การโคลน ลักษณะสมบัติ และการแสดงออกของยีนที่ถูกกระตุ้นได้ด้วยความร้อนที่เกี่ยวข้องกับภาวะความเครียดของกุ้งกุลาดำ

นางสาว ปรานี เปียดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CLONING, CHARATERIZATION, AND EXPRESSION OF HEAT INDUCIBLE GENES RELATING TO STRESS CONDITION OF BLACK TIGER SHRIMP, *Penaeus monodon*

Miss Pranee Peaydee

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

Faculty of Science
Chulalongkorn University
Academic Year 2009
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Thesis Title	CLONING, CHARACTERIZATION, AND EXPRESSION OF HEAT INDUCIBLE GENES RELATING TO STRESS CONDITION OF BLACK TIGER SHRIMP *Penaeus monodon*
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Field of Study	Biotechnology
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4972362023 : MAJOR BIOTECHNOLOGY
KEYWORDS :*Penaeus monodon* / HEAT INDUCIBLE / STRESS TOLERANCE

PRANEE PEAYDEE : CLONING, CHARACTERIZATION, AND EXPRESSION OF HEAT INDUCIBLE GENES RELATING TO STRESS CONDITION OF BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR : PROFESSOR PIAMSAK MENASVETA, Ph.D.THESIS CO-ADVISOR : NARONGSAK PUANGLARP, Ph.D., 157 pp.

Stress tolerance was determined in heat induced *P. monodon* exposed to pathogenic and environmental stresses. Shrimp initially induced by heat (35 ℃, 2 h) were subjected to WSSV challenge and ammonia exposure. The results showed that the survival rates of heat induced shrimp exposed to WSSV and ammonia were 15 and 20 %, respectively, higher than that of un-induced shrimp, indicating that stress tolerance to WSSV and ammonia can be enhanced by heat acclimation. Consequently, expression patterns of *P. monodon* genes including aquaporin (PmAQP1), glucosamine-6-phosphate deaminase (PmGluN6P-deaminase), and C-type lectin domain containing protein (PmCLP) were determined. Full length cDNA sequences of PmAQP1 (786 bp ORF encoding 261 amino acids), PmGluN6P-deamiase (948 bp ORF encoding 315 amino acids), and PmCLP (678 bp ORF encoding 225 amino acids) were firstly identified and characterized. Expression patterns of these 3 genes in various tissues of juvenile *P. monodon* were investigated using RT-PCR and quantitative analyses of the expression levels of the target genes were determined. The constitutive levels of Aquaporin (PmAQP1) were detected in hepatopancrease, gill, ovaries, testes, intestine, stomach and heart. The level in gill appeared to be greater than others tested tissues. The expression of PmAQP1 was not detected in haemocyte, epidermis, lymphoid organs and muscle. The result of PmAQP1 expression in shrimp exposed to ammonia stress indicated that the up-regulation of PmAQP1 was detected in heat induced shrimp much sooner than that of un-induced shrimp, indicating that the expression of PmAQP1 might be involved in the enhancement of ammonia stress tolerance in the shrimp. Expression of PmGluN6P-deaminase was also determined in ammonia exposed shrimp. The result revealed no significant difference of the expression level was detected between heat-induced and un-induced shrimp, indicating no relation in PmGluN6P-deaminase expression and ammonia stress tolerance. The involvement of PmCLP in WSSV tolerance was determined. The up-regulation of PmCLP in heat induced shrimp after exposed to WSSV for 72 h indicated the potential functional role of PmCLP in pathogenic stress tolerance of *P. monodon*. In addition, recombinant PmAQP1 was conducted and successfully produced using pET17b System. The recombinant protein will be useful for antibody construction and further used as biological tool for determining health status of the shrimp.

Student's Signature ....

Field of Study : Biotechnology Advisor's Signature ....

Academic Year : 2009 Co-Advisor's Signature ....

ปรานี เปียดี : การโคลน ลักษณะสมบัติและการแสดงออกของยีนที่ถูกกระตุ้นได้ด้วยความร้อนที่เกี่ยวข้องกับภาวะความเครียดของกุ้งกุลาดำ (CLONING, CHARACTERIZATION, AND EXPRESSION OF HEAT INDUCIBLE GENES RELATING TO STRESS CONDITION OF BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ. ดร. เปี่ยมศักดิ์ เมนะเศวต, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. ณรงค์ศักดิ์ พ่วงลาภ 157 หน้า.

ศึกษาการทนทานต่อภาวะความเครียดต่อเชื้อโรคและแอมโมเนียของกุ้งกุลาดำที่ถูกกระตุ้นด้วยความร้อน โดยเริ่มจากการกระตุ้นกุ้งด้วยความร้อน (35 °C เป็นเวลา 2 ชั่วโมง) ก่อนสัมผัสกับไวรัสโรคตัวแดงดวงขาวและแอมโมเนีย อัตราการรอดของกุ้งที่ถูกกระตุ้นด้วยความร้อนก่อนสัมผัสกับไวรัสโรคตัวแดงดวงขาวและแอมโมเนีย สูงกว่าอัตรารอดของกุ้งที่ไม่ได้รับการกระตุ้นด้วยความร้อน 15 และ 20 เปอร์เซ็นต์ ตามลำดับ แสดงว่าการกระตุ้นด้วยความร้อนสามารถเพิ่มความทนทานต่อภาวะความเครียดจากไวรัสโรคตัวแดงดวงขาวและแอมโมเนียได้ จากนั้นศึกษารูปแบบการแสดงออกยีน PmAQP1, PmGluN6P-deaminase และ PmCLP ของกุ้งกุลาดำโดยเริ่มจากทำการค้นหา full length cDNA sequence ของยีน PmAQP1 (786 bp ORF คิดเป็น 261 amino acid), PmGluN6P-deaminase (948 bp ORF คิดเป็น 315 amino acids), และ PmCLP (678 bp ORF คิดเป็น 225 amino acids) ซึ่งเป็นการรายงานครั้งแรกในกุ้งกุลาดำ จากนั้นทำการศึกษารูปแบบการแสดงออกของยีนทั้ง 3 ยีนในเนื้อเยื่อต่างๆ ในกุ้งกุลาดำขนาด 20-25 g ด้วยเทคนิค RT-PCR และ quantitative analysis จากผลที่ได้พบว่าการแสดงออกของยีน PmAQP1 ตรวจพบได้ในตับ, เหงือก, รังไข่, อัณฑะ, ลำไส้, กระเพาะ, และหัวใจ ในเหงือกจะมีการแสดงออกที่มากกว่าในเนื้อเยื่ออื่นๆ และไม่พบการแสดงออกในเม็ดเลือด, ผิวหนังชั้นนอก, lymphoid organs, และกล้ามเนื้อ ผลระดับการแสดงออกของยีน PmAQP1 ในกุ้งที่สัมผัสกับภาวะความเครียดด้วยแอมโมเนีย แสดงให้เห็นการเพิ่มการแสดงออกของยีน PmAQP1 โดยกุ้งที่ได้รับการกระตุ้นด้วยความร้อนจะมีการแสดงออกที่เร็วกว่ากุ้งที่ไม่ได้รับการกระตุ้นด้วยความร้อน ผลที่ได้แสดงให้เห็นว่า PmAQP1 เกี่ยวข้องกับการเพิ่มขึ้นของระดับความทนทานต่อแอมโมเนียในกุ้ง ส่วนการแสดงออกของยีน PmGluN6P-deaminase ต่อแอมโมเนียนั้น ไม่มีความแตกต่างอย่างมีนัยสำคัญของระดับการแสดงออกระหว่างกุ้งที่ถูกกระตุ้นด้วยความร้อนกับกุ้งที่ไม่ได้รับการกระตุ้น แสดงให้เห็นว่า PmGluN6P-deaminase ไม่น่าจะเกี่ยวข้องกับการเสริมสร้างความทนทานในกุ้ง ส่วนความเกี่ยวข้องของยีน PmCLP ในการทนทานต่อไวรัสโรคตัวแดงดวงขาว พบการเพิ่มระดับการแสดงออกของยีนในกุ้งที่ถูกกระตุ้นด้วยความร้อนหลังสัมผัสไวรัสโรคตัวแดงดวงขาวเป็นเวลา 72 ชั่วโมง แสดงให้เห็นบทบาทหน้าที่ของ PmCLP ในการทนต่อความเครียดที่เกิดจากเชื้อโรคของกุ้งกุลาดำ นอกจากนี้ยังได้ศึกษาการสร้างโปรตีนลูกผสมของยีน PmAQP1 เบื้องต้น การผลิตโปรตีนสำเร็จได้โดยใช้เวกเตอร์ pET17b โปรตีนนี้มีประโยชน์นำไปใช้สร้างแอนติบอดีในอนาคต เพื่อใช้เป็นดรรชนีชีวภาพสำหรับตรวจสอบสุขภาพของกุ้ง

