD/-Leu, SD/-Leu/-Trp, and SD/-Leu/-Trp/X-α-Gal/AbA.

2.10.4 Y2H library screening

After pGBKT7-RBD-A2M plasmid and cDNA library of *V. harveyi* infected *P. monodon* hemocytes were constructed, the Y2H assay was then carried on as described below.

2.10.4.1 Yeast mating

A 3 mm large colony of the Y2H Gold containing the pGBKT7-RBD-A2M plasmid was inoculated in 50 ml of SD/-Trp broth with shaking incubation at 250 rpm, 30 °c until the OD_{600} reached 0.8. The cell was then harvested by centrifugation at 1,000 xg for 5 min and later resuspened in SD/-Trp broth to make a cell suspension with cell density of $1x10^8$ cells per ml counted by a hematocytometer. To mate the transformant containing the bait with the Y187 containing the hemocyte cDNA library of 6 and 48 h post *V. harveyi*-challenged shrimp, all of the cell suspension was combined with 1 ml aliquot of the library, and the mixture was transferred to 2 liter flask. Fourty five milliliter of 2x YPDA liquid medium supplemented with 50 µg/ml kanamycin was then added to the mixture followed by incubation at 30°C with shaking at 50 rpm overnight. The cell of mated yeasts was centrifuged at 1,000 xg for 10 min, the cell was rinsed with 50 ml of 0.5x YPDA broth supplemented with 50 µg/ml kanamycin and finally resuspended with 10 ml of the same solution. Tne hundred microliter of the mated yeasts with 1/10, 1/100, 1/1,000, and 1/10,000

dilutions were plated on SD/-Leu/-Trp/X-α-Gal/AbA. The plates were incubated upside down at 30 °c for 3-5 days. The selected transformants growing on the plates and appearing a blue colony were later patched onto the high stringency medium, SD/-Ade/-His/-Leu/-Trp/X-α-Gal/AbA.

2.10.4.2 Determination of the mating efficiency

Mating efficiency in the Y2H screening should not less than 2%. The mating efficiency was calculated by the following equation;

$$\frac{\text{No. of cfu/ml of diploids x 100}}{\text{No. of cfu/ml of limiting partner}} = \text{\% Mating efficiency}$$

The No. of cfu/ml of diploids was calculated from the number of colony growing on the SD/-Leu/-Trp selective plate. The No. of cfu/ml of limiting partner was calculated from viability of prey library which was the number of colony growing on the SD/-Leu selective plate.

2.10.5 Isolation of interacting prey(s)

2.10.5.1 Segregation of library prey plasmid

The selected transformants were later segregated on SD/-Leu/-Trp/X-α-Gal for 2-3 times. At each time, a single blue colony was picked for subsequent steaking. The SD/-Ade/-His/-Leu/-Trp/X-α-Gal media was lastly used to select the positive transformants.

2.10.5.2 Prey plasmid extraction and selection

Positive yeast tranformants were patched on SD/-Leu and they were grown for later prey plasmid extraction. To extract the prey plasmids from the positive transformant, a half of the patched yeast were dissoved in 100 µl of lysis solution (8% (w/v) sucrose, 1 M Tris.HCl pH 8.0, 0.5 M EDTA and 5% (v/v) Triton-X-100) containing 0.45 mm sterile glassbeads. Then, the cells were broken by vigorous vortexing for 5 min and additional 100 µl of the lysis solution was added followed by boiling for 5 min and chilling on ice for 1 min. The cell debris was seperated by centrifugation at 8,000 rpm for 10 min and the supernatant was collected. Prior to remove impurities by centrifugation at 12,000 rpm for 10 min, 100 µl of 7.5 M ammonium acetate was added and the solution was incubated at -20 °C for 1 h. The plasmid DNA in the collected supernatant was precipitated by adding 500 µl of pre-cold

absolute ethanol and incubated on ice for 10 min. After centrifuged at 12,000 rpm for 15 min at 4°C, the pellet was washed by 1 ml of 75% ethanol and finally dissoved in 20 µl of sterile DI water. Ten microliter of isolated prey plasmids was later introduced to *E. coli* strain XL-1-Blue and the transformants were selected by LB broth containing 100 µg/ml ampicillin.

2.11 Confirmation of positive interactions using Co-transformation technique

After isolation of prey plasmids from the selected transformant discovered from the Y2H assay, 100 ng of each prey plasmid were co-transformed with either 100 ng of the empty pGBKT7 or 100 ng of the pGBKT7-RBD-A2M into the Y2HGold in order to confirm the protein-protein interaction. The transformation was conducted, using the Yeastmaker™ Yeast Transformation System 2 (Clontech) as described in section 2.10.1.2. One hundred microliter of the transformants with 1/10 and 1/100 dilutions was then plated on SD/-Leu/-Trp/X-α-Gal and SD/-Ade/-His/-Leu/-Trp/X-α-Gal/AbA. The media were then incubated at 30 °C for 3-5 days. The prey plasmids identified as authentic interacting protein partners with the RBD of A2M was subjected to DNA sequencing and the DNA sequences were later searched against the GenBank database using the BlastX program.

2.12 Production of recombinant *Pm*A2M and anti-r*Pm*A2M polyclonal antibody

2.12.1 Construction of *Pm*A2M-recombinant pET-22b(+)

The forward primer (5'-CGCGGCCATATGCTTGTCCACGAATTCACC-3') and the reverse primer (5'-CATATGCTCGAGTTTGCTGCCGTCCACCTCGTA-3') with flanking *Nde*I and *Xho*I restriction sites at 5' ends, respectively, were used to amplify the RBD of A2M from the pGBKT7-RBD-A2M plasmid using the Advantage®2 Polymerase Mix (Clontech) with the PCR condition as following; the pre-denaturation step at 94 °C for 5 min, 30 cycles of 1) the denaturation step at 94 °C for 30 sec, 2) the annealing step at 70 °C for 30 sec and 3) the elongation step at 72 °C for 30 sec, and then a final extension at 72 °C for 10 min. After that, the PCR product was purified by a NucleoSpin Extract II kit (Clontech) according to the manufacturer's instruction and later digested with the *Nde*I and *Xho*I restriction enzymes at 37 °C overnight. The *Nde*I and *Xho*I digested product was cloned in frame with the pET-22b (+) expression vector (Novagen) cut with the same enzymes using T4 DNA ligase (New England Biolabs) by incubation at 16 °C overnight. The recombinant plasmid was electroporated into *E. coli* strain XL-1-Blue as described in section 2.10.1.1 and the transformant was selected by LB agar medium supplemented with 100 µg/ml ampicillin at 37 °C overnight. Afterwards, the plasmid was extracted from the positive clones and

purified QIAprep spin Miniprep Kit (QIAGEN) according to the manufacturer's manual followed by subjecting to DNA sequencing using T7 promoter at Macrogen INC., South Korea in order to verify the correctness of the inserted RBD of A2M sequence.

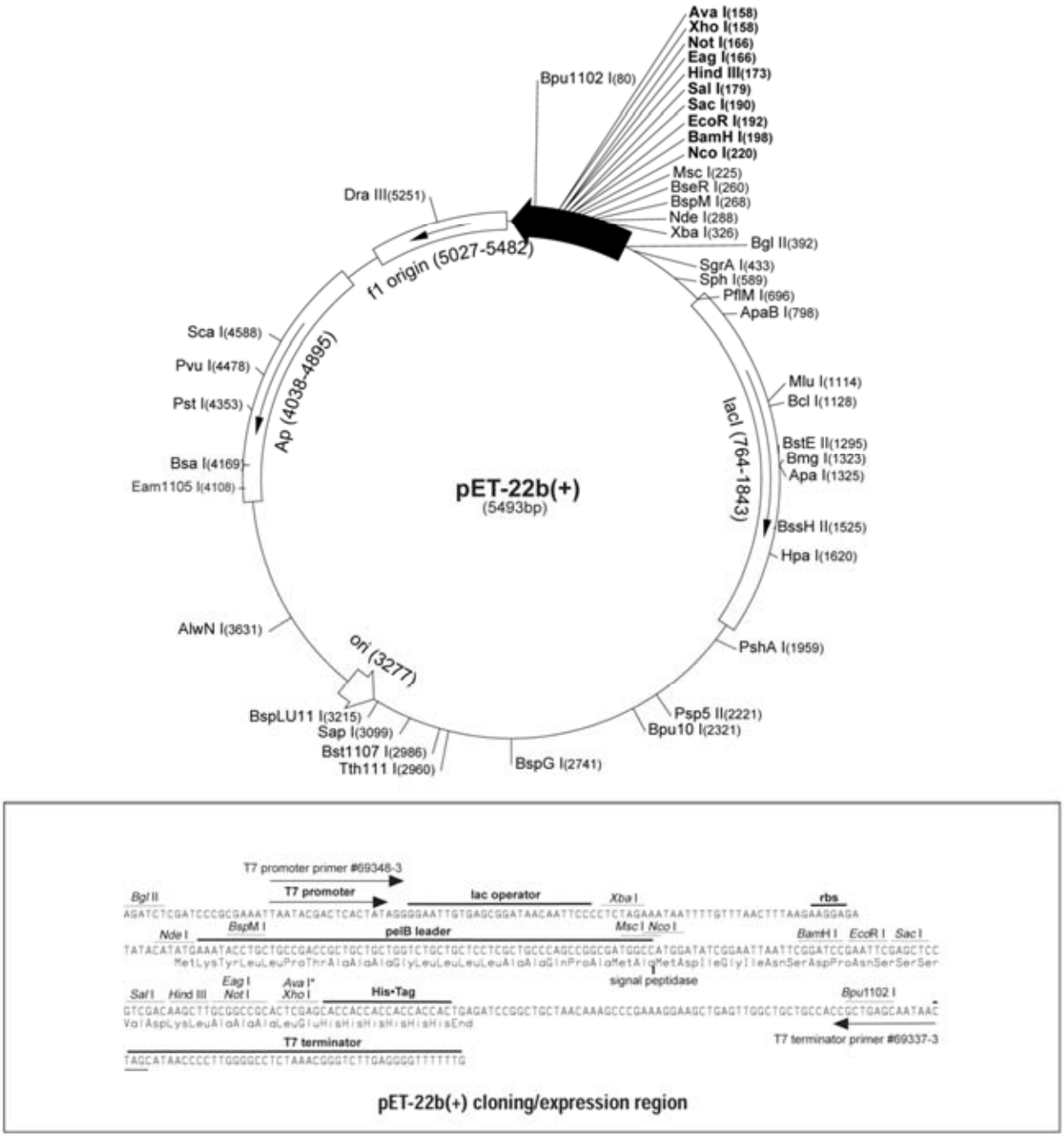


Figure 2.3 Map of the pET-22b(+) expression vector (Novagen)

2.12.2 Over-expression and solubility analysis of the r*Pm*A2M

To produce the recombinant *Pm*A2M protein, the recombinant plasmid (pET-RBD-A2M) was introduced into the *E. coli* strain BL21(DE3), an expression host by electroporation and the transformant was selected by LB agar plate supplemented with 100 µg/ml ampicillin at 37 °C overnight. The selected transformant containing the pET-RBD-A2M plasmid was then inoculated in LB broth containing 100 µg/ml ampicillin until the OD_{600} of the culture reached 0.6. To induce the recombinant protein expression, IPTG (Isopropyl β-D-1- thiogalactopyranoside) was added to final concentration of 1 mM. The bacterial cell was then collected at 0, 2, 4, and 6 h after IPTG induction by centrifugation at 8,000 x g for 10 min. The collected cells were dispersed in SDS-PAGE sample buffer and the solution was incubated in boiling water for 10 min. The over-expressed recombinant *Pm*A2M protein was later resolved by 15% (w/v) acrylamide SDS-PAGE followed by coomassie brilliant blue staining.

For solubility analysis of the r*Pm*A2M, the IPTG-induced cell were harvested at 6 h post induction by centrifugation at 8,000 x g for 10 min. The pellet was later resuspended in phosphate buffered saline pH 7.4 (PBS) followed by sonication to break the cell. After centrifugation at 8,000 x g for 10 min, the inclusion bodies were seperately dissoved in buffers with different pH; 5.2, 8.0, 10.3 and 12.0, and the solutions were vigorously shaked at 4 °C overnight. The solubility of the r*Pm*A2M was examined by 15% (w/v) acrylamide SDS-PAGE followed by coomassie brilliant blue staining.

2.12.3 Protein purification and antibody production of the r*Pm*A2M

After the recombinant *Pm*A2M protein was over-expressed by IPTG induction and its inclusion bodies were solubilized by 20 mM sodium phosphate buffer pH 10.3 overnight, the mixture of solubilized proteins was purified using a Ni-Sepharose™ 6 Fast Flow affinity column (GE Healthcare), according to the manufacture's manual. After that, the $(His)_6$-tagged recombinant protein bound to the column was eluted by 20 mM phosphate buffer pH 8.0, 0.1 M NaCl, 500 mM Imidazole and the eluted fractions were analyzed 15% (w/v) acrylamide SDS-PAGE followed by either coomassie brilliant blue staining or western blotting using the anti-HIS tag antibody (GE Healthcare).

2.12.4 Antibody purification of anti-r*Pm*A2M polyclonal antibody

The rabbit polyclonal and mouse polyclonal antibodies against the r*Pm*A2M was commercially raised in experimantal animals at a commercial service of the Biomedical Technology Research Unit, Chiang Mai University, Thailand.

2.13 Subcellular localization analysis of *Pm*A2M protein in shrimp hemocytes by immunofluoresence

To study the localization of the *Pm*A2M protein in shrimp hemocytes, hemolymph was aspirated from three individuals of the normal shrimp and then pooled. The hemocytes were later centrifuged at 800 x g for 10 min at 4 °C and the hemocyte pellet was immediately fixed in 4% (w/v) paraformaldehyde in PBS. A hemocytometer was used to count the number of hemocytes and to make 1×10^5 cells/ml of hemocytes diluted by the fixing solution. Using cytospin centrifugation, hemocytes were attached onto a polylysine coated microscope slide (Polysine slides, Thermo Scientific). To permeabilize the hemocyte membrane, the slide was washed by PBS for 5 min and later covered by 300 µl of 0.1% (v/v) Triton-X 100 in PBS for 5 min at ambient temperature. After rinsing the slide in PBS for 5 min, it was immersed in 200 µl of blocking solution (10% (v/v) FBS in PBS) for 1 h at room temperature followed by washing in PBS. Purified Rb anti-*Pm*A2M polyclonal antibody diluted 1:500 in PBSF (PBS plus 1% (v/v) FBS) was applied onto the slide subsequently incubated at 37 °C for 1 h. Prior to further incubate with a 1/500 dilution of Alexa Fluor® 488 goat anti-Rb IgG antibody (Invitrogen) in PBSF, the slide was washed in PBS for three times. The reaction was kept in dark for 1 h at room temperature followed by three times washing. Nuclei of hemocytes were eventually detected by 1:1,500 PBS-diluted TO-PRO-3 (Invitrogen) for 10 min at ambient temperature and then washed twice followed by direct addition of Prolong Gold Antifade Reagent (Invitrogen). Next, the slide was mounted and kept in dark at 4 °C until it was visualized under a confocal fluorescence microscope (Olympus).

2.14 Investigation of *Pm*A2M protein contents in *V. harveyi*-infected shrimp hemocytes by immunofluoresence

To investigate the *Pm*A2M protein expression in the *V. harveyi*-infected shrimp hemocytes, immunofluorescent staining of *Pm*A2M protein was conducted. The experimental shrimp were divided into 2 groups; 0.85% (w/v) normal saline-injected (control group) and *V. harveyi*–injected groups (challenged group). At 10 min after bacterial injection, hemolymph of both groups was collected and hemocytes were then harvested by

centrifugation at 800 x g for 10 min at 4 °C. The detection of the *Pm*A2M protein content was then performed by immunofluoresence as described in section 2.13.

2.15 Western blot analysis of *Pm*A2M levels in hemocytes and cell-free hemolymph

Hemocytes and hemolymph of *V. harveyi*-infected shrimp were individually collected at 0, 6, 24, 48 and 72 hpi, as well as those from the SSS injected shrimp at 0 hpi. Hemocytic proteins were extracted as previously described by Somboonwiwat et al. (30), with the data at each time point being derived from the pooled samples from seven individuals. The protein concentration was measured using the bradford assay. Fifteen µg of the pooled hemocyte protein extract and 150 µg of the pooled hemolymph protein at each time point were separated by resolution through a 12.5% (w/v) acrylamide PAGE and transferred onto a PVDF membrane (GE Healthcare). The western blot analysis was carried out by incubating the membranes with rabbit anti-*Pm*-RBD-A2M polyclonal Ab (1:10,000 dilution in 1x PBS-0.1% tween 20 supplemented with 1% skim milk) to detect the *Pm*A2M protein at room temperature for 3 h, washing 3 times in 1x PBS-0.1% tween 20, and then incubating with alkaline phosphatase-conjugated goat anti-rabbit IgG Ab (Immuno Research) at room temperature for 1 h. After washing 3 times in 1x PBS-0.1% tween 20, the protein bands were detected by developing the membranes in NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution).

In a similar manner, β-actin, used as the protein loading and transfer reference, was detected as above except using a 1:3,000 dilution of mouse anti-β-actin mAb (millipore) and alkaline phosphatase-conjugated goat anti-mouse IgG Ab (Immuno Research) as the primary and secondary Abs, respectively, whilst the detection of the $(His)_6$-tagged proteins (see above) was performed in the same way except using the anti-$(His)_6$ primary Ab (GE Healthcare).

2.16 Co-localization of *Pm*A2M and clottable proteins (CPs) on the clot matrix by immunofluoresence

2.16.1 Preparation of clot matrix

To prepare the clot matrix for further examination, the Hanging drop technique (Bidla et al., 2005) was performed. Briefly, 200 µl of shrimp hemolymph was aspirated from a ventral cavity using a sterile syringe with a 23G needle containing 50 µl of anticoagulant solution (10% (w/v) sodium citrate). Five microliter of 1 M $CaCl_2$ was then added directly to the collected hemolymph to activate the blood clotting, followed by gently mixing and

incubation at room temperature for 5 min. Next, the reaction was ended by the addition of 25 µl of 0.5 M EDTA. Two hundred fifty microliter of the clotting activated plasma was later dropped onto the polylysine-coated slide and the slide was incubated upside down in a humid chamber at room temperature for 30 min. After that, the slide was transferred in the same position and allow the plama surface to attach a new polylysine-coated slide without touching of each other. Subsequently, the slide with the clot matrix was processed.

2.16.2 Immunofluorescent detection

After the Hanging drop technique was done, the samples were air-dried at 37 °C. To detect the *Pm*A2M and clottable proteins on the clot matrix, the immunofluoresent technique as described in a part of "Subcellular localization analysis of PmA2M protein in shrimp hemocytes by immunofluoresence" was performed with slight modification. Firstly, the purified Ms anti-*Pm*A2M polyclonal IgG antibody at a 1/150 dilution in PBSF and the purified Rb anti-CP polyclonal IgG antibody at a 1/250 dilution in PBSF were simultanaeously applied to the samples followed by incubation at 37 °C for 1 h. Before immersing in PBSF of a 1:500 dilution of Alexa Fluor® 568 goat anti-mouse (Invitrogen) and Alexa Fluor® 647 goat anti-rabbit Abs (Invitrogen) for additional 1 h in dark, the sample was rinsed three times with PBS. After mounting, the slides were kept in dark at 4 °C until they were visualized under a confocal fluorescence microscope.

2.17 Sequence analysis and phylogenetic analysis of *Pm*ATP synthase beta subunit

Amino acid fragments of the highest up-regulated protein derived from the nano-LC-ESI-MS/MS were analyzed by MASCOT MS/MS Ions Search program (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS) and the best matched amino acid sequence was later searched against the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th/home.jsp). Using the Genetyx program and the online SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen J.D. et al., 2004), the deduced open reading frame (ORF) and the predicted amino acid sequences and signal peptide region were analyzed. The deduced amino acid sequence was subsequently searched against the GenBank database using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul and Lipman, 1990). According to the BLASTx result, amino acid sequences of the six best hit proteins were selected for multiple alignment analysis by the online ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin et al., 2007). Signature motifs of

those sequences were finally identified by the NCBI Conserved Domain Database website (Marchler Bauer et al., 2011) and the Expasy Proteomics Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

ATP synthase beta subunit amino acid sequences among various organisms were obtained from the GenBank database and aligned using the ClustalX program (Chenna et al., 2003). A Neighbor-joining distance based phylogenetic tree with a selected outgroup was then produced by the PHYLIP (Phylogeny Inference Package) program using the default settings (Felsenstein, 1993).

2.18 Transcript distribution analysis of *Pm*ATP synthase beta subunit in shrimp tissues

In order to investigate the mRNA expression levels of the ATP synthase beta subunit in eleven different shrimp tissues, semi-quantitative RT-PCR was performed. The first strand cDNA was synthesized from the DNaseI-treated total RNA of the eleven different tissues collected from unchallenged shrimp (n = 3), that is the antennal gland, epipodite, eye stalk, gills, heart, hemocyte, hepatopancreas, intestine, LO, stomach and hematopoietic tissue. The ATP-F/R primer pair (Table 1) specific to the ATP synthase beta subunit was used to amplify a 235 bp gene fragment. The EF1-α was used as the internal control. The PCR reactions contained 2 (for ATP synthase beta subunit gene) or 1 (for EF1-α) µl of a 10-fold dilution of the cDNA template, 100 nM dNTP, 100 nM of each gene specific primer, 1X RBC buffer and 1.25 unit of Taq DNA polymerase (RBC Bioscience) in a 25 µl final volume. The gene amplification was carried out at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 30 sec, 57 ºC (for ATP synthase beta subunit) or 55 ºC (for EF1-α) for 30 sec and 72 ºC for 30 sec, and then a final extension step at 72 ºC for 10 min. The PCR products were verified by running on a TBE-1.5% (w/v) agarose gel electrophoresis and visualized by UV-transillumination after staining the gel with ethidium bromide.

2.19 *In vivo* gene knockdown mediated by RNA interference

2.19.1 Production of *Pm*A2M, *Pm*ATP synthase beta subunit and GFP dsRNA

For the negative control used in the RNA interference, dsRNA of the green fluorescent protein (GFP) was amplified from the pEGFP-1 vector (Clontech, USA) using a pair of GFPT7-F and GFP-R primers in order to produce the sense RNA strand and a pair of GFP-F and GFPT7-R primers in order to produce the antisense RNA strand. The primer sequences were listed in Table 2.2.

For the *Pm*A2M and *Pm*ATP synthase beta subunit dsRNA production, cDNA clones and contigs corresponding to the homolog of *Pm*A2M and *Pm*ATP synthase beta subunit were firstly searched from the *P. monodon* EST database (http://pmonodon.biotec.or.th). The dsRNAs of the RBD of *Pm*A2M and *Pm*ATP synthase beta subunit were amplified from clone number HC-N-N01-1034 in contig number 711 and contig number 494, respectively. The separated PCR amplification of a pair of PCR products for each gene as described above was performed using the primer pairs listed in Table 2.2 with the PCR conditions as following: the pre-denaturation step at 94 °C for 3 min, 30 cycles of 1) the denaturation step at 94 °C for 30 sec, 2) the annealing step at 57 °C for 30 sec and 3) the elongation step at 72 °C for 1 min, and then the final extension step at 72 °C for 5 min.