	ลายมือชื่อนิสิต..
สาขาวิชา เทคโนโลยีชีวภาพ............	ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก....................
ปีการศึกษา 2552............................	ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม....................

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LIST OF ABBREVIATIONS

Abbreviations	**Term**
bp	Base pair
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxyribonucleic acid
°C	Degree Celsius
DEPC-H_2O	Diethylpyrocarbonate treated distiooed water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphophate
g	Gram
GenBank	Genetic databank
h	Hour
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani medium
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Milimolar
M	Molar
MW	Molecular weight

Abbreviations	**Term**
µg	Microgram
µl	Microlitre
µm	Micrometre
nm	Nanometre
ng	Nanogram
NCBI	National Center for Biotechnology Information
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNase A	Ribonuclease A
rpm	Rovolution per minute
SDS	Sodium dodecyl sulphate
sec	Second
SDS-PAGE	Sodium dodecyl sulfate-poloyacrylamide gel electrophoresis
Taq	*Thermus aquatucus*
TBE	This borate EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N' –tetraethylenediamine
U	Unit
UV	Ultraviolate
V	Volt
v/v	Volume by volume
w/v	weight by volume

ACKNOWLEDGEMENTS

I would like to express my deepest sense of gratitude to my advisor, Professor Dr. Piamsak Menasveta and my co-advisor, Dr. Narongsak Puanglarp for their guidance, encouragement, valuable suggestion and supports throughout my study.

My gratitude is also extended to Associate Professor Dr. Charoen Nitithamyong, Associate Professor Dr. Somkiat Piyatiratitivorakul, and Dr. Pikul Jiravannichpaisal for serving as thesis committee, for their recommendations and also useful suggestions.

I wish to acknowledge to Center of Excellence for Marine Biotechnology (CEMB), National Center for Genetic Engineering and Biotechnology (BIOTEC) for my financial support.

I especially thank Dr. Rungnapa Leeelatanawit, Miss Sasithon Petkon and Mr Arun Buaklin. They trained me the laboratory techniques that were necessarily used along the thesis. Thanks are also expressed to all my friends, colleagues, and everyone in CEMB, Chulalongkorn University for friendships and helps.

Finally, I would like to express my deepest gratitude to my family for their love, care, and encouragement extended throughout my study.

CHAPTER I

INTRODUCTION

1.1 General introduction

Giant tiger shrimp, *Penaeus monodon*, is one of the most important economic species in Thailand. The country earned billion of baths annually from the shrimp export. Because of the market demand, shrimp culture had increased rapidly and uncontrollably. The consequence of this unprepared expansion was the massive losses of shrimp production. Combinations of various factors were suspected to be the causes. These included contagious pathogens, low quality of broodstock, and toxicity of environmental contaminants. To avoid the problems, other cultured shrimp species such as domesticated Pacific white shrimp, *Litopenaeus vannamei*, has recently been introduced to Thailand and initially contributed to the cultured production significantly. However, after several years, white shrimp production has confronted with similar problems. The reason was simply that those main causes of problems especially the outbreaks of the diseases have never been completely solved. Determination of health status of the shrimp is still limit. The main obstacle is the lack of basic knowledge on the main crucial mechanisms especially the defense system of the shrimp. Therefore, details on the molecular and physiological responses of the shrimp are primarily needed.

Recently, the connection between the stress condition and animal health has gained more attention. A variety of stressful stimuli has been demonstrated to have influence on immune parameters in many invertebrate species. The involvement of stress exposure and immune response has been reported in various numbers of animals.

Acute stressors can be both immunostimulating and immunosuppressive effects depending on species and physiological status of the animal. Animals confront with a range of environmental stresses including abiotic insults such as extreme temperatures, altered water quality or nutrients, and biotic stresses such as pathogens. It has been reported in many cases that animals resistant to one stress are sometimes cross-tolerant to others. This indicates some similarities of their cellular responses to these environmental challenges. The ability of one type of stress to induce protection against unrelated stress will be an important survival strategy for shrimp under

stressful conditions. To understand this phenomenon and to be able to take full advantage of it in shrimp aquaculture, information on cellular and molecular responses of the shrimp defense system during cross tolerance induction and stress challenge will be valuable.

Apart from the main defense mechanisms such as phagocytosis, encapsulation, nodule formation, clotting and agglutination, it is now known that animals are also able to cope with numerous stressors by synthesizing various types of proteins. These proteins, such as heat shock proteins, ubiquitins, and cytochrome P450, have been reported as stress response proteins and they are generally called stress proteins. Most stress proteins act as molecular chaperones that regulate cellular homeostasis. These stress-related proteins have been proposed as biomarkers of cellular aggression. They are also useful for analysis of stress responses in comparison between stress-tolerant and stress-sensitive varieties.

A number of potential stress molecules have been reported to be involved in crucial functions of the cells in many organisms. However, details of stress related proteins in *P. monodon* are scarce. Understanding mechanisms and functions of these genes would provide a new tool for determining health status of the shrimp and for understanding the biological and molecular processes that improve stress tolerance in *P. monodon* which are essential for further development of a sustainable shrimp culture.

1.2 Shrimp farming in Thailand

Shrimp farming started in Thailand since 1970s. Local species, *P. monodon*, was the main culture shrimp as their broodstocks were commonly captured from the sea. Postlarvae were produced in land-based hatcheries. By early 1990s, Thailand emerged as the world's leading farmed shrimp producer and exporter based on *P. monodon* production. A combination of factors such as advanced technologies in hatcheries, farms, feed companies and processing plants, well-organized international marketing, and suitable climate for shrimp growing, has led Thai shrimp farming into fully integrated industry. The main export markets of Thailand were U.S.A., Japan, and European Union (Limsuwan, 2004; Wyban, 2007).

During 2002 to 2007, export value of Thai shrimp decreased (Table 1.1) due to the massive losses of *P. monodon* production. Meanwhile, white shrimp (*P. vannamei*) production started to increase (Fig 1.1) in 2006, *P. vannamei* represented

over 98% of Thailand's total production (Wyban, 2007). The advantage of *P. vannamei* is the complete domestication. Broodstocks are successfully produced in captivity while high quality *P. monodon* broodstocks are wild-caught. However, the disadvantage of *L. vannamei* is that its price remains lower than that of *P. monodon* while the labour cost is in the same level as *P. monodon.* In addition, the labour cost in Thailand is higher than that of other countries (e.g. Vietnam and Chaina) preventing the advantage of competitiveness with the world market. On the other hand, the market of premium-sized *P. monodon* is still open for Thailand because *L. vannamei* is not suitable for that market. Accordingly, *P. monodon* culture is currently promoted for increasing the production of this species.

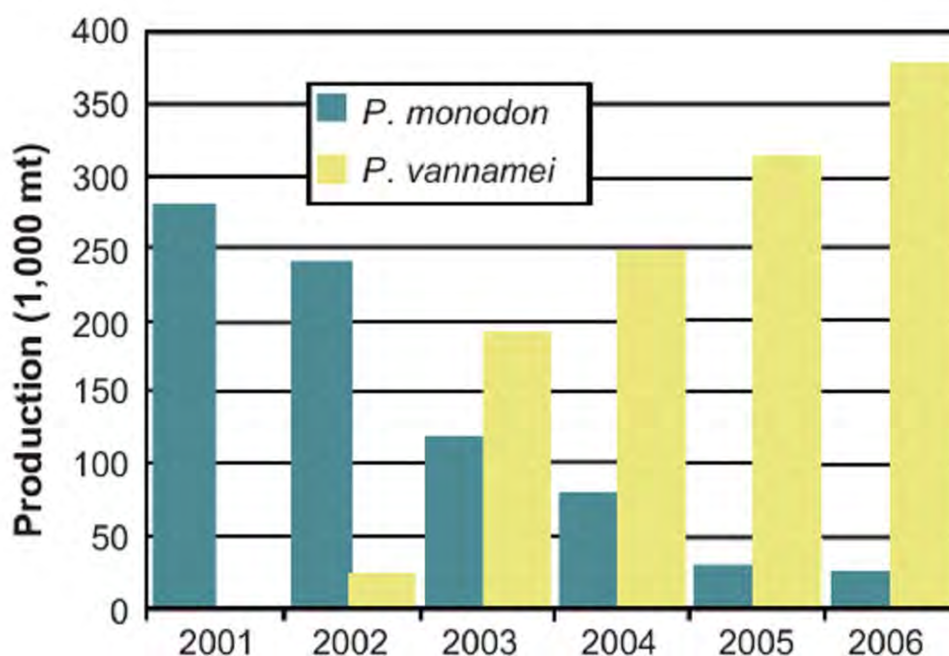

Figure 1.1 Production of *P. monodon* compared to *P. vannamei* (Wyban, 2007)

Table 1.1 Giant tiger shrimp export from Thailand during 2002-2007.

Country	2002		2003		2004		2005		2006		2007	
	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)	Quantity (MT)	Value (MB)
USA	97,681.81	36,011.41	89,115.28	29,032.87	58,365.2	17,206.75	29,116.62	17,206.75	34,537.23	8,847.42	7,979.91	1,909.64
Japan	16,644.6	13,813.33	33,235.52	11,916.87	27,977.27	9,586.59	20,182.85	9,586.59	15,709.39	3,832.31	3,711.32	1,067.25
Canada	6,455.76	3,890.48	11,216.47	3,412.09	6,490.03	2,072.25	3,249.37	2,072.25	2,798.61	744.95	1,762.16	402.68
Singapore	5,251.66	3,138.86	3,317.14	1,258.13	3,383.18	537.88	1,933.5	537.88	1,580.11	236.31	401.47	63.53
Taiwan	4,917.65	1,276.86	3,051.77	799.44	2,964.62	564.58	1,673.65	564.58	607.7	170.12	692.69	194.78
Australia	4,481.25	1,326.06	4,847.5	1,252.31	2,418.19	1,042.02	2,097.76	1,042.02	1,418.36	445.05	658.54	225.13
Hong Kong	1,365.12	533.26	1,437.54	340.42	1,396.98	409.93	1,026.84	409.93	921.88	256.91	1,569	365.91
China	1,049.23	352.68	992.91	214.54	833.1	162.66	1,003	162.66	710.7	85.65	1,629.74	235.57
U. Kingdom	661.07	210.81	184.23	64.11	505.76	181.63	161.79	181.63	241.91	70.54	242.4	73.43
Total	180,615.81	63,822.73	160,986.48	51,524.10	118,343.12	16,629.05	69,168.96	16,629.05	64,565.41	16,178.85	23,933.1	5,922.11

Source: Office of Agricultural Economics, Ministry of Agriculture and Cooperate

1.3 Taxonomy of *P. monodon*

The black tiger shrimp is taxonomically classified as a member of Phylum Arthopoda; Subphylum Crustacea; Class Malacostraca; Subclass Eumalacostraca; Order Decapoda; Suporder Narantia; Infraorder Penaeidea; Superfamily Penaeoidea; Family Peneidae Rafinesque, 1985; Genus *Penaeus* Fabricius, 1798 and Subgenus *Penaeus.* (Bailey-Brook and Moss, 1992)

The scientific name of black tiger shrimp is *Penaeus monodon* where its English common name is black (giant) tiger shrimp.

1.4 Shrimp defense system

Similar to most invertebrates, shrimp internal defenses do not possess adaptive immunity as in vertebrates. However, they survive successfully in pathogen-rich or hostile environments, indicating the effective defense system for combating harmful invaders. Generally, 2 types of immune systems in shrimp were classified; cellular and humoral immunities (Ratchliffe, et al., 1985; Smith and Chisholm, 1992).

1.4.1 Cellular immunity

The cell-mediated immunity of the shrimp involves a number of responses including haemolymph coagulation and clotting, phagocytosis, encapsulation, and cytotoxic interactions (Ratchliffe, et al., 1985). These cellular activities are carried out in 3 morphologically different types of haemocytes; hyaline, semigranular, and granular cells (Johansson, et al., 2000).

All cellular activities in shrimp appear to be influenced by products of the prophenoloxisase activation (proPO) system (Söderhäll and Cerenius, 1998). These products comprise opsonic, cytotoxic, fungicidal, and cell encapsulation promoting activities. There are some evidences that this system is involved in non-self recognition and cellular communication. The proPO activating system consists of several proteins involved in the immune defense in invertebrates leading to melanin production. This also includes cell adhesion, opsonic protein, and peroxinectin from storage granules. Release of peroxinectin can stimulate the phagocytosis by hyaline cell or the encapsulation by semigranular cell (Sritunyalucksana and Söderhäll, 2002).

Semigranular and granular cells can be cytotoxic and lyse foreign eukaryotic cells. This has been shown with both tumorous and non-tumorous cell lines as well as

erythrocytes as target cell (Söderhäll, et al., 1985). The tumor cell targets are essentially susceptible to lysis by mammalian natural killer cell (NK cell). Thus, the invertebrate cytotoxic cells may resemble mammalian NK cells.

1.4.2 Humoral immunity

Humoral defense factors have also been reported in shrimp. Some reports showed that humoral factors could speed up and increase the effectiveness of phagocytic action in an individual animal (Cocknick and Stewart, 1968). Shrimp do not posses immunoglobulins. Humoral factors reported in various species of shrimp include lectin-like proteins, clotting proteins, antimicrobial peptides, agglutinins and precipitins (Ratchliffe, et al., 1985). They are often produced by and act in conjunction with the defense cells.