To prepare 2 complementary ssRNAs, the obtained PCR products were firstly purified using NucleoSpin Extract II kit (Clontech) and then transcribed using *T7 RiboMAX*™ *Express* Large Scale RNA Production System (Promega) in accordance with the manufacturer's manual. After that, equal quantities of complementary ssRNAs were mixed and heated at 70 °C for 10 min. To enable dsRNA forming, the reaction was later cooled down until the temperature was about ambient. To remove the DNA contaminants, the annealed dsRNA was later treated with RQ1 RNase-free DNase (Promega) followed by precipitation by an addition of 0.1 volume of 3 M sodium acetate pH 5.2. The dsRNA pellet was sedimented by centrifugation at 12,000 rpm for 15 min at 4 °C and eventually dissolved in 200 µl of Nuclease-Free H_20. The integrity and quantity of the prepared dsRNA was verified by running in 1.5% (w/v) agarose gel electrophoresis and UV spectrophotometry, respectively.

Table 2.2 List of primers used for RNA interference (RNAi)

Primer name	Sequence (5'-3')
GFP_F	ATGGTGAGCAAGGGCGAGGA
GFP_R	TTACTTGTACAGCTCGTCCA
GFPT7_F	TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA
GFPT7_R	TAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA
ATP_F	AGGCTCACGGTGGTTACTCT
ATP_R	CCTTGGAGGTGTCATCCTTCAG
ATPT7_F	GGATCCTAATACGACTCACTATAGGAGGCTCACGGTGGTTACTCT
ATPT7_R	GGATCCTAATACGACTCACTATAGGCCTTGGAGGTGTCATCCTTCAG
A2M_F	CCATGGAGGGGCAGGGATGC
A2M_R	ATCGCACCCTTCGAGGTACG
A2MT7_F	GGATCCTAATACGACTCACTATAGGCCATGGAGGGGCAGGGATGC
A2MT7_R	GGATCCTAATACGACTCACTATAGGATCGCACCCTTCGAGGTACG

2.19.2 Semi-quantitative analysis of RNAi-mediated *Pm*A2M gene silencing

The Experimental shrimp (approximately 3 grams body weight) were divided into 3 group; 0.85% (w/v) normal saline solution-injected, 10 μg/g shrimp of GFP-dsRNA injected, and 10 μg/g shrimp of A2M-dsRNA injected group. These solutions were adjusted final volume to 50 μl by 0.85% (w/v) normal saline solution. The injection was done twice which the first injection was at 0 h and the second injection was at 24 h post first injection. The hemolymph was then collected after 24 h post second injection (48 h post first injection) and the hemocytes were seperated by centrifugation at 800 x g for 10 min at 4 °C. Total RNA was extracted from the hemocytes and cDNA was synthesized by the First Strand cDNA Synthesis Kit (Fermentas) as mentioned in section 2.9. Semi-quantitative RT-PCR was conducted to assess the *Pm*A2M gene silencing level using the *Pm*A2M gene specific primer as described in section 2.9. The *Pm*A2M gene relative expression was compared to the gene expression of β-actin, the internal housekeeping control gene (Forward and reverse primers: 5'-GCTTGCTGATCCACATCTGCT-3' and 5'-ATCACCATCGGCAACGAGA-3'). Next, the amplified PCR product was resolved by 1.5% (w/v) agarose gel electrophoresis, following by ethidium bromide staining and UV visualization.

2.19.3 Semi-quantitative analysis of RNAi-mediated *Pm*ATP synthase beta subunit

The Experimental shrimp (approximately 3 grams body weight) were divided into 3 group; 0.85% (w/v) normal saline solution-injected, 10 μg/g shrimp of GFP-dsRNA injected, and 10 μg/g shrimp of ATP synthase beta subunit dsRNA injected group. The hemolymph was collected at 24 h post injection and the hemocytes were harvested by centrifugation at 800 x g for 10 min at 4 °C. As described in section 2.9, RNA extraction and cDNA synthesis were carried on. The ATP synthase beta subunit transcription levels were later evaluated by semiquantitative RT-PCR. Afterwards, the relative expression of ATP synthase beta subunit mRNA was normalized by the gene expression of EF-1α gene, the internal housekeeping control gene. The PCR condition and primers specific to ATP synthase beta subunit was previously mentioned in section 2.9. The amplified PCR product was run in 1.5% (w/v) agarose gel electrophoresis, following by ethidium bromide staining and UV visualization.

2.20 Determination of shrimp cumulative mortality after *Pm*ATP synthase beta subunit gene silencing

Ten shrimp (3 grams body weight) were used in each of the three experimental groups as decribed in section 2.19.3. After the dsRNA injections were performed, the cumulative mortality of the experimental shrimp was observed for 5 consecutive days and the dead shrimp number was daily recorded. The result was reported as percent of the cumulative motality $\pm$ 1 SD from 3 independent experiments.

2.21 Investigation of the number of shrimp circulating hemocytes after *Pm*ATP synthase beta subunit gene silencing

Shrimp hemolymph was individually aspirated from 3 experimental groups as decribed in section 2.19.3, at 0 and 12 h after RNAi injection using a sterile 0.5 ml syringe pre-filled with iced-cold 10% (w/v) sodium citrate solution as an anticoagulant. To count the total hemocyte number, 10 μl of the hemolymph was dropped into a hemocytometer and then observed under a light microscope. Three independent experiments were performed and the results were presented as the average total hemocyte number $\pm$ 1 SD.

2.22 Histological study of *Pm*A2M-depleted extracellular blood clots by the scanning electron microscopy

To examine the effect of *Pm*A2M dsRNA-mediated silencing in the histological changes of the shrimp clot in the presence or absence of *V. harveyi*, the Scanning electron microscopy (SEM) was conducted. *V. harveyi* used in the experiment was cultured in 5 ml of TSB broth overnight and then the cells were harvested from 1 ml of the overnight culture by centrifugation at 8000 x g for 10 min, followed by washing with an equal volume of PBS for 3 times. As described in section 2.19.2, 2 experimental groups, which were GFP-dsRNA and A2M-dsRNA injected groups, were prepared and their hemolymph was collected using a sterile 0.5 ml syringe pre-filled with iced-cold 10% (w/v) sodium citrate solution as an anticoagulant. Subsequently, either 1.5 µl of sterile PBS or 1.5 µl of the *V. harveyi* suspended in PBS were introduced into the collected hemolymph and the reactions were mixed gently. The hanging drop technique as mentioned in 2.16.1 was the carried on. Glutaraldehyde 2.5% (v/v) in 0.1 M phosphate buffer pH 7.2 was then used to fix the sample for 1 h followed by washing twice with 0.1 M phosphate buffer pH 7.2 for 10 min of each and a final washing with DI water. The samples were later dehydrated by soaking in 30%, 50%, 70%, 95%, 100%, 100% and 100% (v/v) of the ethanol stepwise for 10 min of each. The remaining H_2O was completely eliminated by a Critical Point Dryer (Balzers, model CPD 020) with CO_2. Aluminum stubs were used as a base for the sample immobilized by electrically conducting carbon tapes and an Ion sputter (Balzers, model SCD 040) was used to sputter gold particles to coat the dried sample which subsequently observed under a JEOL scanning electron microscope, model JSM-6510.

2.23 Identification of the number of systemically disseminating *V. harveyi* in the circulating hemolymph of *Pm*A2M gene silenced shrimp

Freshly prepared *V. harveyi* was diluted by 0.85% normal saline solution to the cell density of 1.5×10^5 CFU/ml. Fifty microliter of diluted *V. harveyi* was intramuscularly introduced into the GFP-dsRNA and A2M-dsRNA injected shrimp. After that, shrimp hemolymph was drawn using a sterile syringe without any anticoagulant solution at 5, 15 and 30 min after bacterial injection and 10 µl of collected plasma was immediately dropped onto TCBS (thiosulfate–citrate–bile–sucrose) agar plates which are a *Vibrio* species selective medium. The 5 replicates were conducted for each sample. After the dropped plama was air-dried, the plates were later cultured at 30 °C overnight.

The disseminating bacterial number was calculated from the colonies growing on the selective media. The data were presented as CFU/ml $\pm$ 1 SD. and were statistically analyzed using the Independent Sample T-test by comparing means. The analyzed data with $p < 0.05$ were accepted as significant changes.

2.24 Microscopic visualization of extracellular blood clots

The appearance of the shrimp clot were observed under the microscope with three different fields; bright field, phase contrast field and differential interference contrast field (DIC). The *V. harveyi* used in the experiment was prepared as following. One colony of bacteria grown on Marine agar plate was transferred to Marine broth (MB) and cultured for overnight at 30°C 180 rpm. After that, one sterile loop of overnight *V. harveyi*-containing MB was sub-cultured in sterile Complete Marine broth (CMB) which later shaking incubated for 3 hours at 30 °C 180 rpm. To harvest the log-phase bacteria, 1 ml of *V. harveyi*-containing CMB was centrifuged at 1,000 xg for 10 min. The cell pellet was washed by 500 µl of Shrimp Sterile solution (SSS) twice and finally resuspended by 500 µl of Shrimp Sterile solution (SSS). The bacteria suspension with concentration approximately 10 x 10^6 cells per ml was obtained. Shrimp plasma was collected without anticoagulant solution and immediately chilled on ice. In the absence of *V. harveyi*, 5 µl of SSS was mixed with 95 µl of the plasma and the solution was immediately dropped onto a sterile slide. The hanging drop technique was then conducted to prepare the clot. Briefly, the slide was incubated upside down in a moist chamber for 10 min. Surface of the drop, where the clot formed, was attached onto a new coverslip. In the presence of the bacteria, 5 µl of log-phase *V. harveyi* in SSS was mixed to 95 µl of the shrimp plasma and the bacterial entrapping clot was prepared as described above. The slides were mounted and then observed under microscope with three different fields.

2.25 Examination of bacterial entrapment efficiency of shrimp hemolymph clots

The log-phase *V. harveyi* was freshly prepared as described above. After collection of shrimp plasma without anticoagulant solution, the plasma was then heated in waterbath at 55 °C for 30 min to obtain unclottable hemolymph. Ten microliter of prepared bacteria was added to 190 µl of either SSS or unclottable hemolymph used as negative control groups. For the experimental group, 10 µl of prepared *V. harveyi* was added to 190 µl of hemolymph and the sample was allowed to clot for 30 min. Other samples were also incubated at room temperature for 30 min. Afterward, 10 µl of the *V. harveyi* containing SSS and *V. harveyi*

containing unclottable hemolymph was transferred to hematocytometer in order to count the bacterial number. On the other hand, to count the number of *V. harveyi* in clottable hemolymph, the clot was firstly removed by a sterile needle and 10 µl of the fluid part remaining in a tube was taken to count the bacterial number using hemocytometer.

2.26 Analysis of fibrinolysis areas occurred in *V. harveyi* entrapping blood clots

Shrimp plasma was collected without anticoagulant solution whereas the log-phase *V. harveyi* was prepared as described above. Fourty five microliter of pre-chilled shrimp hemolymph was mixed with 5 µl of log-phase *V. harveyi* before immediately spreading on the sterile coverslip. The coverslip was placed in 35 mm petri dsih (Corning) and was incubated in a moist chamber for 30 min at ambient temperature to allow the blood to completely clot. After that, the slide was periodically observed by a phase contrast microscope at various time points; 0, 30, 60, 90 and 120 min. The picture and video data were recorded at each time point. To estimate the fibrinolysis areas, borders of swimming bacteria observed in the recorded video data were plotted and the fibrinolysis areas were calculated using Image J program.

2.27 Identification of *V.harveyi* secreted fibrinolytic enzymes

2.27.1 Bacterial movement counting in the presence of protease inhibitors

Prior to set up the blood clot, shrimp hemolymph was aspirated without anticoagulant solution and pre-chilled on ice while the log-phase *V. harveyi* was prepared as described above. Five microliter of log-phase *V. harveyi* was mixed with 45 µl pre-chilled shrimp plasma and spreaded on a coverslip in 35 mm petri dish (Corning) used as the negative control. For positive control, 5 µl of log-phase *V. harveyi* and 1 µl of 100 X HALT protease inhibitor mix (Pierce) was seperately dropped on a coverslip in 35 mm petri dish (Corning). The reaction was then adjusted to final volume of 50 µl by pre-chilled shrimp hemolymph. For the experimental group, final concentrations of 1 mM AEBSF, 15 µM E64, and 10 µM Pepstatin were applied to the reaction. The same volume of log-phase bacteria was used in all groups and final volume of 50 µl was finally adjusted by pre-chilled shrimp plasma. The blood was later incubated in a moist chamber for 30 min to form clots. Next, a perfusion chamber was constructed. The clot attached coverslips were mounted on 4 small pieces of spacer located at 4 corners on a glass slide. Then, the chamber was filled in with Complete Marine broth (CMB) and kept in a humid chamber for 2 h at room temperature (Armstrong and Rickles, 1982).

The samples were visualized under the phase contrast microscope. Figures of each condition were taken by normal exposure time and long exposure time in the same field for 5 different fields. The number of motile *V. harveyi* the clot was counted by considering at the difference of bacteria position in the recorded fields between normal exposure time and long exposure time.

2.27.2 Fibrinolytic activity of *V. harveyi* conditioned medium on clot lysis

The shrimp clots were prepared on coverslips by spreading and dropping technique. Twenty microliter and two microliter of pre-chilled shrimp plasma collected without anticoagulant solution was throughly spreaded and dotted on a coverslip in 35 mm petri dish (Corning). The sample was later left in a humid chamber for 30 min to form blood clots. To prepare *V. harveyi*-conditioned medium, a colony of bacteria grown on Marine agar plate was transferred to 5 ml of Marine broth (MB). The media was incubated shaking for overnight at 30°C 180 rpm. Next, 1 ml of overnight culture was centrifuged at 13,000 rpm for 10 min. The supernatant was collected and used in the experiment as the *V. harveyi* conditioned medium. Afterward, the spreaded clot and dotted clot were covered by the *V. harveyi* conditioned medium with no adding protease inhibitors used as the negative control while those which were covered by the *V. harveyi* conditioned medium added mix inhibitors (HALT protease inhibitor supplemented with 1, 10 phenanthroline) were served as the positive control. For the experimental group, final concentrations of 2 mM 1, 10 phenanthroline, 1 mM AEBSF, 15 µM E64, and 10 µM Pepstatin were used in the reaction. These samples were incubated at 37 °C for 3 h. Next, the coverslips were rinsed with DI-H_2O twice and stained with the GelCode Blue Stain Reagent (Pierce). To visualize the sample in more details, the coverslips were mounted and later observed under the phase contrast microscope with magnification at 400X.

CHAPTER III
RESULTS

I. Identification of *Vibrio harveyi* responsive proteins

3.1 Detection of bacterial infection in the *Vibrio harveyi* injected *Penaeus monodon*

To examine the status of *V. harveyi* infection in shrimp, the experimental shrimp were infeccted with *V. harveyi*. DNA extracted from gill of shrimp was prepared and PCR was conducted in order to detect the gyrB region (molecular marker of *Vibrio* spp.)of *V. harveyi* genome in unchallenged and *V. harveyi*-challenged shrimp. The result revealed that a PCR product fragment of 363 bp corresponded to the positive control was detected in gills of *V. harveyi*-challenged shrimp while the product was not detectable in the unchallenged group (Figure 3.1). This indicated that the bacterial challenge in the experimental shrimp was successful.

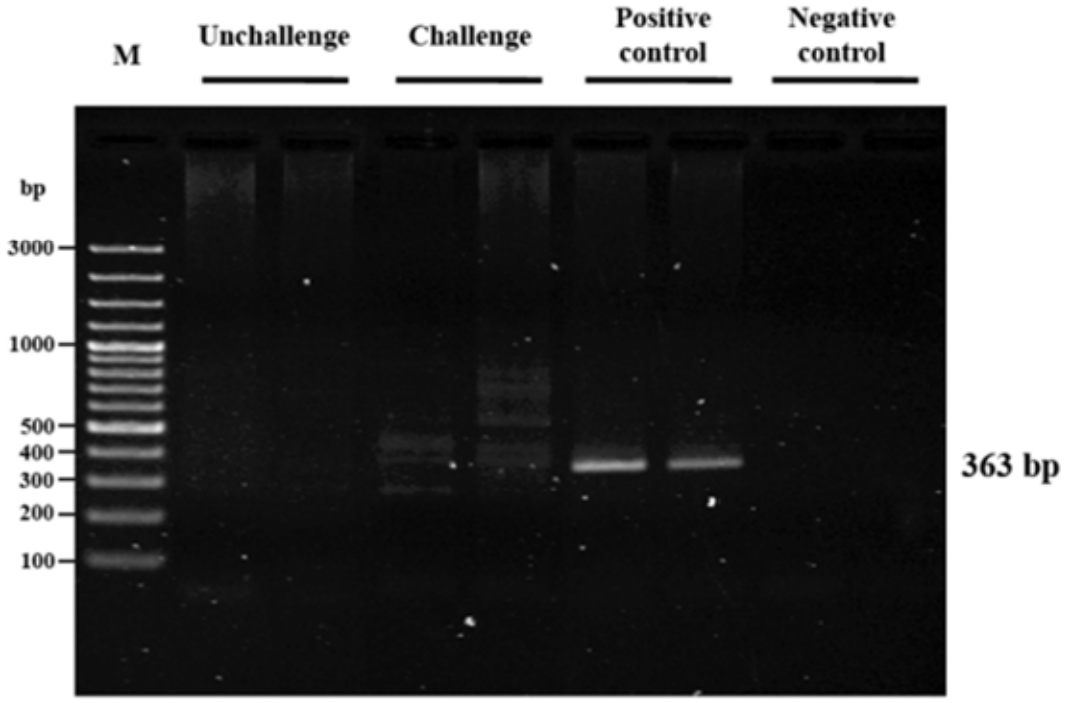


Figure 3.1 Diagnosis of *V. harveyi* infection in the experimental shrimp, *P. monodon*. PCR was used to determine the fragment of the gyrB gene (363 bp) from unchallenged and *V. harveyi* challenged shrimp at 6 h post-infection. Lane M is 100 bp DNA ladder marker. DNA

extracted from the unchallenged and *V. harveyi* challenged shrimp gills was used as PCR templates. PCR grade-water and *V. harveyi* was used as templates for the negative and positive control, respectively.

3.2 Two dimensional gel electrophoresis (2-DGE) analysis and protein annotation of the differentially expressed proteins in the lymphoid organ

To study the shrimp immune system against bacterial infection, diffentially expressed proteins in the lymphoid organ of the shrimp challenged with either 0.85% (w/v) NaCl or 10^5 CFU *V. harveyi* at 6 hpi were identified by 2-DGE. Minimally required for the colliodal coomassie detection was first analyzed. Various amount of extracted proteins was examined by 2-DGE followed by colliodal coomassie staining. The result showed that seven hundred micrograms of total proteins extracted from each experimental group was required for the analyses. About 1,000 protein spots were detected but there were only 48 and 34 protein spots at 6 and 48 hpi, respectively, that seemed statistically eligible for analysis. These eligible protein spots, which exhibited high alteration with more or less than 3 fold expression level, were selected for further analysis by LC-nano ESI-MS/MS (Figure 3.2A). The spots were divided into three groups as following. Four protein spots which showed a more than three-fold higher expression level compared to the control were identified as up-regulated proteins whereas 23 protein spots which showed lower than three-fold expression level as compared to the control were identified as down-regulated proteins. The rest (3 protein spots) that showed less than a 1.5 –fold increase or decrease in the relative intensity compared to the control was categorized as constantly expressed proteins. The LC-nano ESI-MS/MS derived peptide sequences were then searched against the database to annotate the protein types, shown in Table 3.1. Yet, four spots remained unknown because of the lack of the shrimp genome information.

Of those potentially identified protein spots, cytoskeleton proteins such as actin, actin2 and beta-tubulin were prominent. The rest of them varied in biological functions which were grouped as energy balance (ATP synthase beta subunit) (Figure 3.2B), carbohydrate metabolism (triosephosphate isomerase), immune defense mechanism (transglutaminase), stress response (heat shock protein) and signaling pathway (14-3-3 like protein) (Figure 3.2B), chaperone function (protein disulfide isomerase), serine protease inhibitor (alpha-2-macroglobulin) (Figure 3.2B) and transcriptional regulation (calreticulin and tat-binding protein). The proteins from the lymphoid organ with the highest alteration upon *V. harveyi*

infection were actin 2 (16.8-fold decrease at 6 hpi) followed by ATP synthase beta subunit (5.5-fold increase at 6 hpi).

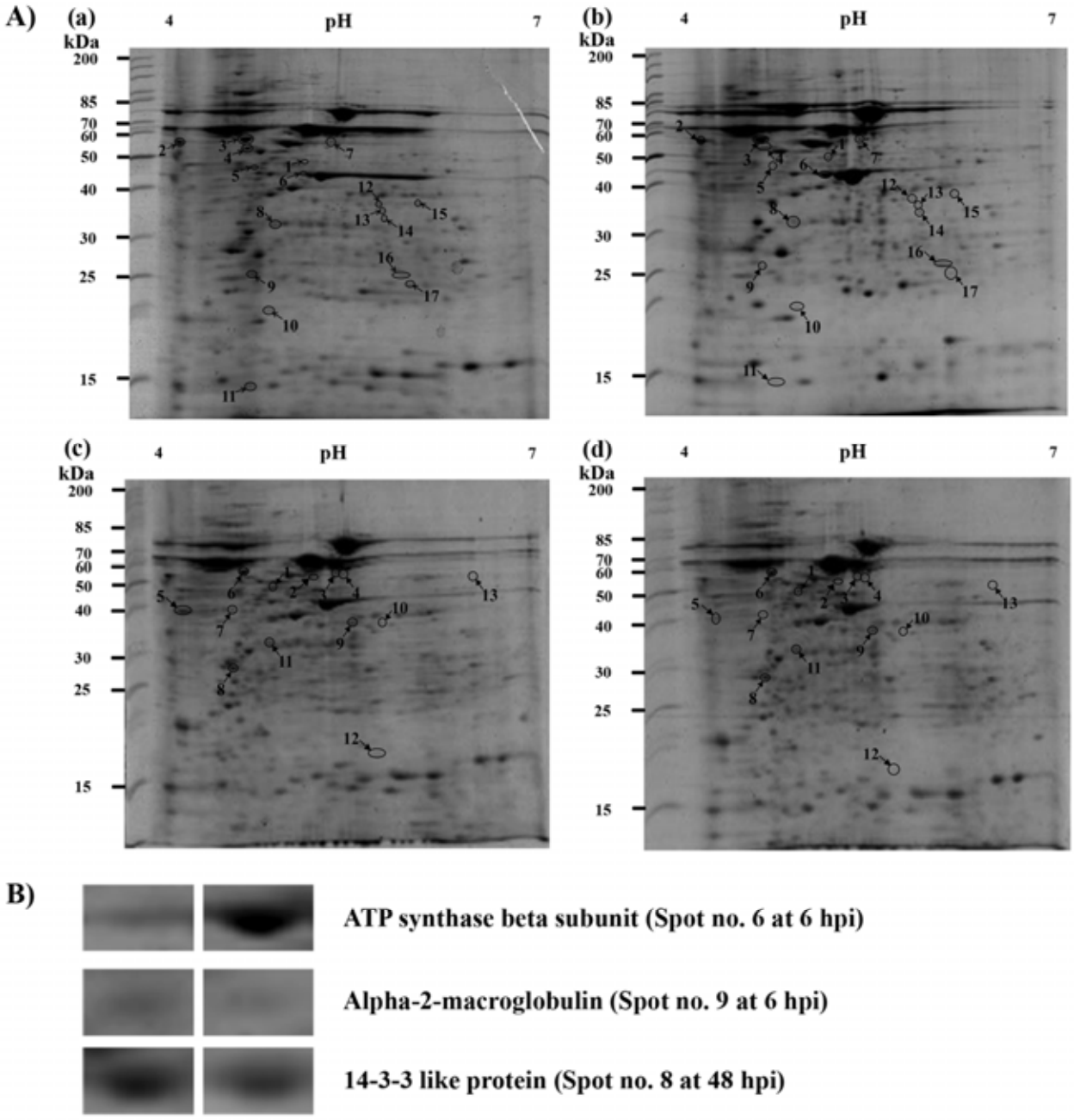


Figure 3.2 The 2-DGE protein expression profiles of the lymphoid organ of *V. harveyi*-infected *P. monodon*. A) The differentially expressed proteins of the lymphoid organ of unchallenged (a, c) or systemically challenged (b, d) shrimp with a lethal dose (10^5 CFU) of *V. harveyi* at (a, b) 6 hpi and (c, d) 48 hpi were compared. The labeled numbers of the protein

spots on the gels correspond with the numbers shown in Table 3.1. The gels are representative of three independent replicates. B) Magnification figures of the interesting protein spots comprising of ATP synthase beta subunit (Spot no. 6 at 6 hpi), alpha-2-macroglobulin (Spot no. 9 at 6 hpi), and 14-3-3 like protein (Spot no. 8 at 48 hpi).