1.5 Stresses

1.5.1 Stress condition

Stress is defined as the response of the organisms exposed to a series of different factors which cause an extension of a physiological state beyond the normal resting state. To survive in this changing environment, organisms require a wide range of fast and adaptive responses, leading to transcriptional activation of genes whose products cope with a given physico-chemical stress. Stress activation is regarded as an essential element in the total adaptive system of the organisms. There is a consensus that the stress response affects most other physiological systems, endocrine systems, autonomic systems, and immune systems, as well as the biochemistry of the brain.

1.5.2 Stressors

The potential stressors are grouped as being environmental (such as temperature, salinity, superoxide anion, toxic substances and waste), physical (such as handling, crowding and transport), and biological or pathogenic stressors (such as Vibrio sp., WSSV, YHY and TSV) (Barton and Iwama, 1991). There are differences in the generalized stress response among different species, and different stocks or races of the same species differ in their tolerance to applied stressors. Stressors come from many sources. They can be classified into 2 main categories.

1.5.2.1 Environmental stressors

Environmental stressors mainly include adverse physical and chemical conditions of the water. Extreme conditions or changes in water quality such as dissolved oxygen, ammonia, hardness, pH, gas content, partial pressures, and temperature can induce stresses. Metals (e.g., copper, cadmium, zinc, and iron) and other contaminants (e.g., arsenic, chlorine, cyanide, various phenols, and polychlorinated biphenyls) in the water can cause severe stress and death. Other potential environmental stressors include insecticides, herbicides, fungicides, and defoliants. Industrial, domestic, and agricultural activities add much of these contaminants to the environment that affect animals at all life stages.

One of the most important limiting factors in intensive culture systems is the build up of toxic nitrogenous wastes, mainly as ammonia and nitrite. Ammonia is the major end-product of protein catabolism in crustaceans and the most common toxicant resulting from excretion of cultured animals and mineralization of organic detritus like unconsumed feed and feces (Chin and Chen, 1987; Lin and Chen, 2001). In intensive shrimp culture system, ammonia increases exponentially over time (Ostrensky and Wasielesky, 1995). Accumulation of ammonia in such confined area deteriorates water quality, slows down growth, increases oxygen consumption and ammonia-N excretion, affects hemolymph and free amino acid levels, and even causes high mortalities (Chen and Lin, 1992; Chen, Chen, and Cheng, 1994). Total ammonia-nitrogen (TAN) is composed of un-ionised (NH3-N) and ionised forms ($NH4^{+}$) (Losordo, Masser, and Racocy 1992; Masser, Rakocy, and Losordo, 1992). It is the un-ionised form which is most toxic to aquatic organisms as it can readily diffuse through cell membranes and is highly soluble in lipids (Chin and Chen, 1987; Frias-Espericueta, et al., 1999). High levels of pH and temperature increase un-ionised form hence increasing the toxicity of the TAN to aquatic animals (Losordo, et al., 1992; Masser, et al., 1992).

Environmental stress from pollutants seems to be an effective factor for the reduction of immunocompetence and is signaled by the appearance or the increased prevalence of disease in crustaceans. Effects include infection pressure from facultative microbial pathogens, and reduced resistance to infection (Sindermann, 1997).

1.5.2.2 Pathogenic stressors

Pathogens and parasites can also be considered as biological stressors. Diseases and outbreaks leading to massive mortalities occur in nature as well as in cultured stocks. Viral pathogenesis has become the main concern for shrimp culture. White spot syndrome virus (WSSV) is a notable infectious pathogen which caused severe loss to shrimp aquaculture industry worldwide including Thailand. WSSV is an unclassified rod-shaped to obviate, enveloped double-stranded DNA virus with a single filamentous appendage. The cellular location is nuclear and the genome size is at approximately 290 kbp. Horizontal transmission may be direct or vectorial and water is the major abiotic vector. Rapid transmission occurs from infected shrimp through the water and by cannibalism of weak or moribund shrimp (Lightner, 1996).

1.6 Stress response

Stress response occurs when there is a difference between what the organism is expecting, and what really exists. Therefore, stress response is an alarm of something missing or a homeostatic imbalance occurring (Levine and Ursin, 1991). The response drives the organism to provide specific solutions to abolish the source of the alarm, as well as the alarm itself. The stress response is an optimal physiological response, where physiological resources are mobilized to improve performance. The response is regarded as a positive and desirable alarm response. The response to the stressors involves all levels of organization, from the cell to the individual organism and to the structure of the population (Barton and Iwama, 1991).

1.6.1 Behavioral stress response

Behavioral response is the immediate signs of a stress. Activities such as food acquisition, predator avoidance, prey capture, migration, and habitat preference are critical to the survival of the organism and thus the population. Alterations may take minutes to weeks to return to pre-stress conditions, depending on the nature and magnitude of the stressor. Behaviors that are most important to the survival of the organism tend to return to normal in the shortest time (Schreck, Olla, and Davis, 1997).

1.6.2 Physiological stress response

In response to a stressor such as handling or crowding, animals will undergo a series of biochemical and physiological changes in an attempt to compensate for the challenge imposed upon it and, thereby, cope with the stress. The stress response has been broadly categorized into the primary, secondary, and tertiary responses (Mazeaud, Mazeaud, and Donaldson, 1977; Wedemeyer, Barton, and McLeay, 1990).

Primary stress response represents the perception of an altered state and initiates a neuroendocrine/endocrine response that forms part of the generalized stress response. This response includes the rapid release of stress hormones, such as catecholamines and cortisol, into the circulation.

Secondary stress response comprises several of biochemical and physiological adjustments associated with stress and it is mediated to some extent by the stress hormones. The composition of blood and tissues is altered and variations in ventilation and heart rate frequently occur. (Mazeaud, et al., 1977)

Tertiary stress response represents whole animal and population level changes associated with stress. Stress induces a reduction in the effectiveness of the immune system, resulting in higher susceptibility to diseases and parasites, decreased growth rate, reduced reproduction and increases mortality (Donaldson, Fagerlung, and McBride, 1988; Passion, 1981). Decreased recruitment and productivity may alter community species abundance and diversity. For example, at high ammonia concentration, fish (*Cyprinus carpio*) approached the surface; activity of the treated fish was decreased, and accompanied by a smaller mean distribution. Blood glucose levels increased, and the treated fish became indifferent to food. (Weinstein and Kimmel, 1998)

Behavioral and physiological responses to a stressor are intimately related. The adaptive behavioral response to a stressor may lessen the energetic demand on the physiological systems that must respond to it. The physiological stress response serves to maintain, direct, and possibly limit the behavioral response (Iwama, 1998).

1.7 Molecular stress response

Animals are frequently exposed to an excess of stress conditions such as low temperature, heat, oxidative stress and heavy metal toxicity. They also face challenges from pathogens including bacteria, fungi, and virus. All these stress factors are a menace for living organisms and prevent them from reaching their full genetic

potential and limit their productivity. In response to these stress factors various genes are up-regulated, which can mitigate the effect of stress and lead to adjustment of the cellular environment and tolerance. It is now well known that the stress signal is first perceived at the membrane level by the receptors and then transuded in the cell to switch on the stress responsive genes for mediating stress tolerance. Recently, some genes of calcium-signaling and nucleic acid pathways have been reported to be up-regulated in response to both cold and salinity stresses indicating the presence of cross talk between these pathways. Since stresses have been categorized into various types including thermal (heat and cold shock), oxidative, environmental, osmotic and pathogenic stresses (Sanders, Venema, and Kok, 1999), the responses of organisms to each stress are, therefore, associated with various numbers of mechanisms and many different types of molecules.

1.7.1 Thermal stress response

Living cells are continually challenged by conditions which cause acute and chronic stress. To adapt to environmental changes and survive different type of injuries, eukaryotic cell have evolved networks of different responses which detect and control diverse forms of stress. One of these responses, known as the heat shock response, has attracted a great deal of attention as a universal fundamental mechanism necessary for cell survival under a variety of unfavorable conditions. Thermal stress caused damage to most macromolecular structures e.g. DNA (depurination), membranes (changes in fluidity) and protein (denaturation).HSFs (heat shock factors) are transcription factors that regulate the expression of HSPs through interaction with a specific heat shock element (HSE) in the promoter region of the *hsp* genes (Morimoto, 1998). Hence, transcription levels of most HSPs are up-regulated in response to many stresses. Members of the HSP70 family appear to play a direct role in the autoregulation of the heat shock response. In larger eukaryotes, HSF1 is present in both unstressed and stressed cells. However, in the absence of stress, HSF1 is expressed as an inert monomer bound to hsp70 and other chaperones and as lacking in transcriptional activity. Both the DNA-binding activity and the transcriptional transactivation domain are repressed through intramolecular interactions and constitutive serine phosphorylation (Morimoto, Kroeger, and Cotto, 1996; Shi, Mosser, and Morimoto, 1998).

Airaksinen, et al., (2003) studied the effect of heat (increase from 28 to 37 °C) and cold (decrease from 28 to 20 °C) shocks on the expression pattern of HSP70 and its co-factors (*zhsf1a*, *zhsf1b*) in embryonic cell line (ZF4) of zebrafish (*Danio rerio*). The result indicated that the expression of HSP70 was up-regulated in heat shock but stable in cold shock while the expression ratios of *zhsf1a* and *zhsf1b* were highly increased in heat shock (10-fold) but slightly increased in cold shock (one half).

Andreas, et al., (2007) studied molecular stress responses of marine *Mytilus gallorpvincialis* during long-term acclimation at increasing ambient temperature. Acclimation to temperatures higher than 24 °C caused an increase in mortality and induced the expression of Hsp72.

In *P.monodon*, expression levels of HSP70 and HSP90 were increased significantly after heat induction while no change in the expression levels of HSP60 was detected (Kanchana Doungpunta, 2004).

1.7.2 Oxidative stress response

Oxidative stress is experienced by all aerobic life when antioxidant defenses are overcome by prooxidant forces, and is the basis of many physiological aberrations. Environmental contaminants may enhance oxidative stress in aquatic organisms, e.g. highly elevated rates of ideopathic lesions and neoplasia among fish inhabiting polluted environments is increasingly related to oxidative stress associated with environmental pollution (Kappus, 1986; Di Giulio, et al., 1989; Malins, et al., 1988). The expression of specific lesions known to arise specifically from oxidative stress, e.g. lipid peroxidation, oxidized bases in DNA, and accumulation of lipofuscin pigments are present in many aquatic animals exposed to contaminants (Bano and Hasan, 1989). Aquatic organisms contain the major antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase. However, there are marked quantitative differences among the various species reported (Winston, 1991).

Kochhann, et al., (2009) studied the effect of chronic thorium (Th) exposure on oxidative parameters, lipid peroxidation levels (LPO), and antioxidant enzymes (GST, SOD and CAT) in the gills of juvenile silver catfish (*Rhamdia quelen*). Chronic Th exposure caused alterations in the oxidative parameters of silver catfish gills, which were correlated with the Th accumulation in this organ. The levels of GST and SOD activity in the gill were decreased while that of LPO was increased.

1.7.3 Environmental stress response

Aquatic organisms are almost always exposed to the mixture of contaminants within the aquatic environment. Even very small amounts of harmful heavy metals such as lead, cadmium, and mercury can cause toxicity in living bodies. Organisms, therefore, possess various physiological mechanisms to defend against many different kinds of toxicities and stresses in order to maintain their homeostasis. Major molecular responses usually involve the regulation of heat shock proteins which are representative stress defense proteins, and the metal-binding protein such as metallothionein (MT). Studies of HSPs in relation to stress and immune responses to heavy metals have been conducted in various species of vertebrates and mollusks.

Choi, Jo, and Choi, (2008) studied the effects of cadmium (Cd) on Pacific oyster, *Crassostrea gigas*. The results revealed time- and dose-related effects on mRNA levels of HSP90 and MT in the gill and digestive gland and changes in enzyme levels in the hemolymph of the exposed oyster.

In case of organisms under captivity or culture, pollutants such as nitrogenous wastes appear to be the main stressors of concern. Ammonia is an unusual toxicant in that it is produced by, as well as being poisonous to, animals. Ammonia is excreted by many aquatic animals and is continually produced as a result of the decomposition of excreted wastes of living organisms and/or the decomposition of dead organisms. Nitrification is a biological process during which nitrifying bacteria convert toxic ammonia to less harmful nitrate. The primary stage of nitrification, the oxidation of ammonia (NH_3) is performed by bacteria such as the *Nitrosomonas* species, which converts ammonia to nitrites (NO_2^-). Other bacterial species, such as the *Nitrobacter*, are responsible for the oxidation of the nitrites into nitrates (NO_3^-). It is important for the nitrites to be converted to nitrates because accumulated nitrites are toxic to aquatics. (Ward, 1996)

Ammonium is a toxic and inhibitory by-product to cells. The effects of ammonium on cell growth, productivity, and glycosylation have been investigated. Elevated ammonium also alters the glycosylation profile of recombinant proteins, and the glycosylation profile of a recombinant protein is fundamental for its efficacy (Goochee, 1991; Jenkins, Parekh, and James, 1996). For instance, elevated ammonium causes lower terminal sialylation of all the glycans, lower galactosylation, lower tetraantennary and tetrasialylated oligosaccharide structures, and increased molecular heterogeneity (Andersen and Goochee, 1995; Borys, Linzer, and

Papoutsakis, 1994; Gawlitzek, Valley, and Wagner, 1998; Thorens and Vassalli, 1986; Yang and Butler, 2002). Many mechanisms have been proposed to explain the relationship between the ammonium toxicity and glycosylation.

Kharbuli, et al., (2006) studied expression of ornithine–urea cycle enzymes, e.g. Glutamine synthetase (GSase), Carbamyl phosphate synthetase III (CPSase III), in early life stages of air-breathing walking catfish *Clarias batrachu*s and the induction of ureogenesis under hyper-ammonia stress. The increase of activities of GSase and CPSase III enzymes in the NH_4Cl-treated (10 mM) fry was accompanied by significant increase of GSase enzyme protein concentration by 95% ($P < 0.01$) and CPSase III enzyme protein concentration by 55% ($P < 0.01$) after 72 h of exposure compared to respective controls.

1.7.4 Osmotic stress response

The ability of animals to regulate osmotic and ionic concentrations is believed to depend upon osmoregulation mechanisms. In crustaceans, osmoregulation and ion acquisition is based on ion transport performed by differentiated osmoregulatory epithelia of the branchial chamber (gills, branchiostegites, epipodites) containing specialized cells or ionocytes (Taylor and Taylor, 1992). The gills are specialized for several functions, including gas exchange, osmoregulation and acid–base balance (Lucu and Devescovi, 1999; Lucu and Towle, 2003).