Table 3.1. The selected lymphoid proteins of *V. harveyi*-infected *P. monodon* identified by 2-DGE and LC-nano ESI-MS/MS at 6 hpi and 48 hpi.

Spot no.	Predicted MW (Da)	Predicted pI	Protein hit	Mowse Score/no. of match peptides	Accession no.	Fold change
6 hours post infection						
Up-regulated protein spots						
1	47598	5.06	PREDICTED: similar to Tat-binding protein-1 [*Apis mellifera*]	362/6	XP_392722	2.98
6	46181	4.90	ATP synthase beta subunit [*Haliotis rufenscens*]	427/6	AAZ30686	5.50
Down-regulated protein spots						
2	46778	4.30	Calreticulin precursor [*Fenneropenaeus chinensis*]	378/11	ABC50166	-3.46
4	58480	4.70	PREDICTED: similar to heat shock protein 1, beta isoform 1 [*Apis mellifera*]	171/2	XP_392456	-3.13
5	41822	5.11	Actin 2 [*Penaeus monodon*]	664/27	AAC78682	-16.75
7	84660	5.51	Transglutaminase [*Penaeus monodon*]	213/6	AAL78166	-3.43
9	32431	5.04	Alpha-2-macroglobulin [*Penaeus monodon*]	252/2	AAX24130	-4.55
10	29673	8.24	Beta tubulin [*Penaeus monodon*]	588/4	ABU49604	-4.00
11	-	-	Unknown	-	-	-3.58
12	41475	5.67	Beta-tubulin [*Cryptocercus punctulatus*]	160/7	ABL11314	-4.05
13	41513	5.30	Actin	339/5	1101351C	-3.72
14	-	-	Unknown	-	-	-3.73
15	-	-	Unknown	-	-	-4.23
16	27484	5.55	Triosephosphate isomerase [*Fenneropenaeus chinensis*]	161/4	ABB81879	-8.65
17	-	-	Unknown	-	-	-3.65
Constant protein spots						
3	55049	4.64	Protein disulfide isomerase [*Litopenaeus vannamei*]	1194/33	ACN89260	1.38
8	41847	5.29	Actin, cytoskeleton 2 (LPC2)	442/7	P53466	1.45
48 hours post infection						
Up-regulated protein spots						
1	-	-	Unknown	-	-	3.33
11	40313	5.56	Cytoplasmic actin Cy II [*Meliocidaris erythrogramma*]	160/3	AAB66304	5.11
Down-regulated protein spots						
2	-	-	Unknown	-	-	-3.98
3	84910	5.61	Transglutaminase [*Fenneropenaeus chinensis*]	78/3	ABC33914	-4.62
4	75092	5.38	Hemocyanin subunit Y [*Marsupenaeus japonicus*]	50/1	ABR14694	-5.25
5	-	-	Unknown	-	-	-5.79
7	-	-	Unknown	-	-	-3.14
8	27834	4.61	14-3-3 like protein [*Penaeus monodon*]	333/15	AAY56092	-3.15
9	41841	5.30	Beta-actin [*Litopenaeus vannamei*]	99/3	AAG16253	-4.67
10	21914	5.78	Cytochrome oxidase subunit I	48/1	ABG46959	-4.38
12	-	-	Unknown	-	-	-3.78
13	-	-	Unknown	-	-	-3.43
Constant protein spots						
6	55049	4.64	Protein disulfide isomerase [*Litopenaeus vannamei*]	585/13	ACN89260	1.30

3.3 Confirmation of the differentially expressed protein upon *V. harveyi* infection by Western blot analysis

One differentially expressed protein was confirmed by western blot analysis. Antibody specific to ATP synthase beta subunit was purchased from Invitrogen. The protein expression of *P. monodon* ATP synthase beta subunit (*Pm*ATP synthase beta subunit) after *V. harveyi* infection was then quantitated by western blot analysis. The estimated size of about 58 kDa *Pm*ATP synthase beta subunit was detected by a commercial monoclonal antibody against ATP synthase beta subunit. The result shows that the protein expression of *Pm*ATP synthase beta subunit in *V. harveyi*-infected shrimp was obviously increased by 3.22-fold at 72 hpi whilst there were no alterations of the protein expression at 6 hpi. However, the result here appeared not correlated well with the proteomic results, where the expression of *Pm*ATP synthase beta subunit protein in *V. harveyi*-challeged was upregulated by 5.5 fold at 6 hpi. Western analysis still confirmed the upregulation of *Pm*ATP synthase beta subunit expression in LO of *V. harveyi*-challenged shrimp. It is possible that the difference of the experimental animal groups used in the experiments might be differently affected by the same lethally bacterial dose resulted in the delay response of some immune related proteins.

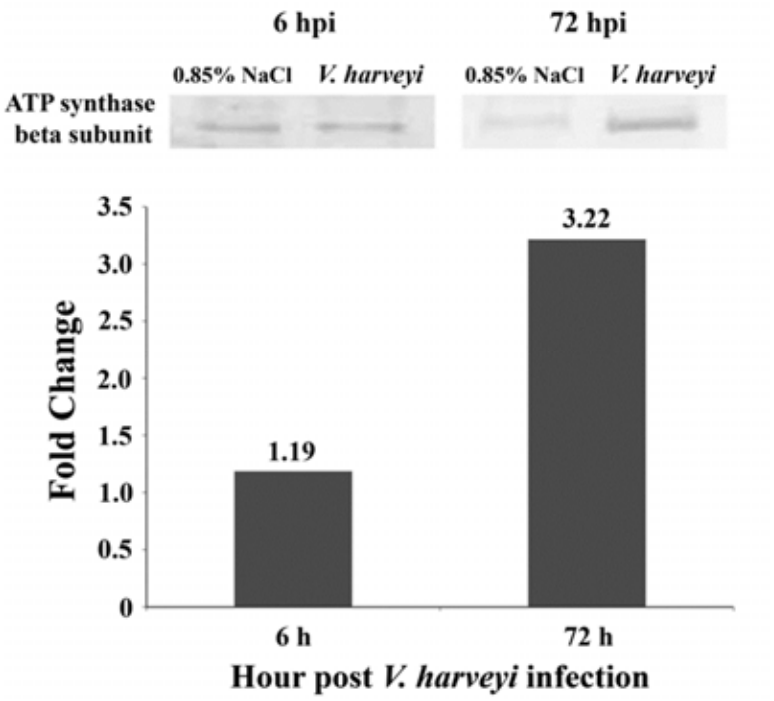


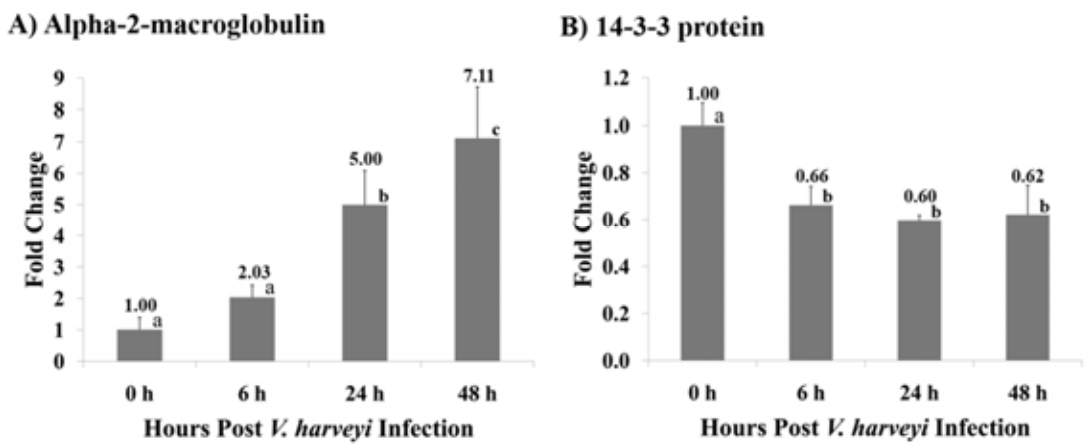
Figure 3.3 Western blot analysis of *Pm*ATP synthase beta subunit protein expression upon *V. harveyi* infection. The extracted proteins of the lymphoid organ were pooled from 3 individuals of either shrimp injected with 0.85% (w/v) NaCl or infected with *V. harveyi* at 6

and 72 hpi. The total protein was separated on 10% SDS-PAGE and subjected to Western blot analysis using anti-ATP synthase beta subunit antibody. The *Pm*ATP synthase beta subunit protein expression at each time point was normalized to that of the control, the saline-injected shrimp.

3.4 Time-course quantitative analysis of the transcripts of differentially expressed proteins in the lymphoid organ upon *V. harveyi* infection

Three differentially expressed proteins which were the 5.5-fold up-regulated ATP synthase beta subunit (observed at 6 hpi), the 3.15-fold down-regulated 14-3-3 like protein (observed at 48 hpi) and the 4.5-fold down-regulated alpha-2-macroglobulin (observed at 6 phi), were subjected to quantitative real-time RT-PCR to examine their transcript level at 0, 6, 24 and 48 hpi. The analyses of these genes were performed using EF1-α transcript as the internal control.

The mRNA expression level of the alpha-2-macroglobulin transcript was substantially and continuously went up over the bacterial infection period from a 2-fold at 6 hpi to a maximum of 7.11-fold at 48 hpi (Figure 3.4A). The expression level of the 14-3-3 like protein transcript slightly decreased (0.6-fold) from 6 hpi and remained at this level throughout the period (Figure 3.4B). Moveover, only a slight up-regulation of the ATP synthase beta subunit transcript expression level was observed (1.21-fold) at 6 hpi (Figure 3.4C).



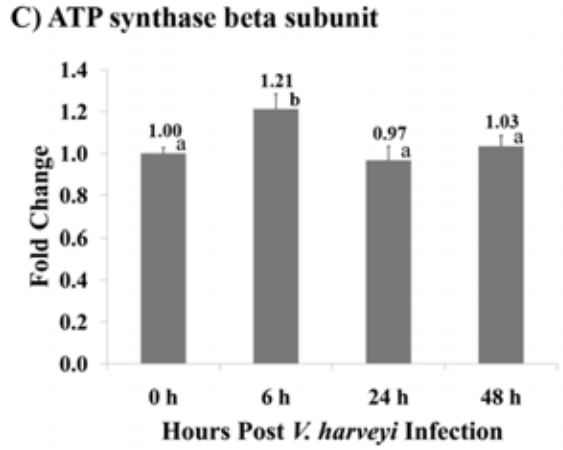


Figure 3.4 Fold change of transcript expression levels of (A) alpha-2-macroglobulin, (B) 14-3-3 like protein and (C) ATP synthase beta subunit, at 0, 6, 24 and 48 hours after *V. harveyi* challenge. The mRNA expression levels were assessed by quantitative real-time RT-PCR and normalized to that of EF1-α using as the internal control. The data are representative of three replications. Means with different lowercase letters (a, b, c) indicate significant difference (P<0.05).

II. Functional characterization of *P. monodon* ATP synthase beta subunit (*Pm*ATP synthase beta subunit) and *P. monodon* alpha-2-macroglobulin (*Pm*A2M)

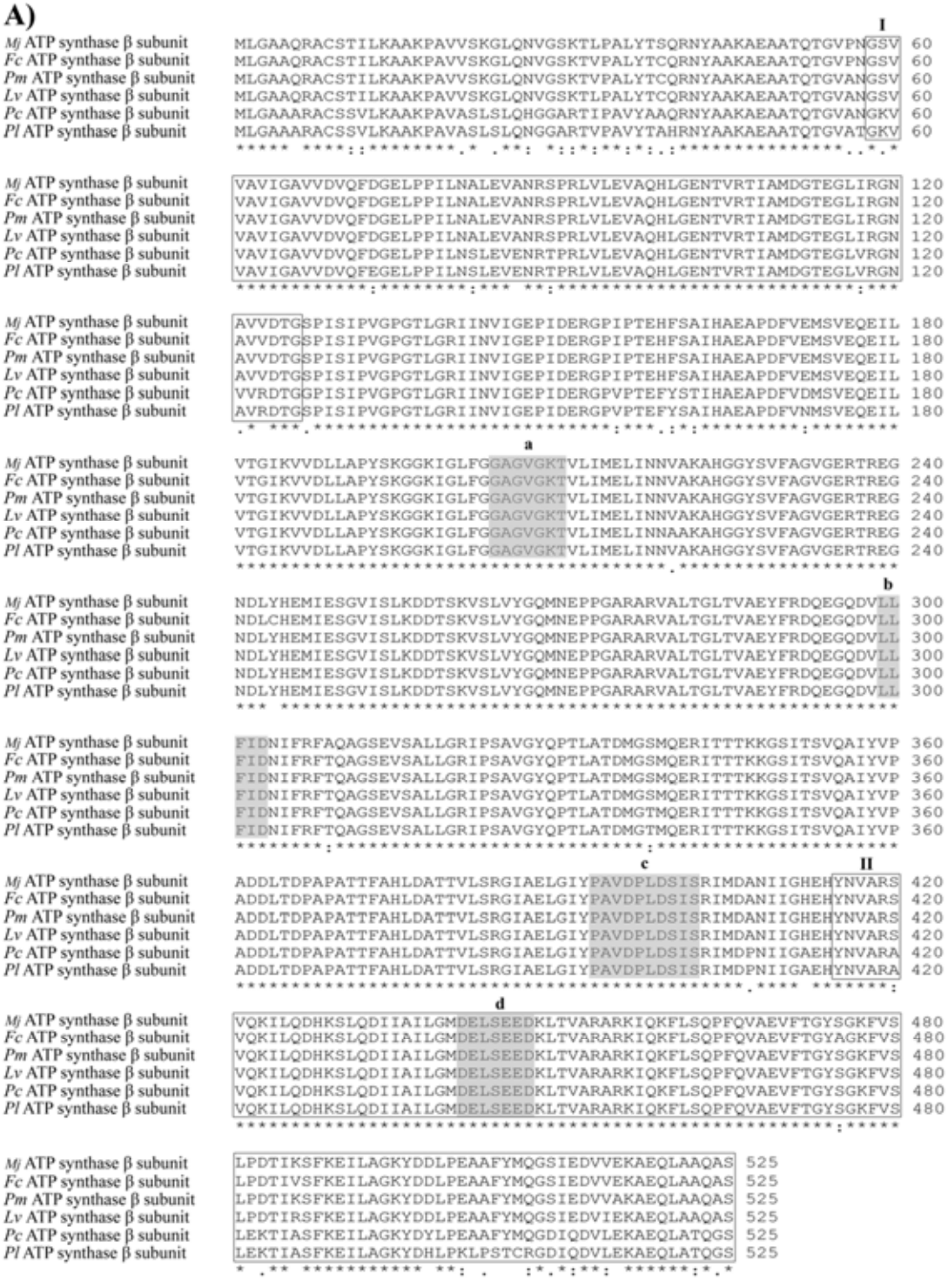
3.5 Sequence and phylogenetic analyses of *Pm*ATP synthase beta subunit

Searching the *P. monodon* EST database using the best matched amino acid sequence obtained from the GenBank and amino acid fragments from the nano-LC-ESI-MS/MS mass spectrometry of the ATP synthase beta subunit as a query identified contig number 494 (CT494) as the best matched. Sequence analysis revealed that the deduced ORF of the *P. monodon* ATP synthase beta subunit contained 1,578 bp and encoded for a predicted 525 amino acid residues. Searching the predicted amino acid sequence against the NCBI Conserved Domain Database identified five conserved domains such as the walker motifs A and B of nucleotide binding domains, DELSEED motif and the carboxy and amino terminal domains. The ATPase alpha/beta signature domain was further identified by the Expasy Proteomics Motif Scan (Figure 3.15A). The *Pm*ATP synthase beta subunit showed the highest amino acid sequence similarity to the homologs from *L. vannamei* (99.8%), *Marsupenaeus japonicas* (99.2%) and *Fenneropenaeus chinensis* (99.2%), and 99.2, 99.0 and 99.0% sequence identity, respectively, while *Procambarus clarkii* and *P. leniusculus*

displayed 95.2% and 94.7% sequence similarity, and 92.0 and 91.4% sequence identity, respectively. The amino acid alignment of *P. monodon* ATP synthase beta subunit with those from *P. leniusculus*, *P. clarkii*, *L. vannamei*, *M. japonicus* and *F. chinensis*, the best matched sequences obtained from the BLAST result, revealed that all predicted domains of ATP synthase beta subunit except the carboxy and amino terminus domains were highly conserved (Figure 3.15A), indicating the likely importance for the their functions.

ATP synthase beta subunit amino acid sequences of various organisms from the GenBank database were used to construct a NJ distance-based phylogenetic tree (Figure 3.15B), with the crustacean ATP synthase beta subunit, including that of shrimp and mollusca, being grouped in the same cluster. There are, in addition, two major groups of ATP synthase beta subunit visible in these analyzed samples, the insect and vertebrate groups.

A)


I
Mj ATP synthase β subunit MLGAAQRACSTILKAAKPAVVSKGLQNVGSKTLPALYTSQRNYAAKAEAATQTGVPNGSV 60
Fc ATP synthase β subunit MLGAAQRACSTILKAAKPAVVSKGLQNVGSKTVPALYTCQRNYAAKAEAATQTGVPNGSV 60
Pm ATP synthase β subunit MLGAAQRACSTILKAAKPAVVSKGLQNVGSKTVPALYTCQRNYAAKAEAATQTGVANGSV 60
Lv ATP synthase β subunit MLGAAQRACSTILKAAKPAVVSKGLQNVGSKTLPALYTCQRNYAAKAEAATQTGVANGSV 60
Pc ATP synthase β subunit MLGAAARACSSVLKAAKPAVASLSLQHGGARTIPAVYAAQRNYAAKAEAATQTGVANGKV 60
Pl ATP synthase β subunit MLGAAARACSSVLKAAKPAVASLSLQNGGARTVPAVYTAHRNYAAKAEAATQTGVATGKV 60
Mj ATP synthase β subunit VAVIGAVVDVQFDGELPPILNALEVANRSPRLVLEVAQHLGENTVRTIAMDGTEGLIRGN 120
Fc ATP synthase β subunit VAVIGAVVDVQFDGELPPILNALEVANRSPRLVLEVAQHLGENTVRTIAMDGTEGLIRGN 120
Pm ATP synthase β subunit VAVIGAVVDVQFDGELPPILNALEVANRSPRLVLEVAQHLGENTVRTIAMDGTEGLIRGN 120
Lv ATP synthase β subunit VAVIGAVVDVQFDGELPPILNALEVANRSPRLVLEVAQHLGENTVRTIAMDGTEGLIRGN 120
Pc ATP synthase β subunit VAVIGAVVDVQFDGELPPILNSLEVENRTPRLVLEVAQHLGENTVRTIAMDGTEGLVRGN 120
Pl ATP synthase β subunit VAVIGAVVDVQFEGELPPILNSLEVENRTPRLVLEVAQHLGENTVRTIAMDGTEGLVRGN 120
Mj ATP synthase β subunit AVVDTGSPISIPVGPGTLGRIINVIGEPIDERGPIPTEHFSAIHAEAPDFVEMSVEQEIL 180
Fc ATP synthase β subunit AVVDTGSPISIPVGPGTLGRIINVIGEPIDERGPIPTEHFSAIHAEAPDFVEMSVEQEIL 180
Pm ATP synthase β subunit AVVDTGSPISIPVGPGTLGRIINVIGEPIDERGPIPTEHFSAIHAEAPDFVEMSVEQEIL 180
Lv ATP synthase β subunit AVVDTGSPISIPVGPGTLGRIINVIGEPIDERGPIPTEHFSAIHAEAPDFVEMSVEQEIL 180
Pc ATP synthase β subunit VVRDTGGPISIPVGPGTLGRIINVIGEPIDERGPVPTEFYSTIHAEAPDFVDMSVEQEIL 180
Pl ATP synthase β subunit AVRDTGSPISIPVGPGTLGRIINVIGEPIDERGPVPTEFYSAIHAEAPDFVNMSVEQEIL 180
a
Mj ATP synthase β subunit VTGIKVVDLLAPYSKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERTREG 240
Fc ATP synthase β subunit VTGIKVVDLLAPYSKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERTREG 240
Pm ATP synthase β subunit VTGIKVVDLLAPYSKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERTREG 240
Lv ATP synthase β subunit VTGIKVVDLLAPYSKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERTREG 240
Pc ATP synthase β subunit VTGIKVVDLLAPYSKGGKIGLFGGAGVGKTVLIMELINNAAKAHGGYSVFAGVGERTREG 240
Pl ATP synthase β subunit VTGIKVVDLLAPYSKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERTREG 240
b
Mj ATP synthase β subunit NDLYHEMIESGVISLKDDTSKVSLVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLL 300
Fc ATP synthase β subunit NDLCHEMIESGVISLKDDTSKVSLVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLL 300
Pm ATP synthase β subunit NDLYHEMIESGVISLKDDTSKVSLVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLL 300
Lv ATP synthase β subunit NDLYHEMIESGVISLKDDTSKVSLVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLL 300
Pc ATP synthase β subunit NDLYHEMIESGVISLKDDTSKVSLVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLL 300
Pl ATP synthase β subunit NDLYHEMIESGVISLKDDTSKVSLVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLL 300
Mj ATP synthase β subunit FIDNIFRFAQAGSEVSALLGRIPSAVGYQPTLATDMGSMQERITTTKKGSITSVQAIYVP 360
Fc ATP synthase β subunit FIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGSMQERITTTKKGSITSVQAIYVP 360
Pm ATP synthase β subunit FIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGSMQERITTTKKGSITSVQAIYVP 360
Lv ATP synthase β subunit FIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGSMQERITTTKKGSITSVQAIYVP 360
Pc ATP synthase β subunit FIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGTMQERITTTKKGSITSVQAIYVP 360
Pl ATP synthase β subunit FIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGTMQERITTTKKGSITSVQAIYVP 360
c
II
Mj ATP synthase β subunit ADDLTDPAPATTFAHLDATTVLSRGIAELGIYPAVDPLDSISRIMDANIIGHEHYNVARS 420
Fc ATP synthase β subunit ADDLTDPAPATTFAHLDATTVLSRGIAELGIYPAVDPLDSISRIMDANIIGHEHYNVARS 420
Pm ATP synthase β subunit ADDLTDPAPATTFAHLDATTVLSRGIAELGIYPAVDPLDSISRIMDANIIGHEHYNVARS 420
Lv ATP synthase β subunit ADDLTDPAPATTFAHLDATTVLSRGIAELGIYPAVDPLDSISRIMDANIIGHEHYNVARS 420
Pc ATP synthase β subunit ADDLTDPAPATTFAHLDATTVLSRGIAELGIYPAVDPLDSISRIMDPNIIGAEHYNVARA 420
Pl ATP synthase β subunit ADDLTDPAPATTFAHLDATTVLSRGIAELGIYPAVDPLDSISRIMDPNIIGAEHYNVARA 420
d
Mj ATP synthase β subunit VQKILQDHKSLQDIIAILGMDELSEEDKLTVARARKIQKFLSQPFQVAEVFTGYSGKFVS 480
Fc ATP synthase β subunit VQKILQDHKSLQDIIAILGMDELSEEDKLTVARARKIQKFLSQPFQVAEVFTGYAGKFVS 480
Pm ATP synthase β subunit VQKILQDHKSLQDIIAILGMDELSEEDKLTVARARKIQKFLSQPFQVAEVFTGYSGKFVS 480
Lv ATP synthase β subunit VQKILQDHKSLQDIIAILGMDELSEEDKLTVARARKIQKFLSQPFQVAEVFTGYSGKFVS 480
Pc ATP synthase β subunit VQKILQDHKSLQDIIAILGMDELSEEDKLTVARARKIQKFLSQPFQVAEVFTGYSGKFVS 480
Pl ATP synthase β subunit VQKILQDHKSLQDIIAILGMDELSEEDKLTVARARKIQKFLSQPFQVAEVFTGYSGKFVS 480
Mj ATP synthase β subunit LPDTIKSFKEILAGKYDDLPEAAFYMQGSIEDVVEKAEQLAAQAS 525
Fc ATP synthase β subunit LPDTIVSFKEILAGKYDDLPEAAFYMQGSIEDVVEKAEQLAAQAS 525
Pm ATP synthase β subunit LPDTIKSFKEILAGKYDDLPEAAFYMQGSIEDVVAKAEQLAAQAS 525
Lv ATP synthase β subunit LPDTIRSFKEILAGKYDDLPEAAFYMQGSIEDVIEKAEQLAAQAS 525
Pc ATP synthase β subunit LEKTIASFKEILAGKYDYLPEAAFYMQGDIQDVLEKAEQLATQGS 525
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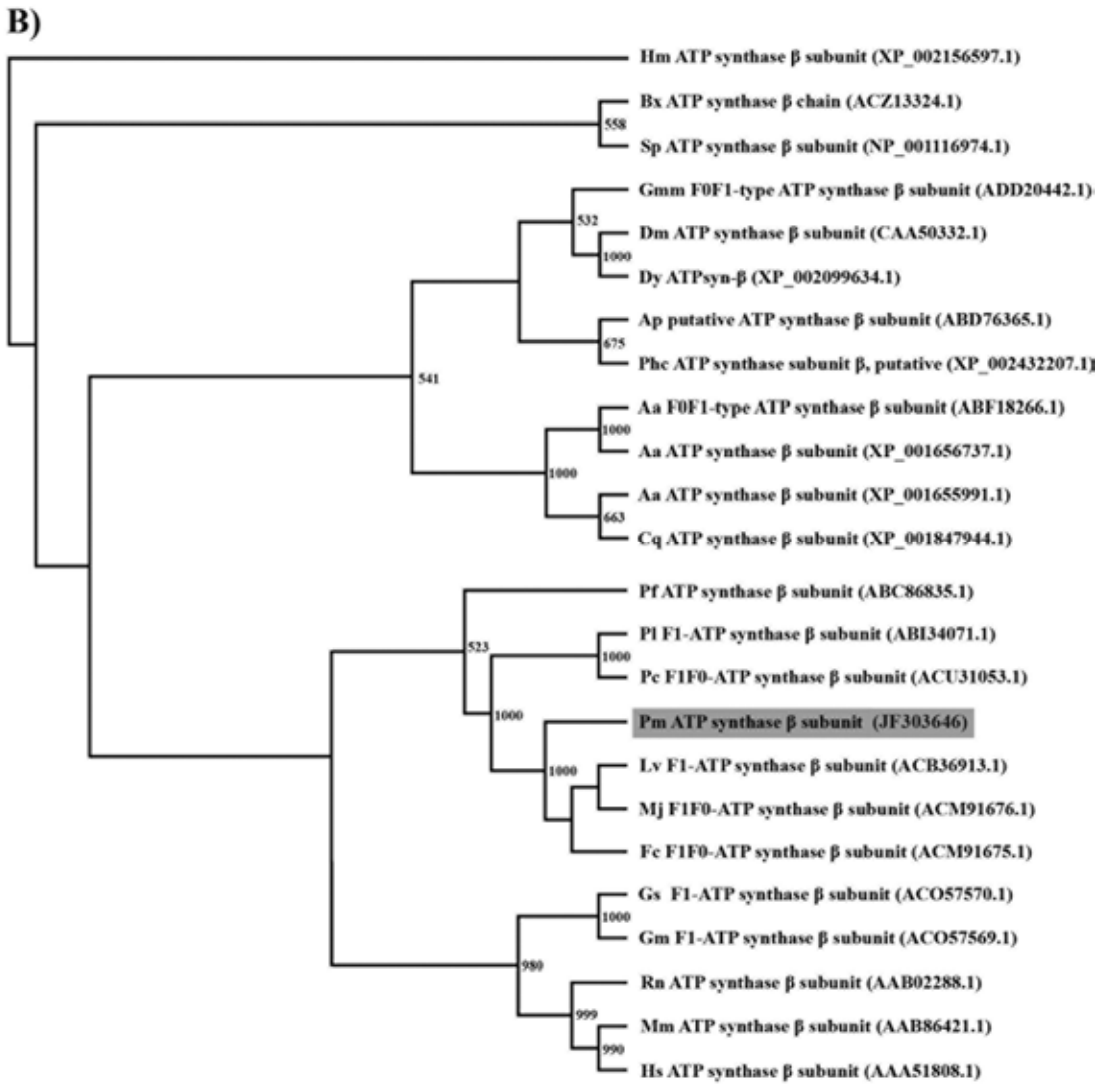

Figure 3.5 *Pm*ATP synthase beta subunit amino acid sequence analysis. (**A**) Amino acid sequence alignment of *Pm*ATP synthase beta subunit with ATP synthase beta subunit from other crustaceans showing the (a) nucleotide-binding domains, (b) the DELSEED motif and (c,d) the ATPase alpha (c)/beta (d) signature domains in grey boxes. The carboxyl (I) and amino (II) terminal domains are shown in open boxes. Amino acid conservation across all aligned sequences is shown as identical (*), conserved (:) and semi-conserved (;). (B) Phylogenetic analysis of ATP synthase beta subunit from *P. monodon* (highlighted in grey), and those from various related organisms; *Hydra magnipapillata* (Hm), *Bursaphelenchus xylophilus* (Bx), *Strongylocentrotus purpuratus* (Sp), *Glossina morsitans morsitans* (Gmm), *Drosophila melanogaster* (Dm), *Drosophila yakuba* (Dy), *Acyrthosiphon pisum* (Ap), *Pediculus humanus corporis* (Phc), *Aedes aegypti* (Aa), *Culex quinquefasciatus* (Cq),

Pinctada fucata (Pf), *P. leniusculus* (Pl), *Procambarus clarkii* (Pc), *L. vannamei* (Lv), *Marsupenaeus japonicus* (Mj), *Fenneropenaeus chinensis* (Fc), *Gillichthys seta* (Gs), *Gillichthys mirabilis* (Gm), *Rattus norvegicus* (Rn), *Mus musculus* (Mm) and *Homo sapiens* (Hs), were analyzed. *H. magnipapillata* (Hm) was selected as an outgroup. Bootstrap support values of more than 50% (out of 1000) are shown for each node. GenBank accession numbers of the ATP synthase beta subunit amino acid sequences in the tree are shown after the name. The nucleotide sequence for *Pm*ATP synthase beta subunit has a GenBank accession code of JF303646.

3.6 Tissue distribution analysis of *Pm*ATP synthase beta subunit

The relative expression level of ATP synthase beta subunit gene transcripts in 11 different shrimp tissues, that is the antennal gland, epipodite, eye stalk, gills, heart, hemocyte, hepatopancreas, intestine, LO, stomach and hematopoietic tissue, were examined. The gene transcripts were found to be expressed in all tissues tested (Figure 3.16), albeit at perhaps relatively low levels in the epipodite.

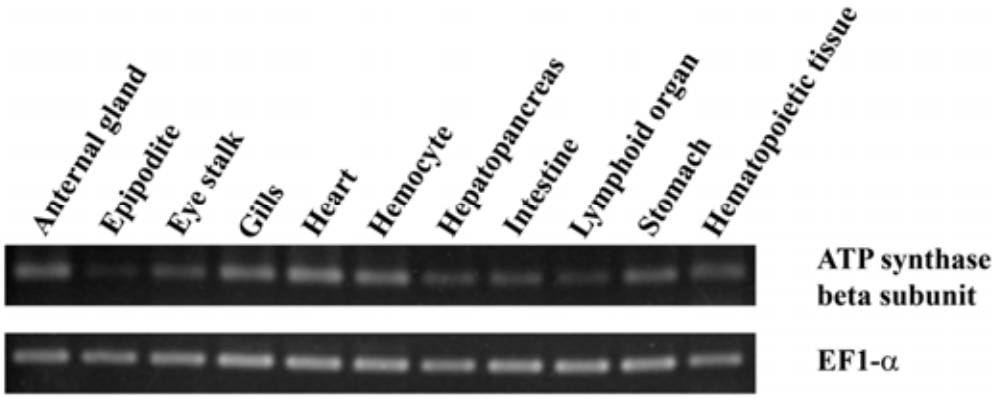

Figure 3.6 Tissue distribution analysis of ATP synthase beta subunit transcripts. Eleven different shrimp tissues (antennal gland, epipodite, eye stalk, gills, heart, hemocyte, hepatopancreas, intestine, LO, stomach and hematopoietic tissue) from unchallenged shrimp were analyzed for the expression of ATP synthase beta subunit mRNA using EF1-α as an internal control.

3.7 The involvement of the *Pm*ATP synthase beta subunit in shrimp survival

Based on the proteomic analysis of the lymphoid organ of *V. harveyi*-infected shrimp, the ATP synthase beta subunit was the highest up-regulated protein among other identified proteins. Therefore, RNA interference of the ATP synthase beta subunit gene was then performed in order to examine the biological function of ATP synthase beta subunit in the innate immunity. The expression level of ATP synthase beta subunit transcript was silenced up to 86.9% in the ATP synthase beta subunit dsRNA injected group compared to that of the control group (Figure 3.17A). This resulted in high cumulative mortality of the knockdown shrimp that rapidly went up to 60% within one day after ATP synthase beta subunit dsRNA injection followed by gradually increase to 73.3% at the rest (Figure 3.17B).

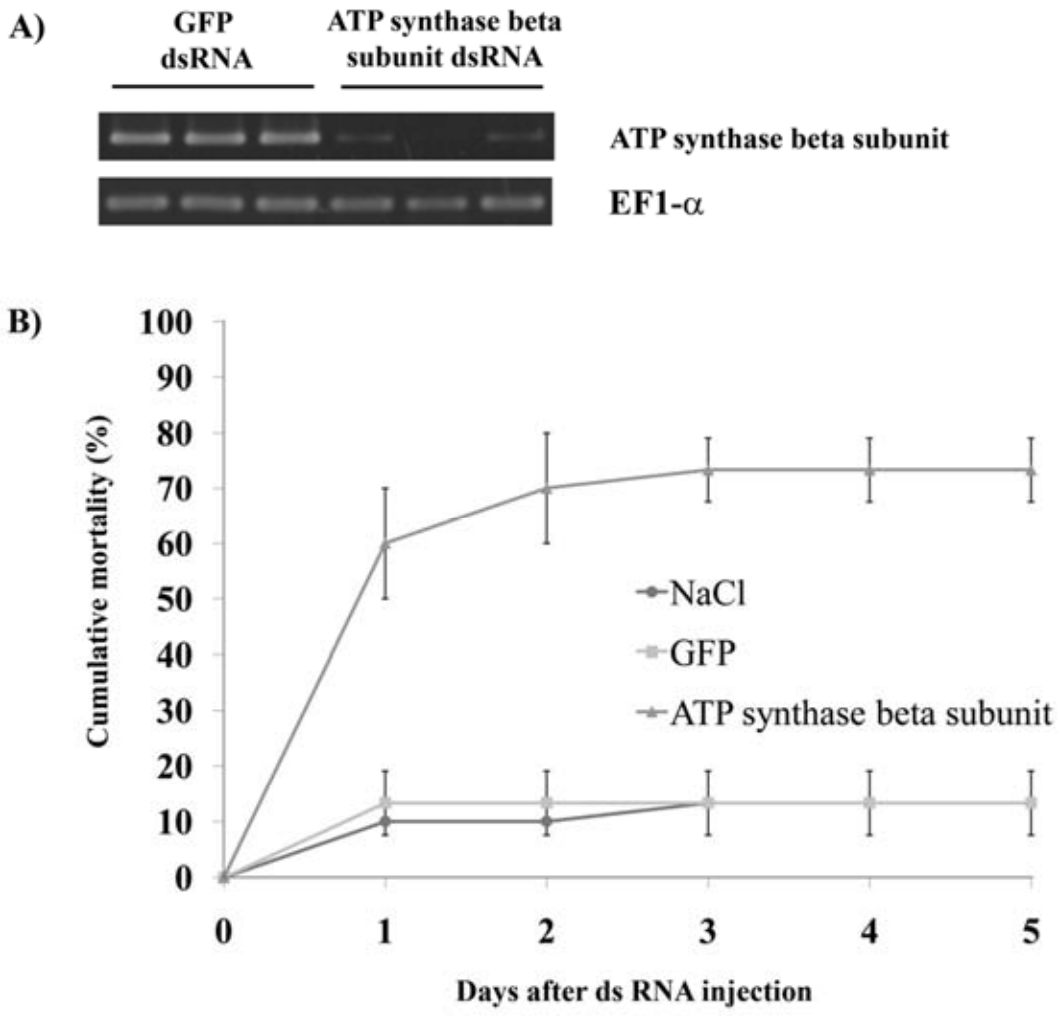

Figure 3.7 (A) Semi-quantitative RT-PCR analysis of the ATP synthase beta subunit gene expression of 10 µg GFP dsRNA (control) and 10 µg ATP synthase beta subunit dsRNA injected shrimp. EF1-α was used as the internal control. Each lane represents individual shrimp and the experiment was run triplicate. (B) The cumulative mortality graph of ATP synthase beta subunit depleted shrimp. The shrimp were divided into 3 groups; sterile saline solution injected or 10 µg of either GFP dsRNA injected (control group) or ATP synthase beta subunit dsRNA injected groups (experimental group). The experiment was conducted for 5 days after the injection. Ten shrimp were used in each group. The result is presented as the mean ± 1 SD and is derived from tripicate.

3.8 Significant decrease in circulating mature hemocyte numbers after *Pm*ATP synthase beta subunit gene silencing

The total number of mature hemocytes in the circulatory system of ATP synthase beta subunit silenced shrimp was verified in order to investigate the biological effect of the ATP synthase beta subunit gene knockdown on shrimp survival. The hemocyte counting in saline solution injected, GFP dsRNA-injected or ATP synthase beta subunit dsRNA-injected shrimp was conducted at 0 and 12 hours post dsRNA injection. Interestingly, the number of circulating mature hemocyte in the ATP synthase beta subunit dsRNA injected shrimp at 12 h after dsRNA injection greatly reduced around 5 fold compared to that at 0 h after dsRNA injection (Figure 3.18). Moreover, the hemocyte number at 0 h after dsRNA injection in all groups was about the same. Altogether, the substantial reduction of hemocyte number caused from the ATP synthase beta subunit silencing contributes to shrimp mortality.

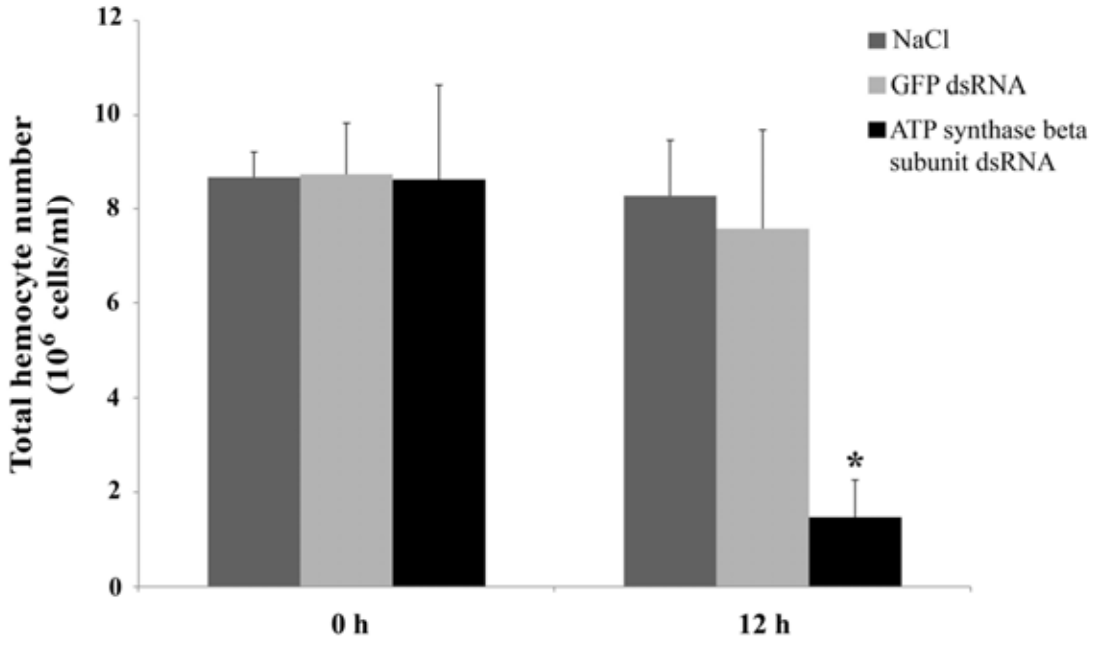

Figure 3.8 Total number of circulating hemocytes counted in saline solution injected, GFP dsRNA knockdown or ATP synthase beta subunit dsRNA knockdown shrimp at 0 and 12 after dsRNA injection. Hematocytometer was used to count the hemocyte number from three individuals in each group. The experimental data are presented as the mean ± 1 SD. Means with an asterisk are significantly change ($p < 0.05$).

3.9 The receptor binding domain (RBD) of *Pm*A2M binds to carboxyl terminus of transglutaminase type II (*Pm*STG II)

A2M is a proteinase inhibitor found in many organisms. In shrimp, there have been no reports on the involvement of A2M in shrimp immunity against bacteria. Previously, the substantial down-regulation of *Pm*A2M protein levels was demonstrated by proteomic analysis of *V. harveyi* infected shrimp hemocytes (Somboonwiwat et al., 2010) and lymphoid organ (Chaikeeratisak et al., 2012). Therefore, Y2H screening was then conducted in order to further investigate the A2M's protein partners in shrimp hemocytes serving potential roles in shrimp innate immunity. In mammals, RBD of A2M is responsible for binding with various receptors for subsequent process, therefore, RBD of PmA2M was selected for this study (Armstrong, 2010). After a DNA fragment encoding the RBD of *Pm*A2M was cloned into pGBKT7, the recombinant *Pm*A2M was then tested for autoactivation of reporter genes and toxicity to host cells. The result showed neither activation of the reporter genes (Figure 3.5A)

nor differences in yeast cell sizes (Figure 3.5B) observed between the control and experimental groups.

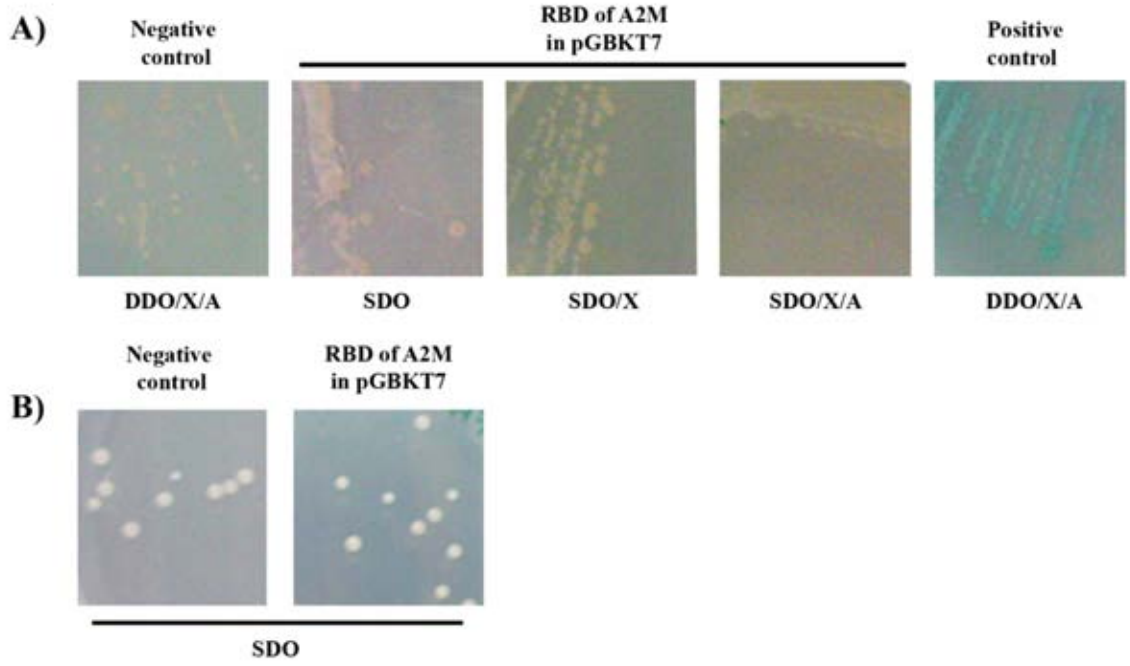

Figure 3.9 Testing bait for autoactivation of the reporter genes (A) and toxicity to the host cells (B). The DNA fragment encoding the RBD of *Pm*A2M was cloned into pGBKT7 and the recombinant *Pm*A2M was transformed into *S. cerevisiae* strain Y2HGold. The host cells containing the recombinant *Pm*A2M and the expression of reporter genes were selected on SDO, and SDO/X with or without A (aureobasidin A) media, respectively. The interaction between the murine p53 and lamin was used as the negative control, while that between murine p53 and the SV40 large T antigen served as a positive control. DDO/X/A media were used to detect the presence of baits and preys in the control group and the expression of reporter genes shown as blue colonies.