Pongsomboon, et al., (2009) identified cDNA sequence of *P. monodon* carbonic anhydrase (*Pm*CA) and studied the role of *Pm*CA in the regulation of salinity stress. The result revealed the changes in the *Pm*CA mRNA expression and total CA activity after shrimp were transferred from 25 to 3 ppt salinities for up to 2 weeks. This indicated that *Pm*CA was significantly up-regulated in shrimp gills within 24 h after hypo-osmotic stress.

1.7.5 Pathogenic stress response

Pathogens can also be considered as biological stressors. The innate immune system of the invertebrates acts as the first-line of defense against pathogens and the system consists of pattern-recognition proteins (PRPs) that recognize specific patterns of foreign pathogens (Iwanaga and Lee, 2005). In animals, some lectins, sugar-binding proteins that bind to the outside of the cell, serve as agglutinins (Drickamer,

1999). Although lectins share the common property of binding to defined sugar structures, their roles in various organisms are not the same.

The importance of lectins in the invertebrate innate defense system and biochemical analyses of these molecules has been reported in several crustaceans (Ratanapo and Chulavatnatol, 1990; Vazquez, et al., 1996; Sricharoen, et al., 2005). For example, in freshwater prawn, *Macrobrachium rosenbergii*, a serum sialic acid-specific lectin is involved in non-self recognition to enhance phagocytosis and bacterial agglutination (Vazquez, et al., 1997). In *Litopeneaeus vannamei*, when juvenile shrimp were challenged with shrimp extracts containing WSSV, the expression levels of *LvLT* decreased initially in the first 2 h and then increased to a much higher level after 4 h (Ma, et al., 2007).

1.8 Cross tolerance

Cells that have been pre-exposed to mildly conditions can acquire a transient resistance against the killing effect of subsequent to a variety of environmental stresses (Kampinga, 1993). Cross protection factors include the treatments of thermal, oxidative, acidic, osmotic and nutrient. Among these factors, most attentions have been paid to the thermal induction within various organisms. The observation that an increase in temperature of a few degrees above the physiological level induced the synthesis of a small number of proteins in *Drosophila* salivary glands has led to the discovery of universal protective mechanism which prokaryotic and eukaryotic cell utilize to preserve cellular function and homeostasis (Linqusit and Craig, 1998).

Heat shock involves the sudden exposure of cells, tissues and organisms to a temperature well above normal, but below lethal. The response usually induces the synthesis of one or more heat shock proteins and commonly results in induced thermotolerance (ITT). That is the induced organisms can survive under temperature conditions that otherwise would be lethal (Nover, 1991; Parsell and Lindquist, 1993). Heat shock proteins, sometimes called stress proteins, are now ones of common molecules associated with molecular stress response in most organisms. These proteins are beneficial to cells in at least two ways: one is by promoting degradation of abnormal proteins, and the other is by acting as molecular chaperones. Molecular chaperones are otherwise unrelated classes of proteins that prevent aggregation and, in some cases, mediate the correct assembly of other proteins (Beissinger and Buchner,

1998). In general, it is believed that these processes are involved in the induction of thermotolerance (Kampinga, 1993).

Clegg, et al., (1998) studied thermotolerance in the pacific oyster by heating them at 37°C for 1 h before lethal heating at 44°C for another 1h. The result indicated that the heat induced oyster revealed 20% mortality while normal oyster had 100% mortality within 14 days.

DuBeau, et al., (1998) studied the involvement of heat induction and osmotic tolerance in Juvenile salmon. The test fish were pre-exposed to heat shock at 26 °C for 15 min before osmotic challenge at 45 ppt. The result revealed 40% mortality with pre-heated salmon while 100% mortality was found in normal fish within 36 h. HSPs were also found to be up-regulated in the fish during the experiment. The result confirms that thermal shock confers protection against osmotic challenge.

Other stressful treatments, such as exposure to ethanol, sodium arsenite, heavy metals and anoxia, can also produce tolerance (Rockman, Hall, and Glavin, 1986; Mittal and Flora, 2006; Tsuji et al., 2002; Zwaan, Cortesi and Cattani, 1995).

The relationship of acid adaptation to tolerance of other environmental stresses was examined in *Salmonella typhimurium*. *S. typhimurium* was adapted to acid by exposing the cells to mildly acidic conditions (pH 5.8). Acid-adapted cells were found to have increased tolerance towards various stresses including heat, salt, and an activated lactoperoxidase system. Acid adaptation increased cell surface hydrophobicity. These results show that acid adaptation alters cellular resistance to a variety of environmental stresses. The mechanism of acid-induced cross-protection involves changes in cell surface properties in addition to the known enhancement of intracellular pH homeostasis (Leyer and Johnson, 1993).

1.9 Genes of interest relating to stress

1.9.1 Aquaporin

Aquaporins (AQP) are a large family of integral membrane proteins. The AQP family can be divided into 2 groups, aquaporins, and aquaglyceroporins. Aquaporins proper facilitate movement of water across membranes, whereas aquaglyceroporins are specific for glycerol and related compounds (Sansom and Law, 2001). Evidence for possible movement of ion and carbon dioxide through the aquaporins, as well as evidence for direct regulation of auqaporin function by posttranslational modification such as phosphorylation (Verkman and Mitra, 2000). To date, 11 isoforms of

aquaporins, AQP0 to AQP10, have been characterized in mammals, (Takata, Matsuzaki, and Tajika, 2004) and more than 200 members of the aquaporins family have been found in plants, microbial, invertebrates and vertebrates (Agre, et al., 2002). Insects were early shown to contain the membrane of the aquaporin gene family in digestive tract and brain. (Le Caherec, et al., 1996).

The expression of some of these AQPs increased near the time of birth and appears to be regulated by grown factor, inflammation, and osmotic stress (Verkman and Song, 2006). The transcriptional regulation of AQPs by alteration of external osmolarity has been demonstrated. In human renal proximal tubule epithelial cells and mouse inner modularly collecting duct cells, AQP1 expression was found to be up-regulated in response to hypertonic stress with NaCl at both the mRNA and protein levels (Jenq, et al., 1998; 1999). Incubation of keratinocytes in sorbitol-added hypertonic medium increased AQP3 mRNA expression (Sugiyama, et al., 2001).

Sugiura, et al., (2008) studies quantified variations of AQPs mRNAs levels in chick kidney after hyperosmotic stimulation (water-deprivation or salt-loading) by real-time RT-PCR analysis. In particular, water-deprivation increased AQP2 and AQP3 mRNAs levels, whereas salt-loading induced a significant increase in AQP1, AQP2 and AQP9 mRNAs levels. AQP4 and AQP7 mRNA levels were not affected by any hyperosmotic stimulations.

1.9.2 Glucosamine-6-phosphate deaminase

Glucosamine-6-phosphate deaminase (GlcN6P-deaminase), formerly known as glucosamine-6-phosphate isomerase, catalyzes the reversible conversion of glucosamine-6-phosphate (GlcN6P) into D-fructose-6-phosphate (Fru6P) and ammonium. It is a hexameric enzyme with the identical subunits with the molecular weight of 29-33 kDa (Nakamura, et al., 2000). GlcN6P-deaminase is an enzyme in glycosylation pathway which may have been influenced by ammonium levels. Numbers of mechanisms have been proposed to explain the relationship between the ammonium toxicity and glycosylation (Andersen and Goochee, 1995; Borys, Linzer and Papoutsakis, 1993; Butler and Spier, 1984; Schachter, 1986; Yang and Butler, 2000).