The Y2H assay was performed against cDNA library of the *V. harveyi* challenged shrimp, with a titer of 1.92 x 10^7 CFU/ml, to determine interacting protein partners of *Pm*A2M. Approximately 7.05 x 10^5 clones were screened between yeast host cells containing either baits or preys with a mating efficiency up to 19.35%. For the primary screening on the DDO/X/A agar plates, hundreds of positive clones were selected and these positive clones were later confirmed by re-streaking on the high stringency media, QDO/X/A. Of those, six

independent positive clones (SB1-4, PB1 and PB2) were eventually selected (Figure 3.6). The prey plasmids from these positive clones were isolated and subjected to DNA sequencing. The DNA sequence identification revealed that SB1-4 were the carboxyl-terminus of transglutaminase type II (*Pm*STG II) (accession number AAV49005.1) whereas PB1 and PB2, were similar to the homologs of the voltage-dependent anion-selective channel isoform 1 from *Tribolium castaneum* (accession number XP_967480.1) and the 40S ribosomal protein S20 from *Rimicaris exoculata* (accession number ACR78697.1), respectively.

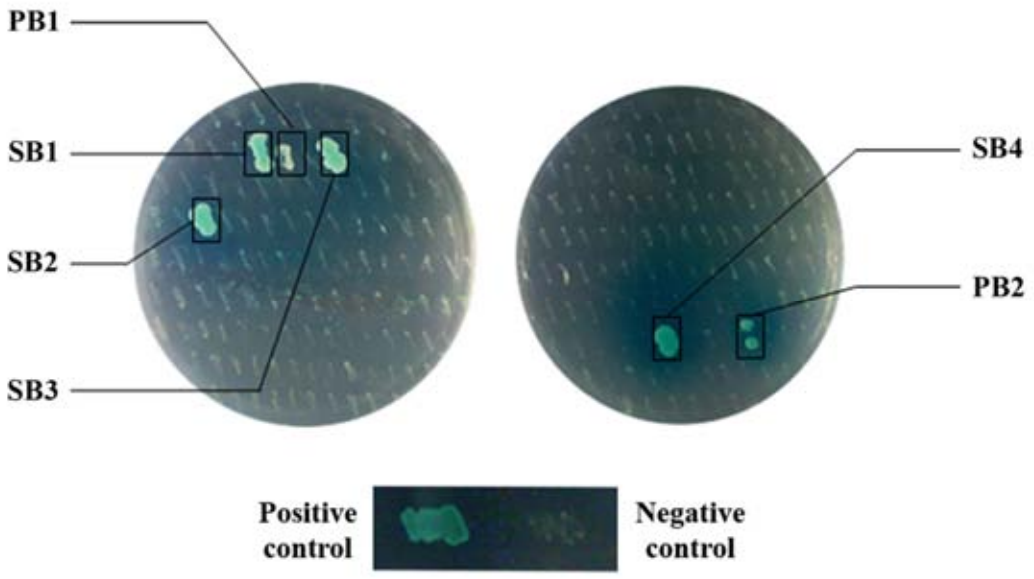

Figure 3.10 Discovery of six independent positive clones, named as SB1-4, PB1 and PB2, on the high stringency media (QDO/X/A) using the Y2H screening between the RBD of A2M (a bait) and the *V. harveyi*-challenged shrimp hemocyte cDNA library (preys). The positive clones can be grown on the media and appeared blue due to the reporter gene expression. The interaction between murine p53 and the SV40 large T antigen served as a positive control, whereas that between the murine p53 and lamin was used as the negative control.

3.10 Confirmation of authentic protein interactions by co-transformation assay

The co-transformation technique was conducted to confirm whether these six purified prey plasmids truly interact with the bait. Each purified positive prey plasmid and either an empty bait plasmid or the RBD of A2M containing bait vector were simultaneously transformd into the *S. cerevisiae* Y2HGold. All hosts which contained the SB1-4 and PB1-2 prey and bait plasmids healthy grew and appeared as blue colonies on both the DDO/X/A and QDO/X/A media resulted from activations of reporter genes, similar to the positive control. The SB1-4 prey and empty bait plasmids containing transformants and the negative control appeared alike which no growth was observed on the QDO/X/A media. In contrast, the yeast cells containing the PB1 and PB2 prey and empty bait plasmids well grew on the QDO/X/A media. This indicate that the PB1 and PB2 clones (voltage-dependent anion-selective channel isoform 1 and 40S ribosomal protein S20 homologs) were false positives (Figure 3.7). Taken together, the results strengthen that the carboxyl-terminus of the *Pm*STG II protein(s) authentically bind(s) with the RBD of *Pm*A2M.

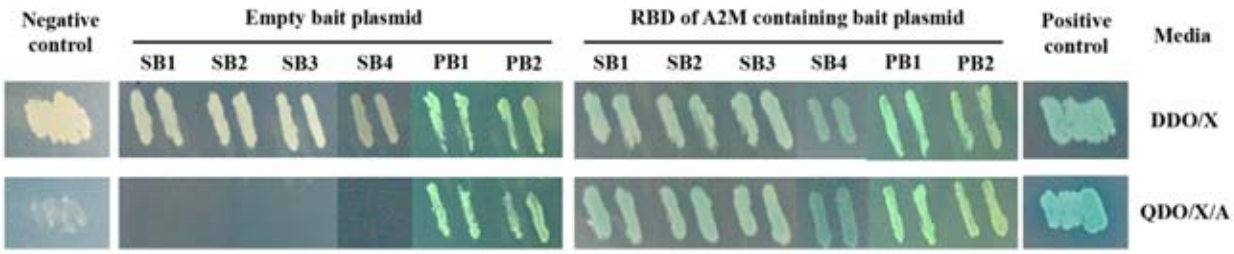

Figure 3.11 Co-transformation assay to confirm positive prey proteins derived from Y2H screening, using the RBD of A2M as a bait. The preys identified as the carboxyl-terminus of the *Pm*STG II (SB1, SB2, SB3 and SB4), and the homologs of the *T. castaneum* voltage-dependent anion-selective channel isoform 1 (PB1) and *R. exoculata* 40S ribosomal protein S20 (PB2), were simultaneously introduced into the *S. cerevisiae* strain Y2HGold with either an empty bait plasmid or a bait plasmid containing the RBD of A2M. The transformants grown on selective media were restreaked onto DDO/X media and QDO/X/A. The negative control and the positive control are the yeast, *S. cerevisiae* strain Y2HGold, containing the murine p53 and either lamin or the SV40 large T antigen, respectively.

3.11 Production of recombinant *Pm*A2M-RBD in *E. coli* expression system

The open reading frame encoding the receptor binding domain (RBD) of *Pm*A2M was firstly cloned into the pET22b (+) expression vector between *Nde*I and *Xho*I cloning sites. The r*Pm*A2M with histidine tag (-Leu-Glu-His_6) at C-terminus was then produced after induction the *E. coli* BL21 (DE3) containing pET22b-*Pm*A2M-RBD expression plasmid with 1 mM IPTG at 0, 2, 4 and 6 h. The whole cells were harvested and analyzed for the overproduced protein by 15% SDS-PAGE and coomassie staining. The result presented that the highest expression of the r*Pm*A2M-RBD was at 6 h post-IPTG induction. The expected r*Pm*A2M-RBD band with the molecular weight of about 15.5 kDa was observed, whereas *E. coli* without IPTG-induction did not over-produce the r*Pm*A2M-RBD (Figure 3.8A). Furthermore, the r*Pm*A2M-RBD was expressed in the inclusion bodies form (Figure 3.8B).

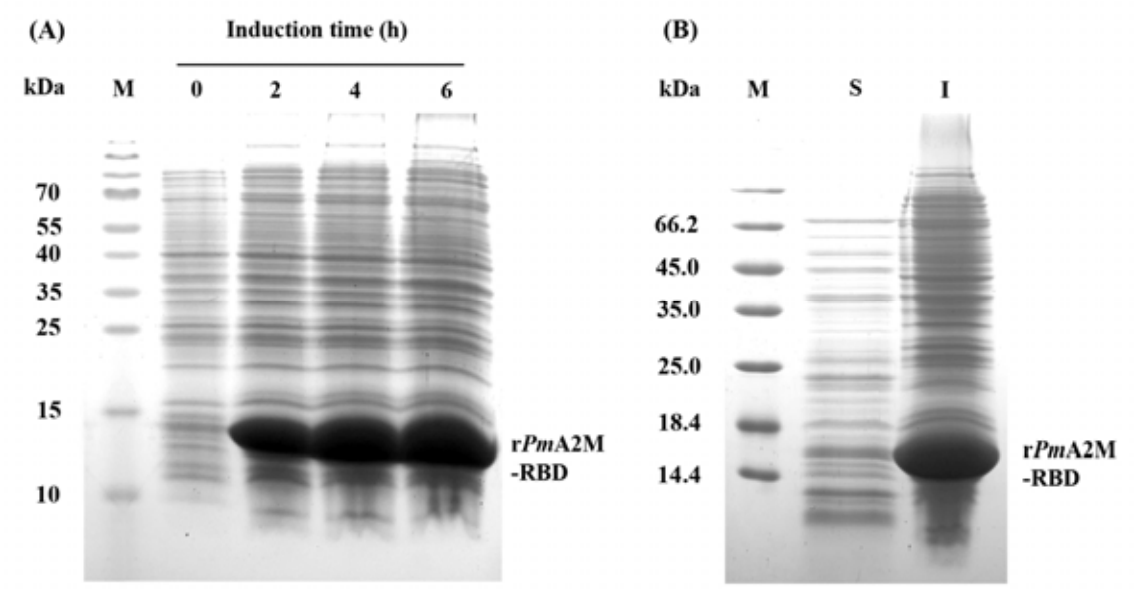

Figure 3.12 SDS-PAGE analysis of r*Pm*A2M-RBD production in *E. coli* BL21(DE3) expression system. (A) Induction of r*Pm*A2M-RBD protein expressed in *E. coli* BL21(DE3) with 1 mM IPTG at 0, 2, 4 and 6 h. The over-expressed r*Pm*A2M-RBD was analyzed by 15% SDS-PAGE followed by coomassie brilliant blue staining. (B) SDS-PAGE analysis of soluble (S) and inclusion bodies (I) fraction of r*Pm*A2M-RBD protein. The expected size of r*Pm*A2M-RBD was around 15.5 kDa. Lane M: Protein marker.

The buffer with different pH values was used to solubilize inclusion bodies and the suitable one was selected. The soluble fraction and the remaining pellet were then analyzed by SDS-PAGE. The result revealed that the inclusion bodies of r*Pm*A2M-RBD were well solubilized with low contaminants by the solution, 20 mM sodium phosphate buffer, pH 10.3 (Figure 3.9).

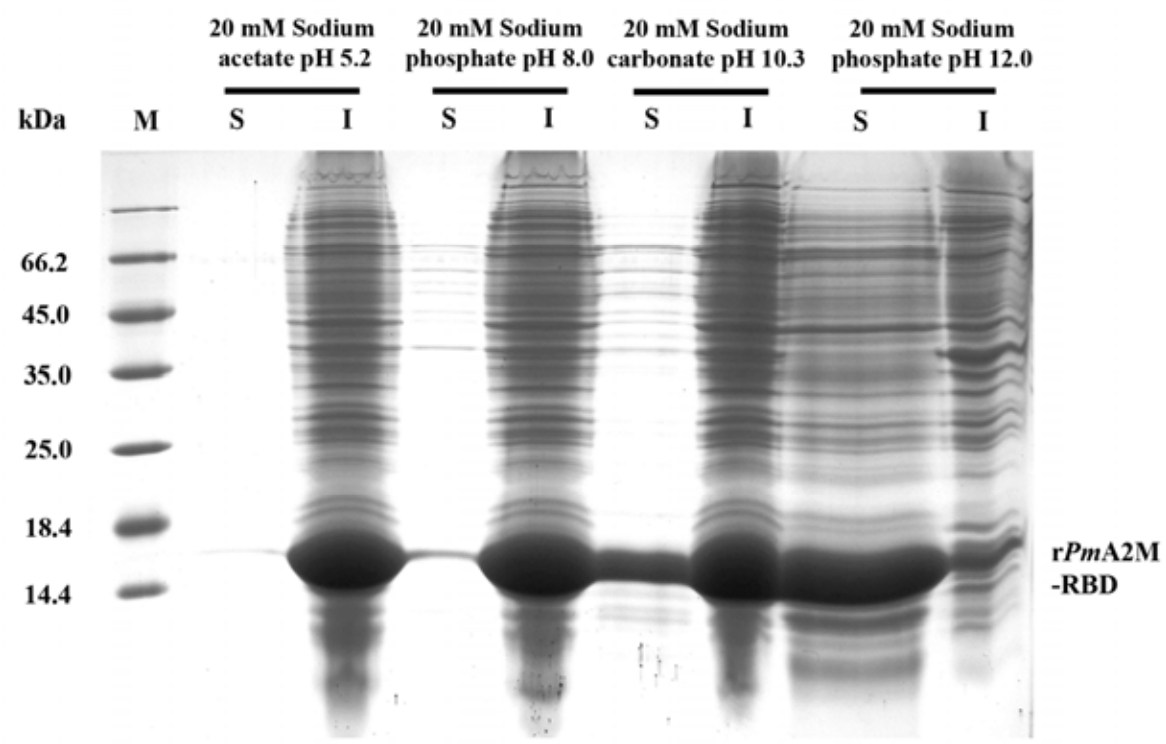

Figure 3.13 Determination of r*Pm*A2M-RBD solubility in various buffers. Soluble (S) and inclusion body (I) parts were run onto SDS-PAGE (15% (w/v) acrylamide) followed by coomassie brilliant blue staining. The expected 15.5 kDa r*Pm*A2M-RBD was shown in the gel. Lane M is the protein marker.

To prepare r*Pm*A2M-RBD for antibody production, large scale over-expression was conducted. The HiTrap chelating HP column was used for protein purification. The soluble protein mixtures were equilibrated with binding buffer (20 mM sodium phosphate buffer, pH 8.0, supplemented with 0.1 M NaCl and 20 mM Imidazole) and were loaded into the column. The column bound proteins were later eluted with the elution buffer (20 mM sodium phosphate buffer, pH 8.0, supplemented with 0.1 M NaCl and 500 mM Imidazole). After dialysis of the purified protein against 50 mM Tris-Cl, pH 8.0, SDS-PAGE and western blot

analysis using anti-His_6 antibody were performed. The single band of the purified protein sized around 15.5 kDa was observed (Figure 3.10).

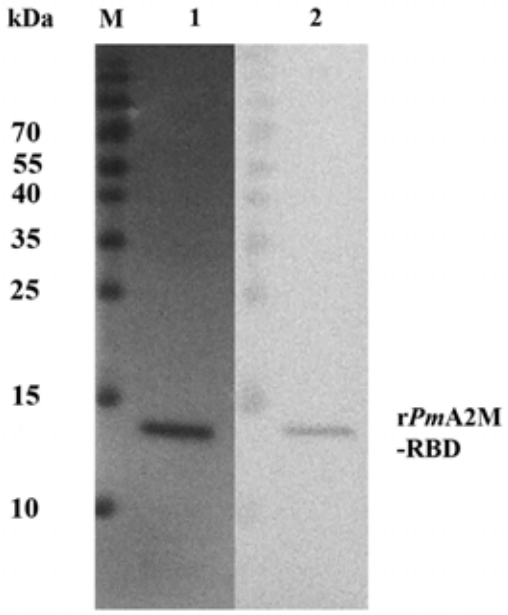

Figure 3.14 SDS-PAGE and Western blotting analysis of purified r*Pm*A2M-RBD-histidine tag fusion protein. Lane M: protein marker; Lane 1: coomassie staining; Lane 2: Western blot analysis. The expected r*Pm*A2M-RBD protein band with estimated size around 15.5 kDa was observed**.**

3.12 *Pm*A2M is mainly localized in semigranular hemocytes

It was revealed in the previous study that A2M is the differentially expressed protein in hemocytes of *V. harveyi*-infected *P. monodon*. To further characterize *Pm*A2M, the localization of *Pm*A2M was then examined in 3 main types of hemocytes categorized based on the number and relative size of granules which are hyaline (agranular), semigranular (small-granular), and granular (large-granular) hemocytes.

The presence of *Pm*A2M protein in hemocytes was assessed by the immunofluorescent staining using anti-r*Pm*A2M Ab and Alexa Fluor® 488 conjugated secondary Ab. The subcellular *Pm*A2M protein were observed as green fluorescense (Figure 3.11). The hemocytic nuclei were stained with TO-PRO-3 iodine (red fluorescense) (Figure 3.11). The result showed that *Pm*A2M seemed to be localized in secretory granules within the semigranular hemocytes, but appeared rare in the other types of hemocytes. (Figure 3.11).

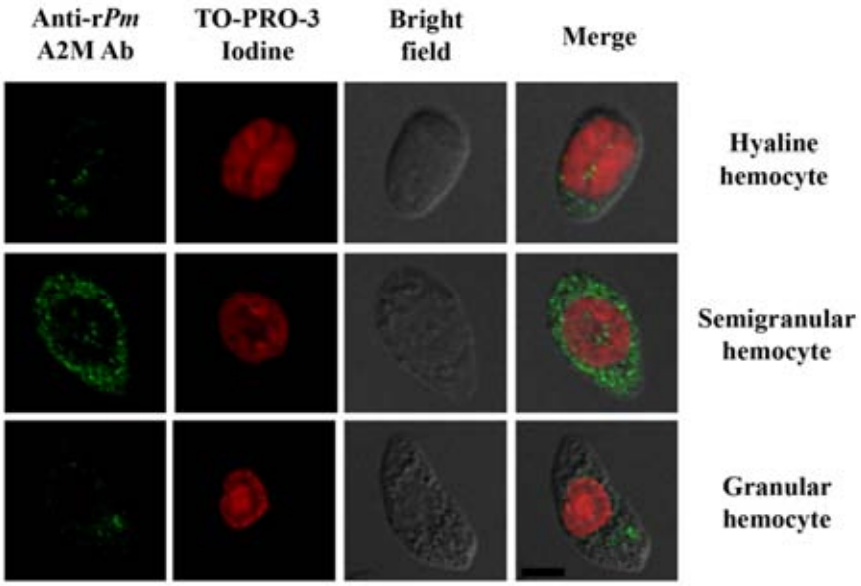

Figure 3.15 Immunofluorescent staining analysis of the *Pm*A2M protein content in three different types of shrimp hemocytes (hyaline, semigranular, and granular hemocytes) observed by confocal microscope. The *Pm*A2M proteins and hemocytic nuclei appeared green (Alexa Fluor 488) and red (TO-PRO-3 Iodine) fluorescense, respectively. The scale bar corresponds to 5 µm. Images are representative of 3 fields of view per sample.

3.13 *Pm*A2M is a rapidly responsive protein upon *V. harveyi* infection

The expression of A2M protein upon *V. harveyi* infection was determined. As shown in the Figure 3.12, the immunofluorescent signal of *Pm*A2M stored in granules of semigranular hemocytes of *V. harveyi*-infected shrimp at 10 min after bacterial injection was much more stronger than that of the control group, the saline injected shrimp (Figure 3.12). This result revealed that the expression of *Pm*A2M rapidly responded to the *V. harveyi* infection within 10 min after bacterial injection. This result implied that the semigranular hemocyte might serve important roles in *Pm*A2M regulation in response to the *V. harveyi* infection and *Pm*A2M is a rapidly responsive protein upon the bacterial infection.

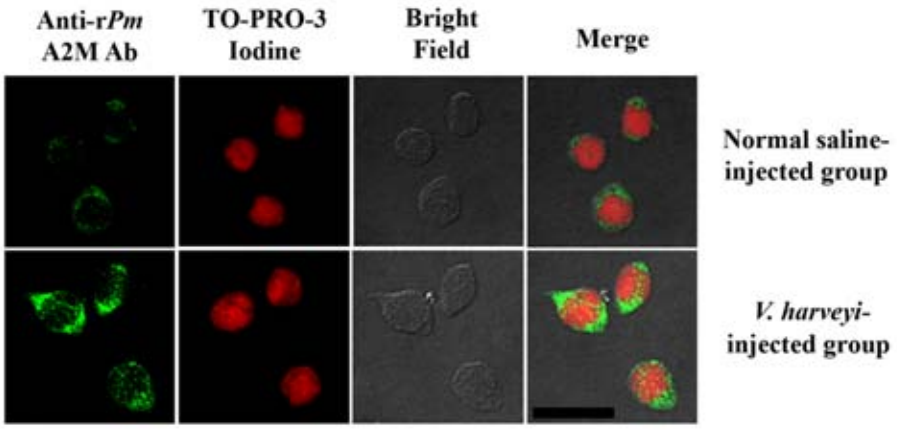

Figure 3.16 Confocal microscope analysis of the *Pm*A2M protein expression in semigranular hemocytes of normal and *V. harveyi*-challenged *P. monodon* using immunofluorescense. Hemocytes of the normal saline injected shrimp (control group) and the *V. harveyi*-injected shrimp at 10 min after bacterial injection from three individual were pooled. 1 x 10^5 cells/ml of the pooled hemocytes were fixed onto slide and stained with Alexa Fluor 488 (green) and TO-PRO-3 Iodine to detect *Pm*A2M proteins and hemocytic nuclei, respectively. The scale bar corresponds to 20 µm. Images are representative of 3 fields of view per sample.

3.14 Secretion of *Pm*A2M into the plasma upon *V. harveyi* infection

In order to investigate the distribution of the *Pm*A2M protein in hemocytes and the cell-free hemolymph of *V. harveyi* infected-shrimp, western blot analysis was performed at 0, 6, 24, 48 and 72 hpi. A dramatic decrease in the *Pm*A2M protein level in the hemocytes of *V. harveyi* infected shrimp was observed at 6 hpi with a low quantity of *Pm*A2M observed at 24 hpi, which correlated to the increased amount of *Pm*A2M protein contents observed in the cell free plasma over the same 6-24 hpi period (Figure 3.13). Furthermore, the *Pm*A2M protein contents in hemocytes increased at 48 hpi and reached the highest level at 72 hpi, whereas that in the plasma declined over this later infection period to essentially undetectable levels by 72 hpi. This result suggested that the A2M protein is released from hemocytes to hemolymph upon the *V. harveyi* infection. In fact, the main source of A2M in the plasma is the blood cells, and cell-free plasma typically has some A2M content but the amount is increased whenever the cells are stimulated.

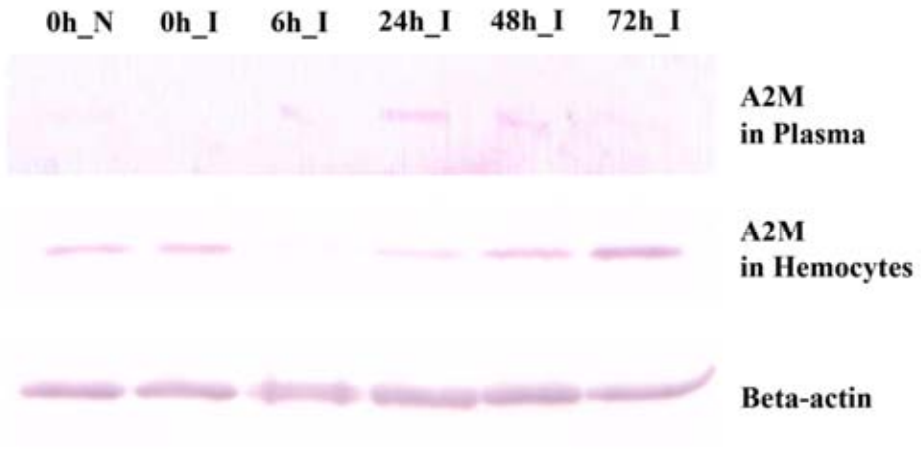

Figure 3.17 Secretion of *Pm*A2M from hemocytes into the hemolymph of *V. harveyi*-challenged *P. monodon*. The A2M contents in the hemocytes and the plasma of the *V. harveyi* infected groups were semi-quantitatively analyzed by western blot analysis at 0 hpi (0h_I), 6 hpi (6h_I), 24 hpi (24h_I), 48 hpi (48h_I) and 72 hpi (72h_I), compared to that of the control group at 0 hpi (0h_N). All samples were pooled from seven individuals. Equal quantities of total plasma (150 μg) and total hemocyte (15 μg) proteins were applied per track. β-actin was used as the internal control for the protein loading / transfer to membranes for hemocyte proteins.

3.15 Location of *Pm*A2M and clottable proteins (CPs) on hemolymph clots

According to the Y2H assay, *Pm*STG II, an important enzyme involved in the blood clotting system, was identified as a protein partner of *Pm*A2M. The co-localization of *Pm*A2M and clottable proteins were then determined on the extracellular blood clots using immunofluorescent staining and confocal microscopy to observe whether *Pm*A2M was also present on the clot. The shrimp clots were prepared on slides using the hanging drop technique. They were stained by mouse anti-r*Pm*A2M Ab and subsequent Alexa Fluor® 568 conjugated goat anti-mouse Ab to detect A2M; while the slides were stained by rabbit anti-rCP Ab and Alexa Fluor® 647 conjugated goat anti-rabbit Ab to detect CPs. The result showed that the clot fibrils developed around hemocyte causing the close association of the cells. Obviously, the *Pm*A2M (green color) was co-localized with the clottable proteins (blue color) shown in the merge field where appeared in cyan color (Figure 3.14).

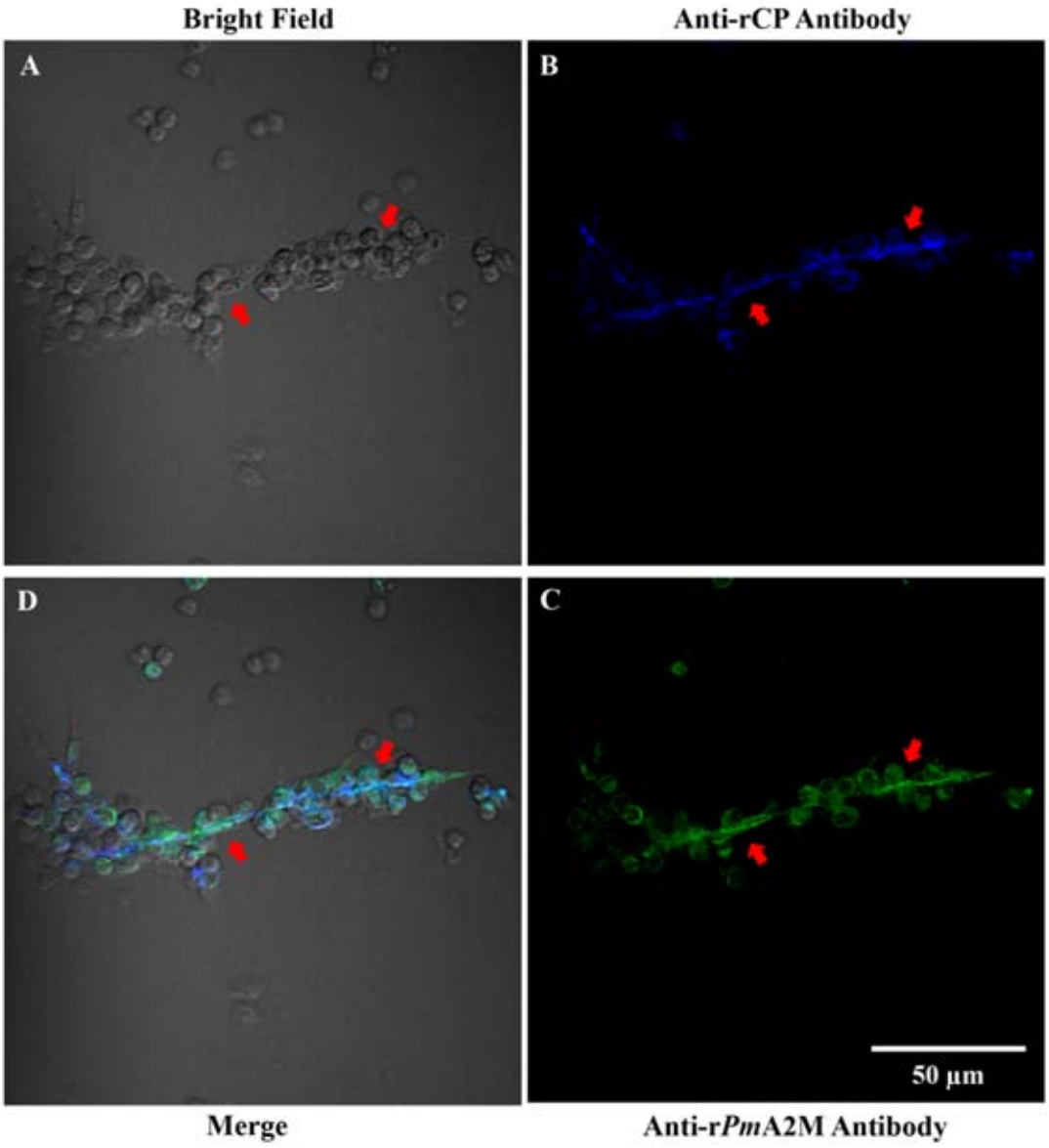

Figure 3.18 Immunofluorescense detection of *Pm*A2M and clottable proteins co-localized on the extracellular blood clots. *Pm*A2M and clottable proteins were probed with anti-r*Pm*A2M and anti-rClottable protein antibodies followed by incubation against Alexa Fluor® 568 (Green color) and Alexa Fluor® 647 (Blue clor) conjugated secondary antibodies, respectively. The co-localization of the proteins was demonstrated by cyan color in the merge field. Arrowheads indicate developing clot fibril. The scale bar corresponds to 50 µm and images are representative of 3 fields of view per sample.

3.16 Partially depleted *Pm*A2M transcript levels resulted in faster fibrinolysis in *V. harveyi* entrapping clots

In order to discover the biological importance of *Pm*A2M, *in vivo* gene silencing was carried on to deplete the expression level of *Pm*A2M mRNA in the shrimp. The semi-quantitative RT-PCR result presented that *Pm*A2M transcripts were significantly reduced approximately 94% after double injection of *Pm*A2M dsRNA compared to the control groups which are normal saline injected and GFP-dsRNA injected shrimp (Figure 3.19A).

The plasma clots of shrimp in the absence and in the presence of *V. harveyi* were prepared by the hanging drop technique and observed under Scanning electron microscope to evaluate the effect of the substantially decreased *Pm*A2M transcripts in the blood clotting system. In the absence of bacteria (Figure 3.19B, panel A and B), the appearance of the shrimp clot in the *Pm*A2M dsRNA injected shrimp appeared fragile compared to the GFP dsRNA injected shrimp, the control group. On the other hand, in the presence of the bacteria (Figure 3.19B, panel C and D), the shrimp clot was much more susceptibility to proteolysis, possibly from *V. harveyi* secreted enzymes as indicated by fibrinolysis surrounding the bacteria. There were, furthermore, some bacteria that had been already escaped from the blood clot, as pointed by arrows shown in Figure 3.19B, panel D. On the contrary, the clot matrix of GFP dsRNA knockdown shrimp still looked flawless and the clot fibril well immobilized the bacterial cell.

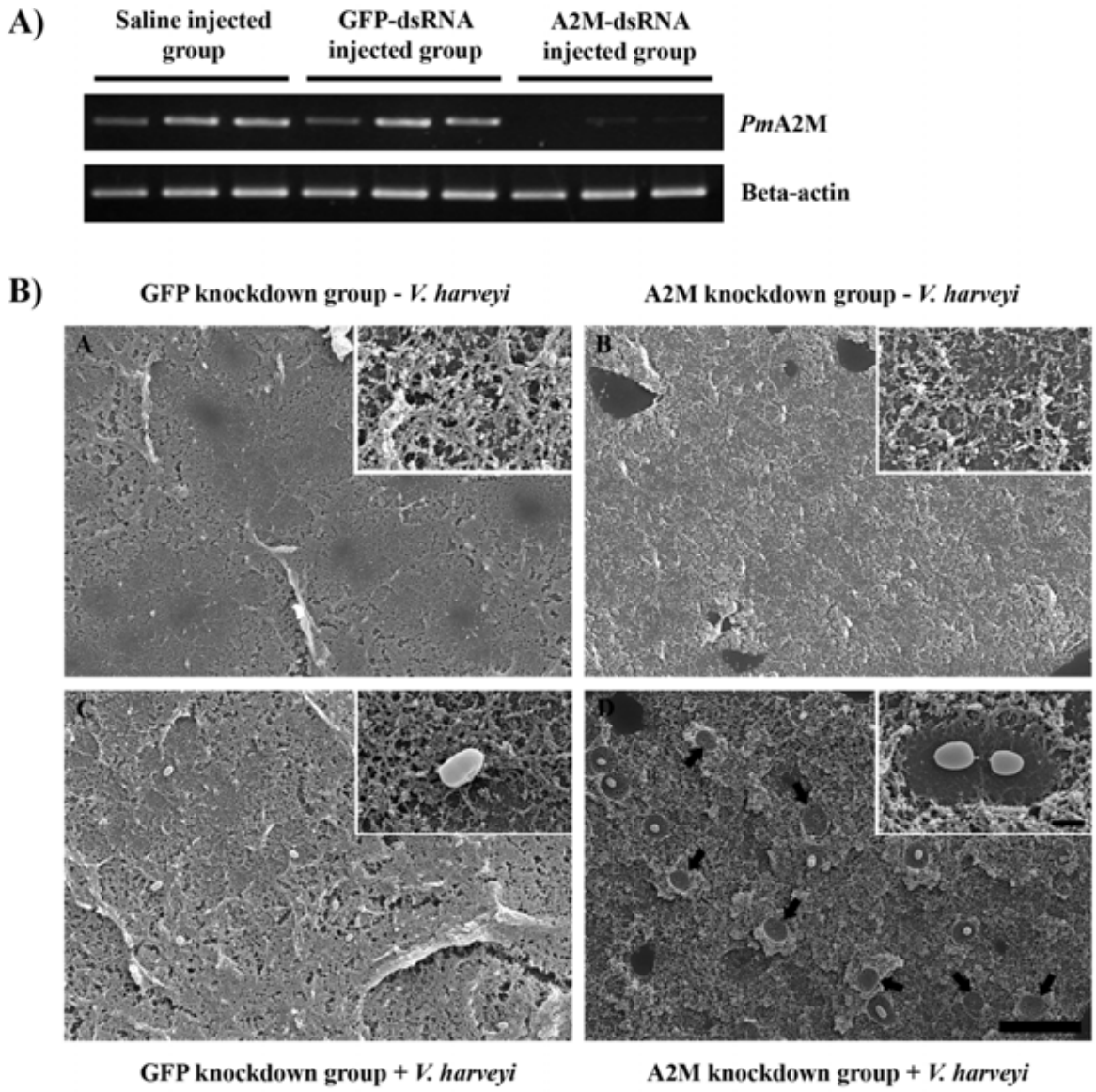

Figure 3.19 A) Semi-quantitative RT-PCR analysis of the *Pm*A2M trascript in hemocytes after dsRNA-mediated gene silencing. Experimental shrimp were divided into 3 groups; normal saline solution injected, 10 µg/g shrimp of GFP-dsRNA injected and 10 µg/g shrimp of *Pm*A2M-dsRNA injected group. Beta-actin was used as the internal control and each lane is the representative of an individual. B) Scanning electron microscopic observation of extracellular blood clots of shrimp. The accelerating voltage at 20 kV was used. Hanging drop preparation was used to prepare the blood clot in the absence (panel A and B) and in the presence (panel C and D) of *V. harveyi* from the plasma of (panel A and C) GFP-dsRNA or (panel B and D) A2M-dsRNA injected shrimp. Fibrinolysis areas that *V. harveyi* had already escaped were pointed by arrowheads. The main panels and the insets were magnified 2,000X

and 20,000X, respectively. Scale bars correspond to 10 µm in the panel and 1 µm in the insets. Images represents at least 3 fields of view per sample.

3.17 Systemic dissemination of *V. harveyi* in the circulatory system of *Pm*A2M gene depleted shrimp

To strengthen the previous finding concerning the *V. harveyi* escape from the blood clots of A2M depleted shrimp, the total amount of *V. harveyi* systemically disseminating in the circulating hemolymph was then determined. After *V. harveyi* injection at 5, 15 and 30 min, the plasma of GFP-dsRNA and A2M-dsRNA knockdown shrimp was aspirated. Strikingly, the result revealed that the number of invading bacteria in the A2M depleted group after 5 min of bacterial injection was 3.3 fold higher than that in the GFP-dsRNA injected group, the control group (Figure 3.20). However, disseminating *V. harveyi* was rapidly eliminated from the circulating hemolymph within 30 min in both groups.

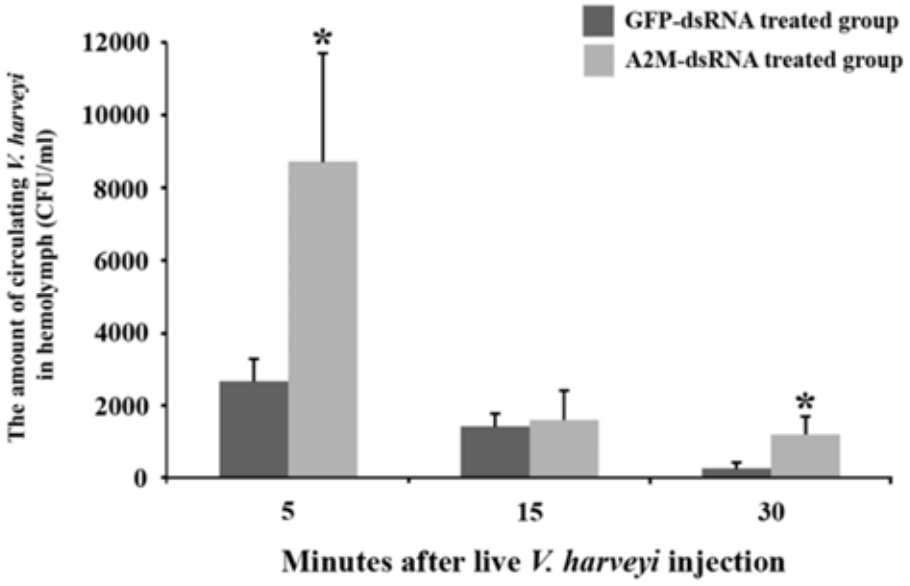

Figure 3.20 The total number of systemically disseminating *V. harveyi* in circulating hemolymph of the A2M dsRNA treated group compared to that of the GFP dsRNA treated group. Hemolymph of *V. harveyi*-injected shrimp was collected at 5, 15 and 30 after bacterial injection and later dotted onto selective media (TCBS). The viable bacteria on the plate were counted and presented as CFU/ml shown as the mean ± 1 S.D. The experiment was done

three replications. Asterisks represent a significant difference between the studied groups ($p < 0.05$).

3.18 Microscopic visualization of extracellular blood clots

To better understand the appearance of the blood clots either in the absence or in the presence of bacteria, histology of normal shrimp clots was next studied using microscopy with different fields; bright field, phase contrast field and differential interference contrast field (DIC). Comparison between those 3 different fields showed that the phase contrast field was the most suitable field used for visualization of the shrimp clot in the presence of bacteria. It was found that the bacterial cells and clot fibrils were well seen in the phase constrast field whereas granules within hemocytes were well observed in the DIC field (Figure 3.21). In both groups, the ruptured cells, which might be involved in the blood clotting system, were observed and, in the presence of the *V. harveyi*, the bacterial cells seemed immobilized in the fibril network.

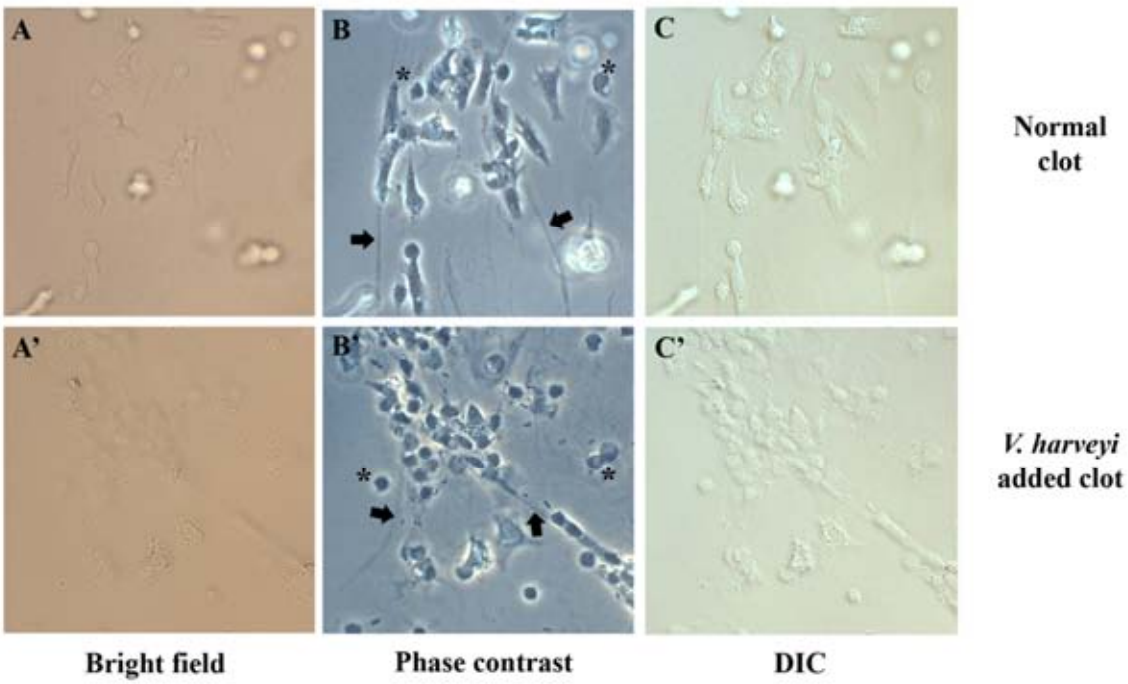

Figure 3.21 Microscopic analysis of shrimp blood clots using three different fields; bright (A, A'), phase contrast (B, B') and differential interference contrast field (DIC) (C, C'). The normal (A, B, C) and *V. harveyi* (A', B', C') added clots were prepared on coverslips by hanging drop technique and were later observed under the microscope. Arrowheads and asterisks indicate the clot fibril and the entrapped *V. harveyi* observed in the phase contrast microscope. Images shown represents at least 3 fields of view per sample.

3.19 Examination of bacterial entrapment efficiency of shrimp hemolymph clots

To investigate the efficiency of the blood clot in bacteria immobilization, the number of *V. harveyi* that remained in the fluid part of the clottable hemolymph was examined compared to that of the unclottable hemolymph. Ten µl of the log-phase *V. harveyi* in Shrimp saline solution (SSS) was added to 200 ul of shrimp plasma. After allowing the blood to completely form hemolymph clots for 30 min, ten µl of fluid parts (using needle to remove clots) was taken for bacterial counting using hemocytometer.

Figure 3.22 demonstrated that the number of *V. harveyi* remaining in Shrimp saline solution (SSS), unclottable plasma (55 °C heated plasma), and the fluid part of clottable plasma was 11.37, 10.97, and 1.72 million cells, respectively. The result revealed that 9.25 million cells of *V. harveyi* were sequestered during the clotting process. Therefore, the entrapment efficiency of the shrimp clot in the presence of bacteria compared to the unclottable plasma was about 84.32%.