Chena and Harcum (2006) studied the effects of elevated ammonium on 12 glycosylation related genes in Chinese hamster ovary cells which were evaluated by quantitative real time RT-PCR. Glucosamine-6-phosphate isomerase increased

linearly with time for the control culture, but it was time insensitive for the ammonium-treated culture. Study in baby-hamster kidney cell line BHK-21 indicated that GlcN6P-deaminase activity was induced under high ammonium concentrations (Gawlitzek, Valley and Wagner, 1998). It was also demonstrated that the disruption of glucosamine-6-phosphate deaminase affected the drug sensitivity and the virulence of *Candida albicans* (Yamada-Okabe and Yamada-Okabe, 2002).

1.9.3 C-type lectin

C-type lectin is a serum protein and is a key molecule in innate immunity. The structure of C-type allows it to bind to various microorganisms including Gram positive and negative bacteria, mycobacterium, virus and fungi. The binding of C-type lectin leads to agglutination of these microorganisms and will help their clearance by phagocytes. Some lectins have been identified in various aquatic invertebrates, such as tunicates (Nair, et al., 2000), sponges (Miarons and Fresno, 2000), crustaceans (Ratanapo and Chulavatnatol, 1990; Cominetti, et al., 2002; Maheswari, Mullaunadhan, and Arumugam, 2002; Alpuche, et al., 2005; Denis, et al., 2003), echinoderms (Hatakeyama, et al., 1994) and clams (Tunkijjanukij and Olafsen, 1998).

Study in human umbilical vein endothelial cells under endothelial oxidative stress increased the expression of human mannose-binding lectin deposition (Collard, Montalto and Stahl, 2000). Clinical studies also showed that levels of mannose binding lectin (MBL), an acute-phase protein, increased during HIV disease (Ji, Gewurz, and Spear, 2005).

Study of Bonura, et al., (2009) showed that *Ci*MBL gene was up-regulated by LPS injection into the ascidian (*Ciona intestinalis*) body wall. By comparing the expression pattern to a housekeeping gene, the result showed an enhanced level in *Ci*MBL mRNA expression after stimulation at each time point. LPS injection up-regulated the gene activity, and *Ci*MBL mRNA level was 6-fold increased at 1 h after the injection, then decreased at 2 and 4 h, and further increased in a gradual fashion within 24 h reaching its maximum peak of expression at 24 h.

1.10 Objectives

The objectives of this study are to:

-clone and characterize heat inducible genes including Aquaporin, Glucosamine-6-phosphate deaminase, and C-type lectin in *P. monodon.*

-determine the expression levels of target genes in heat-induced and un-induced shrimp during normal and stress conditions.

-produce recombinant protein of a target gene using *In vitro* expression technique.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental animals

Giant tiger shrimp, *P. monodon* (weight, 18-20 g) were obtained from local shrimp farm and acclimated to laboratory condition in rectangular tanks for 1 week prior to use. The tank contained approximately 170 l of seawater (salinity: 10-12‰; temperature: 28 °C). Ten shrimp were maintained in each tank and fed twice daily with commercial shrimp diet before the experiment.

2.2 Tissues preparation

Various tissues including gill, heart, hepatopancreas, lymphoid organ, intestine, stomach, epidermis, ovary, testis and muscle were dissected from *P. monodon* specimens. For haemocyte separation, haemolymph was withdrawn from ventral sinus cavity of each shrimp and mixed with equal volume of anticoagulant (10% sodium citrate). The mixture was centrifuged at 3600 g for 5 min, 4 ºC and the pellet containing haemocyte were collected. All tissues were either subjected directly to RNA and DNA extraction or deep frozen in liquid nitrogen and stored at -70 ºC for further use.

2.3 RNA extraction

Total RNA was isolated from dissected tissues using TRI REAGENT® (Molecular Research Center, INC). For haemocyte RNA extraction, haemocyte pellet (5-10 x 10^6 cells) was added to 0.2 ml of TRI REAGENT® and homogenized with a glass homogenizer to obtain the find homogenate. Eight hundred µl of TRI REAGENT® was further added and mixed vigorously. For other tissues, they were ground in the mortar containing liquid nitrogen to obtain the fine power and transferred to microcentrifuge tube containing 500 µl of TRI REAGENT®. The solution was homogenized using a glass homogenizer and added with 500 µl of TRI REAGENT®. The homogenate was vigorously mixed. Following the homogenization, the insoluble materials were removed from the solution by centrifugation at 12,000 g for 5 min at 4 ºC. The clear supernatant was transferred to the new microcentrifuge tube and stored at room temperature for 5 min to permit the complete dissociation of

nucleoprotein complexes. The solution was added with 0.2 ml chloroform per 1 ml of TRI REAGENT® and mixed vigorously for 15 min. The mixture was stored at room temperature for 15 min and centrifuged at 12,000 g for 15 min at 4 ºC. After centrifugation, the upper clear supernatant containing RNA was transferred to the new microcentrifuge tube. The aqueous phase was added with 0.5 ml isopropanal and stored at room temperature to precipitate RNA. The RNA pellet was obtained by centrifugation at 12,000 g for 10 min at 4 ºC. The obtained RNA pellet was washed by removal of the supernatant and adding 1 ml of 75% ethanol. The mixture was subsequently centrifuged at 12,000 g for 5 min. After centrifugation, 75% ethanol was removed and 1 ml absolute ethanol was added. The RNA pellet in absolute ethanol was kept at -80 ºC until use.

2.4 Determination of nucleic acid concentration

2.4.1 Spectrophotometry

DNA and RNA can be quantified by measuring the absorbance at the wavelength of 260 nm (A260). One A260 unit of double strand DNA, single strand RNA, and oligonucleotide equals to 50, 40, and 33 µg/ml, respectively (Sambrook and Law, 2001). The concentration of nucleic acid is calculated using the following equation:

Nucleic acid concentration (µg/ml) = A260 x absorbability coefficient x Dilution factor

The quality of nucleic acid was estimated by the ratio of A260 to A280. The isolated DNA that was free from RNA and protein, the A260 to A280 ratio should be higher than 1.7.

2.4.2 Agarose gel electrophoresis

DNA and RNA can be determined by agarose gel electrophoresis using 1.2 to 1.75 % agarose gels. Generally, agarose gel was prepared by adding agarose powder into 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid, and 2.0 mM EDTA), melt in microwave oven until completely dissolved, and then poured into the gel mould with an appropriate comb. The gel was left to solidify for at least 30 min at room temperature. The comb was gently removed and the gel was transferred into the electrophoresis chamber. TBE (1x) was added to cover the gel. Five µl of DNA or

RNA product was thoroughly mixed with one-tenth volume of 10x loading dye (0.25% bromophenol blue and 25% ficoll) and carefully applied into the gel slot. Two hundred µg of 100 bp DNA ladder and/or *λ-Hind* III was used as standard DNA marker. Electrophoresis was carried out at constant voltage of 100 volts until tracking dye reach about 1 cm from the lower edge of the gel. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for 3 min and destained to remove unbound ethidium bromide by submerging in water for 20 min. The DNA fragments were visualized under the UV light using UV transiluminator. The visible bands of DNA on the stained gel were photographed using camera Pentax K1000 (Asahi Opt. Co, Ltd).