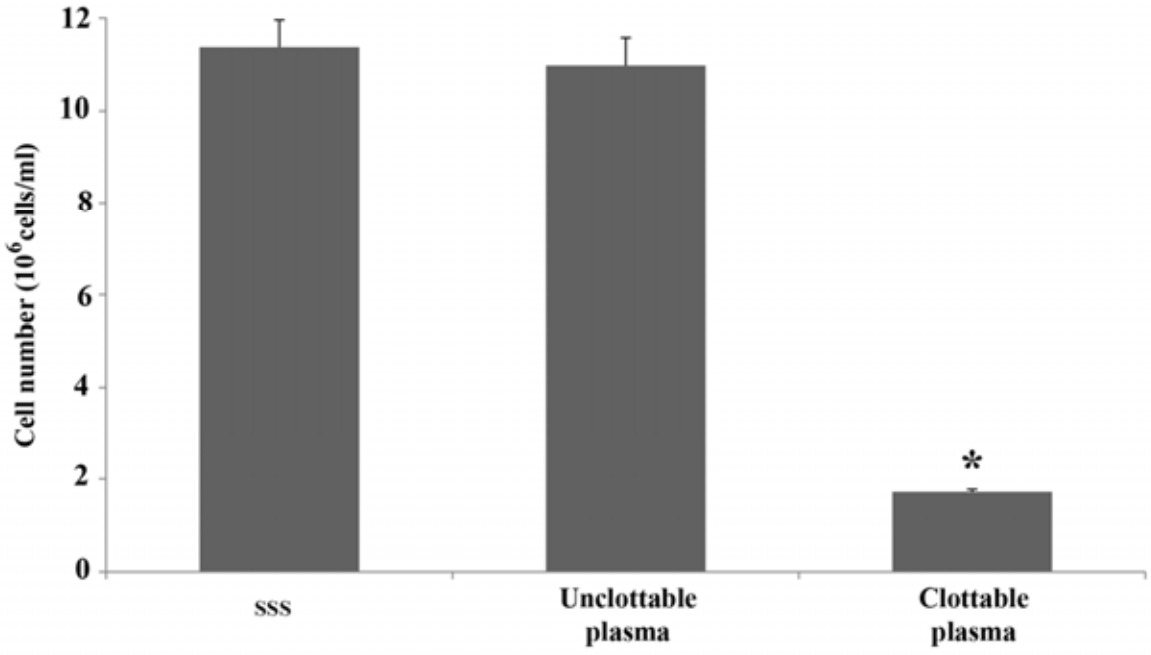

Figure 3.22 Determination of *V. harveyi* entrapment efficiency of blood clots. The freshly prepared *V. harveyi* (10 µl) was added to Shrimp saline solution (SSS), unclottable plasma (55 °C heated plasma), and clottable plasma. The hemolymph was allowed to clot for 30 min. Hemocytometer was used to count the number of the bacteria in the fluid part of all samples. Three trials were done in each group. An asterisk represents a significant difference among the experimantal groups ($p < 0.05$).

3.20 Analysis of fibrinolysis areas occurred in *V. harveyi* entrapping blood clots

Based on our previous result showing that *V. harveyi* was able to escape from the A2M depleted clot mediated fibrinolysis, presumably caused by proteases released from the bacterial cells, the occurrence of bacterial escape in the *V. harveyi* entrapping blood clots was then visualized by time-lapse phase contrast microscopy. Figure 3.23A showed that the bacteria entrapped within the shrimp clot were held firmly at 0 min. They appeared alive because the bacterial proliferation was obviously exhibited over the observed time. Apparently, the proliferating *V. harveyi* regained movement at 60 min and finally escaped from the fibrinolysis area within 90 min.

Calculated by Image J program, the fibrinolysis areas occurred in *V. harveyi* entrapping blood clots at 0, 30, 60, 90 and 120 min were 0.015, 0.104, 0.486, 0.926, and 1.448 mm^2, respectively. Total area in the observed field is 7.237 mm^2. The graph presented

that the fibrinolysis area slightly increased for 0.21% within 30 min and gradually went for up to 1.44% at 1 h, followed by exponential increase for 20% at 2 h (Figure 3.23B). In fact, the graph showed that the longer time, the bigger fibrinolysis areas.

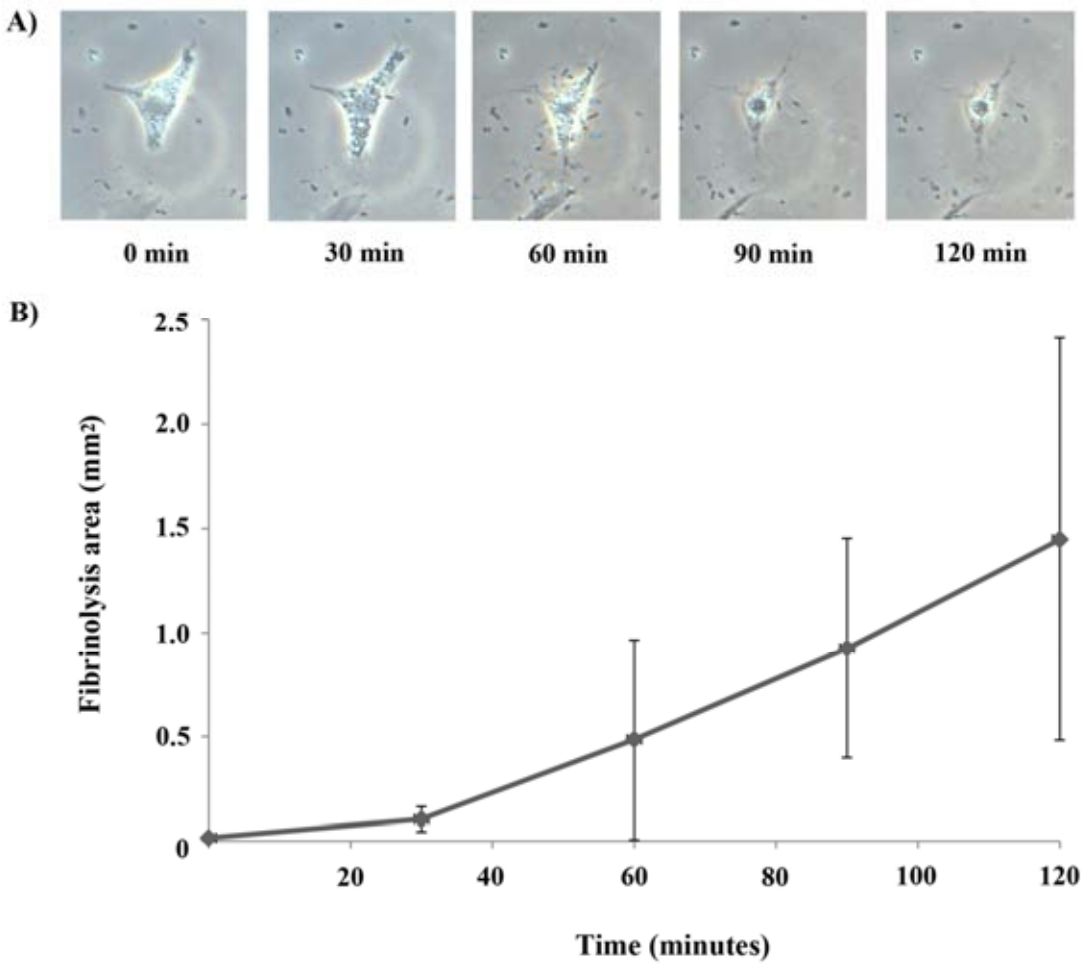

Figure 3.23 (A) Time-lapse microscopic visualization of *V. harveyi* entrapping blood clots at 0, 30, 60, 90 and 120 min. The hemolymph clot in the presence of *V. harveyi* was prepared on a coverslip and observed under the phase contrast microscope. Images represents at least 3 fields of view per sample. (B) Fibrinolysis zones occurred in the *V. harveyi* entrapping clots within 2 hours. The areas of fibrinolysis were calculated by Image J program based on the area within which individual bacterium could move. The areas were 0.015, 0.104, 0.486, 0.926, and 1.448 mm^2 at 0, 30, 60, 90 and 120 min, respectively. Total area in the observed field is 7.237 mm^2. The experiment was done tripicate and the data were shown as the mean ± 1 S.D.

3.21 Identification of *V.harveyi* secreted fibrinolytic enzymes

V.harveyi secreted fibrinolytic enzymes were then identified by examination the percentage of the bacterial movement in the shrimp clot at 2 h in the presence of various kinds of protease inhibitors such as AEBSF (Serine protease inhibitor), E64 (Cysteine protease inhibitor), and pepstatin (Aspartyl protease inhibitor). The result demonstrated that the percentage of moving *V.harveyi* was significantly decreased to 5.36% and 7.11% in the presence of protease inhibitor mix called HALT or with AEBSF, respectively (Figure 3.24). Therefore, the result ascertained that *V. harveyi* uses proteases mediating fibrinolysis and this fibrinolytic enzyme is catagorized in the serine protease group.

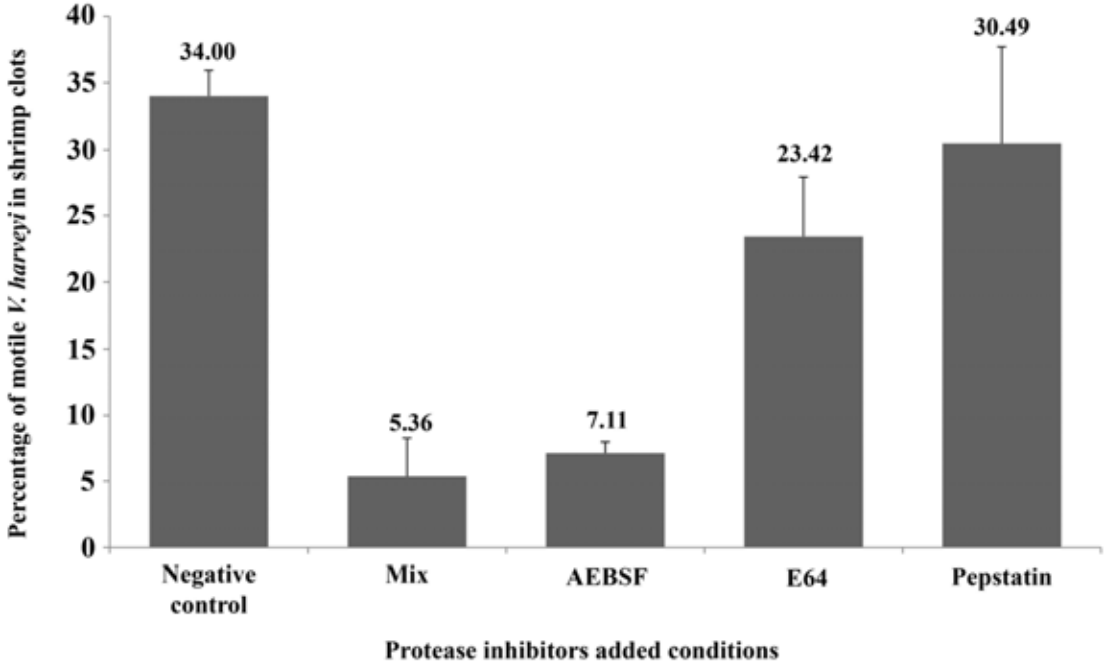

Figure 3.24 Percentage of motile *V. harveyi* in the hemolymph clot at 2 hours in the conditions supplemented with various protease inhibitors. The hemolymph in the presence of *V. harveyi* was allowed to clot on the coverslip and then incubated in the moist chamber for 2 hours. The bacterial movement in the clot was counted by looking at the difference of bacteria position in the figures taken by normal exposure time and long exposure time in the same field. Negative control was the normal *V. harveyi* added clot and MIX was a protease inhibitor mix, called the Halt, which composes of AEBSF (Serine protease inhibitor), E64 (Cysteine protease inhibitor), pepstatin (Aspartyl protease inhibitor), leupeptin (Broad spectrum protease inhibitor), aprotinin (Plasmin inhibitor), and bestatin (Amino protease inhibitor).

The three replications were conducted per group and the data were reported as the mean ± 1 S.D.

A parallel experiment, which determined the effect of various protease inhibitors on the fibrinolytic activity of *V. harveyi*-conditioned media, prepared from *V. harveyi* overnight culture. The shrimp clots were prepared on cover slip by spreading and dropping. The plasma allowed to clot for 30 min and the samples were covered by *V. harveyi* conditioned media with either lacking or adding protease inhibitors.

This result revealed that the fibrinolytic activity of *V. harveyi* conditioned media was completely inhibited when adding 1,10 Phenanthroline (Metallo-protease inhibitor) or AEBSF (Serine protease inhibitor) (Figure 3.25A). Otherwise, the clot was apparently digested in the positive control, which was bacterial conditioned culture with no adding inhibitors. Control levels of fibrinolysis were seen in the presence of E64, a cysteine protease inhibitor, and pepstatin, an aspartyl protease inhibitor. This suggested that both metallo-proteases and serine proteases released by the bacteria are required for the fibrinolysis.

Moreover, the appearance of the digested clots were visualized under the phase contrast microscope with 400 magnification. It was found that the *V. harveyi* conditioned media exhibited the hemocyte lysis activity indicating that there are some cytolytic substances secreted by the bacteria in the media. Hemocyte cytolysis may be involved in the pathogenicity of this bacteria (Figure 3.25B).

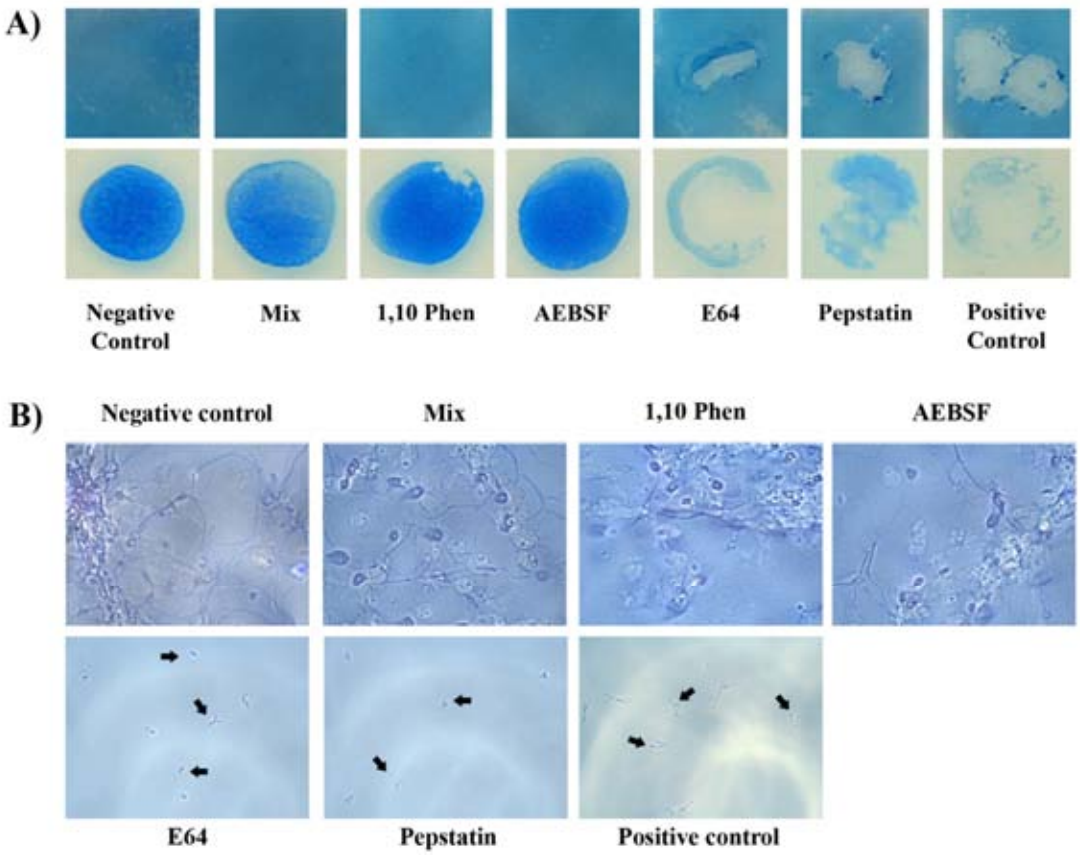

Figure 3.25 (A) Verification of the fibrinolytic activity of *V. harveyi* conditioned media in the presence of various protease inhibitors; MIX (Halt supplemented with 1,10 phenanthroline), 1,10 phenanthroline (Metallo-protease inhibitor), AEBSF (Serine protease inhibitor), E64 (Cysteine protease inhibitor), and pepstatin (Aspartyl protease inhibitor)**.** The clots were prepared on coverslips. After covered the clots by *V. haveyi* conditioned media with either no adding protease inhibitors or adding protease inhibitors, the samples were incubated in moist chamber at 37 °c for 3 hours. Negative control was sterile media and positive control was *V. harveyi* conditioned media with no adding inhitors. The images shown are representative of three independent results. (B) Microscopic analysis of shrimp clots after incubated for 3 hours with *V. haveyi* conditioned media with either no adding protease inhibitors or adding different protease inhibitors. The blood clots were stained by GelCode Blue Stain Reagent (Pierce) and observed under phase contrast microscope with magnification at 400X. Images shown are representative of three independent results.

CHAPTER IV
DISCUSSION

Up to date, the proteomic approach was extensively applied to investigate cellular changes in several aspects. The common techniques used consist of two-dimensional electrophoresis (2-DGE) and mass spectroscopy (MS), which enable us to identify the responsive proteins. Due to the usefulness of this approach, immune responses of the black tiger shrimp, *P. monodon*, against *V. harveyi*, a devastating bacterium, are consequently investigated.

Here, we examined the protein expression profiling of the lymphoid organ of *V. harveyi* infected *P. monodon* at 6 and 48 hpi (Chaikeeratisak et al., 2012). Highly altered proteins after infection with more than 3 fold were selected and sequenced. According to protein expression profile results, 21 out of total 30 eligible protein spots were successfully identified whilst the remaining proteins were still unknown. Some examined proteins remained anonymous owing to information lacking of shrimp genome sequences. The known proteins were varied in biological functions such as metabolism and biogenesis, immune related proteins, stress response, cell signaling, chaperone function, serine protease inhibitor and transcriptional regulator, but mainly involved in cellular structure.

According to the 2-DGE result, after the *V. harveyi* infection, significant down regulations in protein expression level of several cytoskeleton proteins involved in cellular structure such as beta-tubulin (spot no. 10 and 12 observed at 6 hpi), beta-actin (spot no. 9 observed at 48 hpi), actin (spot no. 13 observed at 6 hpi), and actin 2 (spot no. 5 observed at 6 hpi) were prominently observed. The down regulation of the cytoskeleton proteins, especially beta-actin and actin, effects the cellular structure and results in destabilization of the microfilament system in the cells (Guenal et al., 1997). The type III secretion system (T3SS), which is a pathological mechanism for bacterial dissemination, can trigger apoptosis in the host cell (Grassmé et al., 2001). Also, the T3SS was found as a pathological mechanism of *V. cholerae* for penetrating the host cells by affecting the host cytoskeleton protein (Tam et al., 2007). It was previously noticed that depolymerization of microtubules, which are polymers of tubulins, is rapidly occurred at the early phase of programmed cell death (Moss et al., 2006). These alteration of cytoskeleton component can then affect the cellular stability finally mediating programmed cell death. Therefore, the substantial change in cytoskeleton protein's

expression level observed in the lymphoid organ suggested that the apoptosis in the organ was triggered. Moreover, an increase of cytoplasmic calcium concentration serves an important role in pathological conditions through apoptosis (Nicotera et al., 1992). Calreticulin, which resides within the endoplasmic reticulum (ER), can regulate the calcium (Ca^{2+}) homeostasis by binding to the Ca^{2+} via its Ca^{2+} binding domains and by acting as a cargo transferring Ca^{2+} between cytoplasm and the ER (Luana et al., 2007). Interestingly, our proteomic result showed the down-regulated calreticulin protein expression level (spot no. 2 observed at 6 hpi). The result implied that the increase of intracellular calcium concentration in cytoplasm possibly resulted in apoptosis of the cell. This assumption was well corresponded to the high cumulative mortality of the *V. harveyi* challenged shrimp at 6 hpi which often reached about 50% at 6 hpi observed in the experiment. Taken together, it is possible that the shrimp death at the early phase of *V. harveyi* infection caused by the bacterial pathogenicity in order to widespread disseminate into the host system via programmed cell death. However, one of the structural protein family, cytoplasmic actin Cy II (spot no. 11 observed at 48 hpi), was found to be up-regulated at the late phase of bacterial infection. This suggested that the up regulation of this protein might facilitate recovering of the cellular structure in the lymphoid organ of those shrimp that still survived.

Another interesting shrimp immune-related protein observed after the bacterial infection was 14-3-3 like protein (spot no. 8 observed at 48 hpi). The 14-3-3 like protein, which involves in many cellular processes such as apoptosis, stress response, signal transduction, and cell cycle, can regulate a targeted protein through an interaction between its binding grooves and the specific phosphorylated sites of the targeted protein. The binding contributes to a conformational change of the targeted protein resulting in either activation or inactivation of the protein functions (Mackintosh, 2004; van Hemert et al., 2001). The down-regulation in transcriptional level of the 14-3-3 like protein was also determined and it seemed broadly correlated with the down-regulation in its translational level observed in the proteomic result. Additionally, the transcriptional expression level of the 14-3-3 like protein was also found to be down-regulated after yellow head virus infection in the black tiger shrimp, *P. monodon* (Pongsomboon et al., 2008). Therefore, the change of the 14-3-3 like protein might directly or indirectly participate in the shrimp immunity in response to the bacterial infection.

One mechanism to survive in the severe condition of bacteria infected hosts is to depress key metabolic pathways, in particular glycolysis pathway (Michael, 2004). As shown

in the proteomic study, triosephosphate isomerase (spot no. 16 observed at 6 hpi) which is an enzyme serving important roles in the glycolysis pathway and energy production was found to be down-regulated upon the *V. harveyi* infection. This change might cause the decrease in energy production through glycolysis. Not only affecting the energy production, the bacterial infections also disrupt the oxygen consumption. These studies also exhibited the down regulation of cytochrome oxidase subunit I (spot no. 10 observed at 48 hpi) which is a key enzyme in aerobic metabolism. Due to these circumstances, lactic acid was later accumulated leading to anaerobic metabolism to compensate the energy imbalance (Scholnick et al., 2006).

Nevertheless, ATP synthase beta subunit (spot no. 6 observed at 6 hpi), another key enzyme involved in aerobic respiration, was found to be the highest up-regulated protein among other immune responsive proteins after the *V. harveyi* infection. As studied at transcriptional level, a slight but significant increase of the ATP synthase beta subunit mRNA expression seemed correlated to the increased translational level observed in the proteomic result. This indicates that the response to *V. harveyi* infection (at least to this level of a systemic infection) was mainly controlled at the translational / posttranslational level. Even though the western blot analysis appeared not well corresponded with the proteomic results due to the delay response of the ATP synthase beta subunit to the bacterial infection, the up-regulation of this protein in the lymphoid organ after *V. harveyi*-challenge was still confirmed. Possible explanations of the alteration of ATP synthase beta subunit expression level are that the protein was more expressed in order to balance the energy losses occurred during the bacterial infection or the protein might exhibit any other biological functions related to the innate immunity against bacterial infection. However, the up-regulation of ATP synthase beta subunit in shrimp in response to *V. harveyi* observed here suggested the likely important function of the protein at the early phase of the infection.

The ATP synthase beta subunit has previously been identified as a highly altered protein expression level in response to various severe shrimp pathogens (Bourchookarn et al., 2008; Chongsatja et al., 2007; Somboonwiwat et al., 2010; Wang et al., 2007; Zhang et al., 2010). In WSSV infected shrimp, the up-regulation of proteins related to the energy production are probably important for the infected cells to meet the requirement of energy during rapid virus replication and it might be also essential for several energy-dependent biological processes that facilitate cell survival. Recently, there is a study reporting that the

binding of WSSV to ATP synthase beta subunit and showed that the recombinant protein could attenuate WSSV infection in the shrimp *L. vannamei* (Liang et al., 2010).

Residing in the inner membrane of mitochondria, the membrane of chloroplast thylakoid, and the cell membrane of some bacteria, ATP synthase is an enzyme complex which composes of 2 main parts; the F_1 sector (a soluble portion) and the F_0 sector (a membrane bound portion). The F_1 sector contains a catalyzing site for ATP hydrolysis and ATP formation. This sector comprised of large portions of 3 subunits of each alpha and beta and a variety of different subunits. Even though it is widely known that ATP synthase serves necessary roles in ATP production of the cell, some extra functions of the ATP synthase have just been revealed (Devenish et al., 2008). Other biological functions as well as locations of the ATP synthase have been regarded as the external ATP synthase (eAS). The eAS locates on the surface of many cell types which act as cell-surface receptors and the organization of its subunit might be varied depending on cell types and their specific biological functions (Champagne et al., 2006; Chi and Pizzo, 2006).

The deduced amino acid sequence of contig 494 (CT494) from the *P. monodon* EST database had three well conserved regions; nucleotide binding domains, DELSEED motif and the ATPase alpha/beta signature domain, in accord with the encoding of this *Pm*ATP synthase beta subunit. The nucleotide binding domain consisted of two important domains, motif A (GXXXXGKT/S) and motif B (LLFID), that serve a crucial role in ATP synthesis and hydrolysis (Wiese et al., 2006). According to the crystal structure studies, motif A forms a loop structure (the P-loop) around nucleotides. Utilizing lysine (K) and threonine (T) residues inside the motif, the loop interacts with phosphate-oxygen atoms of the nucleotide (Ramakrishnan et al., 2002). The structure is stabilized by hydrogen bonds formed between motif A and a highly conserved aspartate (D) residue within motif B with a bound water molecule (Wiese et al., 2006).

At the carboxyl terminal domain of the beta subunit, the DELSEED motif was observed. This motif is highly conserved among crustaceans and is supposed to be responsible for binding to the gamma subunit (γ) causing the mechanical rotation of F_1 portion synthesizing ATP. However, several recent studies suggest that the clusters of acidic and negatively charged amino acids within the DELSEED motif allow itself to form a bridge with the epsilon subunit (ε) making the enzyme inactive (Hara et al., 2000; Noji et al., 1997). When the bridge is broken, due to ATP binding inside the beta subunit (β), the enzyme then

becomes active (Hara et al., 2001). The ATPase alpha/beta signature sequences (P-S/A/P-L/I/V-D/N/Hx-{F}-{S}-S-x–S) are also found at the carboxyl-terminus (Liu et al., 2007).

Despite ATP synthase beta subunit mRNA expression normally found in all examined tissues, its mRNA was found to be mainly expressed in antennal gland, gill, heart, hemocyte, stomach and hematopoietic tissue in normal shrimp. To further elucidate the function of ATP synthase beta subunit transcripts, its transcripts were knocked down. The mass mortality of ATP synthase beta subunit knockdown shrimp was apparently observed within 24 hours. This supported that ATP synthase beta subunit is important for shrimp survival independent of any role or not in responses to pathogen infections.

In the fresh water crayfish, *P. leniusculus*, ATP synthase beta subunit was identified as a receptor for the cytokine astakine 1 and localized on the plasma membrane of hematopoietic tissue (Lin et al., 2009). Furthermore, the injection of *P. monodon* astakine, which is phylogenetically related to crayfish astakine 1, is able to activate cell proliferation of the hematopoietic tissue (Hsiao and Song, 2010). Therefore, the ATP synthase beta subunit perhaps plays essential roles in the hemocyte production in the black tiger shrimp as well because the silencing of ATP synthase beta subunit mRNA causes a considerable decrease in the total number of hemocytes, probably resulted in shrimp death. Taken together, gene suppression revealed that the presence of ATP synthase beta subunit was important to shrimp survival that might be due to its essential function in energy production as well as its possible involvement in hemocyte production.

Another immune responsive proteins observed during the *V. harveyi* infection period was alpha-2-macroglobulin (A2M) (spot no. 9 observed at 6 hpi). It was found that the A2M protein expression level was highly down-regulated but its transcript expression level continuously and considerably went up over the period of bacterial infection and reached maximum level at 48 hpi. This result suggests that the A2M protein content in the lymphoid organ might be reduced during the infection.

Of those important immune responsive proteins, A2M was always found to be highly altered after pathogenic infections. So far, there have been many other studies reporting the responses of A2M expression in major immune related tissues in response to various severe shrimp's pathogens. When shrimp was challenged with viruses such as white spot syndrome (WSSV) and yellow head viruses (YHV), the change of A2M expression level was noticed at transcriptional level but not at translational level (Bourchookarn et al., 2008;

Pongsomboon et al., 2011; Rojtinnakorn et al., 2002; Wang et al., 2007). Otherwise, the considerable alteration in both levels of the A2M was obviously seen after a bacterial challenge, especially *V. harveyi* (Ma et al., 2010; Somboonwiwat et al., 2010) . For example, according to the proteomics study of differentially expressed proteins in the *V. harveyi*-challenged *P. monodon*, the A2M protein expression level was the highest down-regulated among other immune related proteins observed (Somboonwiwat et al., 2010).

Alpha-2-macroglobulin (A2M), which is a wide spectrum-protease inhibitor, mainly acts as a controller of exogenous proteases that are virulence factors released from invading pathogens, and endogenous proteases that are mainly involved in the host immune system (Armstrong and Quigley, 1999). Gollas-Galván et al. (2003) recently revealed that shrimp A2M exhibits the protease inhibitory activities to many different proteases which are representative of bacterial secreted proteases, such as aminopeptidase and elastase (Gollas-Galván et al., 2003). Thus, it is possible that the considerable change verified in A2M expression is responsible for the host immune defense against bacteria-released proteases. However, the biological pathways, in which A2M involves, to defend those bacterial proteases in shrimp remain unclear.

In order to gain more insights concerning the immune defense mechanisms of A2M upon the *V. harveyi* infection, the interacting protein partners of *Pm*A2M were then examined using Y2H assay. According to the assay, the RBD of *Pm*A2M was found to bind with the carboxyl-terminus of transglutaminase type II of *P. monodon* (*Pm*STG II). To date, there have been at least 3 types belonging to the transglutaminase enzyme family identified in *P. monodon* which are transglutaminase type I (AY074924) (Huang et al., 2004), transglutaminase type II (AY771615) (Chen et al., 2005), and transglutaminase type II (AF469484) (Yeh et al., 2006). However, the amino acid sequences of the C-terminus of both *Pm*STG II (AY771615 and AF469484) were totally alike; hence, either of which *Pm*STG II or both are an interacting partner of *Pm*A2M. Since both *Pm*STG IIs were determined as a key enzyme involving in the blood coagulation system (Chen et al., 2005; Yeh et al., 2006), the interaction between *Pm*A2M and *Pm*STG II suggested the possible roles related to the hemolymph clotting. According to the polymerization activity of transglutaminase that activates the acryl group-transfer reaction between glutamines (Gln) and lysine (Lys) residues, not only four conserved lysine residues (Lys 1324, Lys 1387, Lys 1421, and Lys 1425) were discovered in the RBD of *Pm*A2M (Lin et al., 2007) but also deduced transglutaminase cross-linking glutamine residues were identified in human A2M

(Gln 670 and Gln 671) (Sottrup-Jensen et al., 1984). A2M of crayfish, *P. leniusculus*, which is examined as a substrate of the tranglutaminase enzyme, also exposes free glutamine and lysine residues. Moreover, A2M of American horseshoe crab, *Limulus polyphemus*, is found to decorate in the plasma clot in the presence of calcium. (Armstrong and Armstrong, 2003; Hall and Söderhäll, 1994; Theopold et al., 2004). Therefore, it is possible that the *Pm*A2M also acts as a substrate of the tranglutaminase enzyme, which can be cross-linked with other clotting related proteins, and plays crucial roles in the blood coagulation system in the black tiger shrimp. As extensively studied in the mammal, the RBD of the mammalian A2M can interact with the LRP receptor-associated protein (RAP) involved in the signal transduction and the low-density lipoprotein receptor-related protein A2M receptor (LRP-A2MR), which the binding of these proteins involves in the elimination of inhibitor–protease complexes from the host's blood circulatory system (Armstrong, 2010). Yet, until now, there are no reports on the invertebrate's A2M regarding to these receptors. We assumed that the RBD of *Pm*A2M does not contain a receptor binding region called the KPTVK motif (Lin et al., 2002); therefore, shrimp occupies other immune pathways to eliminate the inhibitor–protease complexes from the blood circulation.

The blood clotting in invertebrates typically processes in plasma by incorporation of several hemocyte released molecules without any direct participation of hemocytes (Cerenius et al., 2011). The hemolymph clotting system in shrimp and crayfish, *P. leniusculus*, is proposed to be alike (Chen et al., 2005). A key clotting enzyme, transglutaminase, is released into the hemolymph via cytolysis of hyaline hemocytes and then catalyzes the plasma clotting by cross-linking clottable proteins (CPs) which are an abundant protein in hemolymph, mainly produced in gills, the central nervous system, and the lymphoid organ. (Vargas-Albores F. and Jiménez-Vega, 1998; Yeh et al., 2007). Our results presented that *Pm*A2M was an early responsive protein to the bacteria, and was also secreted from semi-granular hemocytes into plasma at the period of the bacterial infection from 6 to 48 hours. This is the first report in shrimp that clearly demonstrate the involvement of *Pm*A2M in the coagulation and the hemocyte released A2M was incorporated onto the clots serving as one of the major backbones on the clot matrix as well as the CPs. However, the A2M contents in plasma were not detected as much as we expected at the early phase of *V. harveyi* infection. In the American horseshoe crab, methylamine-reacted A2M was found to be rapidly cleared out from the blood circulatory system within 30 min (Melchior et al., 1995). This matter might

imply that bacterial protease-inhibitor complexes were instantly eliminated from the clots or the hemolymph via some clearance pathways in shrimp.

To better understand the biological involvement of *Pm*A2M in the hemolymph coagulation, the effect of A2M transcript knockdown on bacterial sequestration in the plasma clots was then investigated using Scanning electron microscope (SEM). The partially A2M-depleted clots appeared fragile observed in their physical structure and susceptible to the proteases secreted from *V. harveyi*. Moreover, our result demonstrated the inefficiency of the partially A2M-depleted clots in sequestering the invading *V. harveyi* resulting in the faster bacterial dissemination into the blood circulation. Therefore, this revealed the first direct evidence on the biological function of *Pm*A2M in shrimp immunity indicating that A2M decorated on the shrimp clots serves a potential role in inhibition of *V. harveyi* secreted proteases causing timely fibrinolysis on the shrimp clots; thus, the protease-mediated bacterial escape was retarded.

In general, the molecular contraction of A2M (the physical entrapment mechanism) consists of 3 major steps. The first step is called the protease recognition; a protease digests one or more of the peptide bonds of the bait region, which activates the molecular compaction of the molecule to immobilize the protease. The second step is named the capture step; the thiol group within the A2M molecule is then cleaved resulted in covalent linking between the reactive glutamyl group and the protease (mammalian A2M) or to cross-link the chains of A2M itself (Limulus A2M). The final step is that the RBD which is exposed to the outside of the A2M molecule due to the conformation change is then interacted with its surface receptor mediating the endocytotic clearance pathway (Armstrong, 2010; Armstrong, 2001). Based on the process of molecular compaction of A2M, it appears that only a thiol-reacted A2M, in which the RBD is exposed, will be next recognized and cleared out of the circulatory system whereas the unreacted A2M is immune from the clearance pathway and remains in solution. As previously studied, the clot decorated A2M in the American horseshoe crab, *L. polyphemus,* was tested whether it exhibited the protease inhibitory activities or not, but the result showed that A2M on the fibrils appeared as a reacted form (Armstrong and Armstrong, 2003). Otherwise, *Pm*A2M decorated on the shrimp plasma clots was still able to inhibit the proteolysis activity of *V. harveyi* released proteases. This suggested the exposure of the *Pm*A2M's RBD linked on shrimp clots before entrapping any proteases. It is interesting that the molecular structure reorganization of *Pm*A2M is possibly different from other isoforms or A2Ms in other species and so further investigations

concerning this matter are yet needed. In fact, our finding clearly uncovers an essential role of *Pm*A2M in the blood clotting system, which is the most proficiently fundamental immune defense against *V. harveyi* invasion, by retarding the fibrinolysis mediated by bacterial released proteases.

Our former studies lead us to be mainly interested in the blood coagulation system of shrimp and in the pathogenic mechanisms of the bacteria to escape from being entrapped by the host clotting system. So far, little has been known about the shrimp clotting system in the presence of bacteria, therefore, microscopy based techniques, which have been mostly applied to study the clotting appearance of insects (Bidla et al., 2007; Bidla et al., 2005; Grégoire, 1951; Ratcliffe and Gagen, 1977; Rowley and Ratcliffe, 1976), was then firstly conducted for studying the shrimp clotting system as well as the escape of entrapped *V. harveyi*.

Open circulatory system is one of the most apparent characteristics of invertebrates. After getting injured, the hemolymph clotting instantaneously begins to seal the wounds in order to prevent blood losses and to trap any invading pathogens from entering and disseminating throughout the circulation (Dushay, 2009). In shrimp, the plasma clotting system potentially entraps the invading *V. harveyi* up to 84.32% and this clotting process homogeneously occurs in the whole collected plasma resulting in a lump of clotted hemolymph (unpublished data). We assume that the bacteria that are not immobilized within the clots and left in a fluid part might be subsequently eliminated by other immune defenses. Otherwise, observed in the American horseshoe crab, *L. polyphemus*, the process is distinct from shrimp. The clot fibrils of bacteria infected hemolymph rapidly contracts itself to form a smaller nodule, possibly entrapping blood cells (amebocytes) and the bacteria inside, and this compression causes a remaining fluid part underneath the clot (Conrad et al., 2006). Seen in the *V.harveyi* entrapping clots using phase contrast microscopy, ruptured hyaline hemocytes release the transglutaminase enzyme and other coagulating materials in a circular coagulation island to originate the blood clotting (Grégoire, 1951; Omori et al., 1989; Vargas-Albores F. and Jiménez-Vega, 1998). The meshy clot fibrils throughly develop resulting in the close association of many cell types of hemocytes and invading bacteria. The granules containing immune substances are discharged from those bacteria-surrounding hemocytes suggesting the shrimp immune responses against the bacterial infection. Taken together with our previous study showing that the *Pm*A2M is localized in shrimp semi-granular hemocytes, it seems that *Pm*A2M is also released during the clotting and the infections. Likewise, degranulation of

amebocytes in the American horseshoe crab, *L. polyphemus*, and exocytosis of granules containing hemocytes in the Freshwater crayfish, *P. leniusculus*, are intensely triggered in response to bacterial endotoxins (Armstrong and Rickles, 1982; Johansson and Söderhäll, 1985). This suggests the secretion of A2M in both species in response to the endotoxins as well (Armstrong et al., 1990; Liang et al., 1992). In fact, the hemolymph coagulation system of shrimp is a primarily effective immune defense which facilitates the better cooperation of humoral and cellular factors to obstruct the pathogen invasions before entering into their circulatory system.

To gain more knowledge concerning the *V. harveyi* escape from the plasma coagulation system, the time-course observation of *V. harveyi* entrapped in shrimp clots was revealed here for the first time. The bacteria immobilized within the clots were initially held firmly indicating that the clot fibrils closely attach to the bacterial cells, but within 30 min, many bacteria regained movement and were swimming within little free spaces suggesting that fibrils surrounding the bacterial cells had been destroyed. We speculate that the most efficient period of shrimp clots to entrap the *V. harveyi* is no longer than half an hour and fully active A2M ,which is linked on the clot, perhaps inhibits the fibrinolytic enzymes secreted from those entrapped bacteria. Although some dead bacteria were seen, most of them remained alive and continue to proliferate followed by escape. In accord with this observation, bacteria caught within the hemolymph clots of the moth, *Galleria mellonella*, and the fruit fly, *Drosophila melanogaster*, are also viable; even though, the prophenoloxidase system and melanization has already functioned in the bacteria-associating clots (Bidla et al., 2005; Ratcliffe and Gagen, 1977). This suggests that not only the prophenoloxidase system and melanization but also a variety of host immune defense mechanisms might be involved in the bacterial killing process. Furthermore, the fibrinolysis areas predicted by the moving *V. harveyi* were exponentially and stably increased after an hour of bacterial entrapment. It is possible that A2M linked on the clots becomes saturated by *V. harveyi* secreted enzymes and thereby fibrinolytic enzymes can better digest fibril proteins.

The escape of *V. harveyi* from the shrimp clot was protease-mediated. Either 1,10-phenanthroline or AEBSF inhibited the escape, suggesting that both metallo-proteases and serine proteases released from the entrapped bacteria were required for substantial fibrinolysis. To date, several extracellular proteases belonging to *V. harveyi* have been identified as follows; a 22 kDa cysteine protease, a major exotoxin, (Liu et al., 1997; Liu and Lee, 1999) and three alkaline metal-chelator-sensitive proteases (Fukasawa and Osumi, 1988;

Fukasawa, 1988). Nevertheless, there is only a 35 kDa novel zinc metalloprotease, named Pap6, exhibiting specific protease activity against a broad range of host extracellular matrix, particularly fibrinogen, which is typically converted to fibrin acting as a monomer to form blood clots in mammals (Blomback et al., 1978; Teo et al., 2003). Metalloproteases, vEP and vEP-MO6, which are produced from *V. vulnificus*, a bacterium causing diarrhea, also show a broad spectrum of proteolytic activities to clotting-associated proteins (Chang et al., 2005; Kwon et al., 2007). Although extracellular serine proteases of *V. harveyi* have not been well studied, a 50 kDa novel alkaline serine protease, named AKP-Vm, has most recently been discovered as an extracellular toxin of *V. metschnikovii*, a bacterium causing pneumoni. Interestingly, the AKP-Vm serine protease displays strong fibrinolytic activities to several blood coagulation-associated proteins (Park et al., 2011). Altogether, this ascertains that metallo-proteases and serine proteases of *V. harveyi* determined in our study are necessary for its escape from the shrimp clotting process via fibrinolysis. Further investigations are yet needed to identify which of those fibrinolytic enzymes are specifically responsible for proteolysis of the extracellular blood clots. Also, enzyme kinetic as well as protein-protein interactions between A2M and the fibrinolytic enzymes should be verified. The finding in our research will definitely fulfill and extend knowledge based on the shrimp immune defense, especially the blood coagulation system, and pathogen interactions.

CHAPTER V

CONCLUSIONS

1. Proteomic analysis has successfully identify the differentially expressed proteins in the lymphoid organ of *V. harveyi* infected *P. monodon* and some of the substantially altered proteins were also examined their expression at the transcriptional level using real-time RT-PCR.
2. Two *V. harveyi* responsive proteins, *Pm*ATP synthase beta subunit and *Pm*A2M, were selected for further characterization.
3. *Pm*ATP synthase beta subunit transcribed in many shrimp tissues probably serves important roles in the hemocyte production as well as shrimp survival in the black tiger shrimp, *P. monodon*.
4. *Pm*A2M interacted with structural elements of the extracellular blood clots by transglutaminase type II activity and so involved in the blood coagulation system of shrimp.
5. *Pm*A2M was a rapidly responsive protein in hemocytes of *V. harveyi* infected *P. monodon* and was released from semi-granular hemocytes into hemolymph during the period of infection.
6. *Pm*A2M protected the clot against bacterial protease-mediated fibrinolysis and retarded the bacterial dissemination.
7. *V. harveyi* released proteases required for substantial fibrinolysis were catergorized in the metallo-protease and the serine protease families.

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Appendix

Presentations

1. The 35th Congress on Science and Technology of Thailand (STT35). Science and Technology for a Better Future. October 15-17, 2009. The Tide Resort (Bangsaen Beach), Chonburi, Thailand. "Proteomic Analysis of Responsive Proteins in Lymphoid Organ of *Vibrio harveyi* Infected *Penaeus monodon*" (Oral presentation)

2. The 10th International Symposium on Genetics in Aquaculture (ISGA X). June 22-26, 2009. Bangkok Convention Center & Sofitel Centara Grand, Bangkok, Thailand. "Identification of Proteins Differentially Expressed in *Penaeus monodon* hemocyte in Response to *Vibrio harveyi* infection" (Poster presentation)

3. The 18th Science Forum 2010. March 11-12, 2010. Faculty of Science, Chulalongkorn University, Bangkok, Thailand. "Identification of Differentially Expressed Proteins in Lymphoid Organ of *Vibrio harveyi* Infectd *Penaeus monodon*" (Oral presentation)

   Award: The best oral presentation award in the biological science session of the 18th Science Forum 2010 at Chulalongkorn University, Thailand

4. The 7th National Symposium on Marine Shrimp. September 7-8, 2010. Twin Lotus Hotel, Nakhon Si Thammarat, Thailand. "Identification of binding partners of alpha-2-macroglobulin in the black tiger shrimp using yeast-2-hybrid screening" (Oral presentation)

5. The 15th Biological Sciences Graduate Congress. December 15-17, 2010. University of Malaya, Kuala Lumpur, Malaysia. "Yeast Two-Hybrid Screening for Identification of Interacting Proteins with Alpha-2-Macroglobulin in the Black Tiger Shrimp, *Penaeus monodon*" (Oral presentation)

Award: The third runner up oral presentation award in theme: Cell and Molecular Biology of The 15th Biological Sciences Graduate Congress at University of Malaya, Kuala Lumpur, Malaysia.

6. The RGJ-Ph.D. Congress XIII. April 6-8, 2012. Jomtien Palm Beach Resort, Pattaya, Chonburi, Thailand. "Alpha-2-macroglobulin serves an essential role in protecting the shrimp clots against protease-mediated bacterial escape" (Oral presentation)

   Award: The outstanding oral presentation in the biological science session of the RGJ-Ph.D. Congress XIII.

7. The 20th Science Forum 2012. April 19-20, 2012. Faculty of Science, Chulalongkorn University, Bangkok, Thailand. "Alpha-2-macroglobulin serves an essential role in protecting the shrimp clots against protease-mediated fibrinolysis resulting in bacterial escape" (Oral presentation)

   Award: The best oral presentation award in the biological science session of the 20th Science Forum 2012 at Chulalongkorn University, Thailand

Publication

1. Somboonwiwat, K., V. Chaikeeratisak, H. C. Wang, C. F. Lo, and A. Tassanakajon. 2010. Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after *Vibrio harveyi* infection. *Proteome Sci.* 8: 39.

2. Chaikeeratisak, V., K. Somboonwiwat, H. C. Wang, C. F. Lo, and A. Tassanakajon. 2012. Proteomic analysis of differentially expressed proteins in the lymphoid organ of *Vibrio harveyi*-infected *Penaeus monodon*. *Mol Biol Rep.* 39: 6367-6377

BIOGRAPHY

Mr. Vorrapon Chaikeeratisak was born on April 28, 1984 in Bangkok. He graduated with the degree of Bachelor of Science from the Department of Bichemistry, Faculty of Science, Chulalongkorn University in 2006. He has studied for the degree of doctor of philosophy of Science at the Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2007.

He had published his works in the research journals of the Proteome Science and the Molecular Biology Reports on the topics of "Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after *Vibrio harveyi* infection" and "Proteomic analysis of differentially expressed proteins in the lymphoid organ of *Vibrio harveyi*-infected *Penaeus monodon*", respectively.

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