2.5 Isolation and characterization of the full length cDNA of target genes using rapid amplification of cNDA ends-polymerase chain reaction (RACE-PCR)

2.5.1 Preparation of the 5' and 3' RACE template

Total RNA extracted from shrimp using TRI REAGENT® (Molecular Research Center, INC) as described in 2.3 was subjected to mRNA purification using oligo(dT)-cellulose (Amersham Biosciences®, UK). The purified mRNA was further reversed transcribed to RACE-Ready cDNA using a BD SMART™ RACE cDNA Amplification Kit (BD Clontech). RACE-Ready cDNA was prepared by combining 1 µg of mRNA with 1 µl of 5' CDS primer and 1 µl of 10 µM SMART II A oligonucleotide for 5' RACE-PCR and 1 µg of mRNA, 1 µl of 3' CDS primer A oligonucleotide for 3' RACE-PCR. The solution was gently mixed and briefly centrifuged. The reaction was incubated at 70 ºC for 2 min and immediately placed on ice for 2 min. The reaction tube was briefly centrifuged and added with 2 µl of 5X first strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase. The reaction was gently mixed and briefly centrifuged. The reaction was then incubated at 42 ºC for 1.5 h. The first strand reaction product was diluted with 125 µl of Tricine-EDTA buffer and heated at 72 ºC for 7 min. The first strand cDNA template can be stored at 20 ºC for up to three months.

2.5.2 Primers design

Gene specific primers (GSPs), including aquaporin, glucosamine-6-phosphate deaminase, and C-type lectin like protein were designed from the obtained nucleotide

sequences resulting from cloning and sequencing analysis. Antisense and/or sense primers were designed for 5' RACE-PCR and 3' RACE-PCR, respectively (Table 2.1-2.2).

Table 2.1 Primers sequences for first strand cDNA synthesis and RACE PCR**.**

Primer	Sequence
SMART II Oligonucleotide	5' AAGCAGTGGTATCAACGCAGAGTACGCGGG 3'
3' RACE CDS Primer A	5' AAGCAGTGGTATCAACGCAGAGTAC(T)$_{30}$V N 3' (N=A,C,G, or T; V=A, C, or G)
5' RACE CDS Primer	5' (T)$_{25}$V N 3' (N=A,C,G, or T; V=A, C, or G)
10X Universal Primer A Mix (UPM)	Long: 5' CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT 3' (0.4 µM) Short: 5' CTAATACGACTCACTATAGGGC 3' (2 µM)
Nested Universal Primer A (NUP)	5' AAGCAGTGGTATCAACGCAGAGT 3' (10 µM)

Table 2.2 Sequence, length, and the melting temperature of gene specific primers for RACE-PCR**.**

Gene	Sequence	Length (bp)	Tm
1.Aquaporin	3' RACE F: 5' GAC GAC CCA CAC CAT CTC AT 3' 5'RACE R: 5' GAG GAG ACC GCC CAT GAT AG 3'	20 20	59.85 61.90
2. C-type lectin like protein	3' RACE F: 5' CGG AAA ATG AAT CGG ACG AAG CAG AC3' 5'RACE R: 5' CGG AGG TCA GGT CAG CAT TCT CGT TC 3'	26 26	63.57 66.72
3. Glucosamine-6-phosphae deaminase	FL-GNPDAF: 5' ATG CGT CTG GTT ATC TTA GAC GAT3' FL-GNPDAR: 5' CAT TAC CGT ATT CGG AAT ATG ATT T 3'	24 25	58.55 55.42

2.5.3 RACE-PCR

Master mix of 5’ and 3’ RACE PCR reaction was prepared in a volume of 42.75 µl for each reaction. The mixture contained 5 µl of 10X Advantage 2 PCR buffer, 1 µl of dNTP mix (10 µM each) and 1 µl of 50X Advantage 2 polymerase mix. The PCR reaction condition for 5’ and 3’ cDNA ends are shown in Table 2.3.

When Tm of GSP>70 ºC, the reaction was carried out for 5 cycles composing of a 94 ºC for 30 sec, and 72 ºC for 2 min, 5 cycles composing of a 94 ºC for 30 sec, 70 ºC for 1 min and 72 ºC for 2 min, 20 cycles composing of 94 ºC for 30 sec, 68 ºC for 1 min and 72 ºC for 2 min. The final extension was carried out at 72 ºC for 7 min.

When Tm GSP< 70 ºC, the reaction was carried out for 25 cycles composing of a 94 ºC for 30 sec, 65-68 ºC for 1 min and 72 ºC for 2 min. The final extension was carried out 72 ºC for 7 min. The 5’ and 3’ RACE-PCR products were analysed using agarose gel electrophoresis.

Table 2.3 Component of RACE-PCR

Component	5’ RACE-Sample (µl)	3’ RACE-Sample (µl)
5’ RACE-Ready cDNA	1.25	-
5’ GSP (10 µM)	1	-
3’ RACE-Ready cDNA	-	1.25
3’ GSP (10 µM)	-	1
UPM	0.5	0.5
H_2O	1	1
Master mix	42.75	42.75
Final volume	50	50

2.5.4 Elution of DNA fragments from agarose gels

After electrophoresis, the desired DNA fragment was excised from the agarose gels (200-300 mg) using a sterile scalpel and placed in a pre-weighed microcentrifuge tube. DNA was isolated using HiYield ™ Gel/PCR DNA Extraction kit (RBC; Real

Biotech Corporation). DF buffer (0.5 ml) was added and mixed by vertex. The mixture was incubated at 55 ºC for 10-15 min or until the gel slice was completely dissolved. The mixture was transferred into a DF column inserted in a collection tube and centrifuged at 8,000 rpm for 30 sec. The flow-though solution was discarded. After this step, 0.5 ml of wash buffer was added to the DF column and centrifuged as above. The flow-though solution was discarded. The column was re-centrifuged to remove the trace amount of the washing solution. The DF column was then placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted out by an addition of 15 µl of the elution buffer or H_2O to the center of the DF membrane and left for 1 min before centrifugation at 12,000 rpm for 2 min.

2.5.5 Ligation of PCR product to pGEM-T easy vector

The ligation of DNA product to pGEM-T easy vector (Promega, USA) was performed in a final volume of 10 µl containing 3 µl of gel-eluted PCR product, 25 ng of pGEM-T easy vector, 5 µl of 2x rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM DTT, 2 mM ATP and 10% PEG 8000) and 3 Weiss unit of T4 DNA ligase. The ligation solution was gently mixed by pipetting and then incubated at 4 ºC overnight.

2.5.6 Transformation of ligation product to competent cells

2.5.6.1 Preparation of competent cells

Competent *E. coli* strain JM109 cells were prepared following Ausubel, et al. (1989) with modification. A single colony of *E. coli* was inoculated into 5 ml of sterile LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) and incubated at 37 ºC overnight with shaking. The culture was sub-inoculated by adding 1 ml of the culture into 50 ml of LB broth and incubated with vigorous shaking at 37 ºC until the OD600 was approximately 0.4-0.6. The culture was then placed on ice for 30 min and centrifuged at 3,000 rpm for 10 min. The supernatant was discarded before the cell pellet was resuspended with 30 ml of chilled $MgCl_2$-$CaCl_2$ solution (80 Mm $MgCl_2$ and 20 Mm $CaCl_2$) and kept on ice for 45 min. The solution was then centrifuged at 3,000 rpm for 10 min. The supernatant was discarded and cell pellet was resuspended with 2 ml of chilled 0.1 M $CaCl_2$ solution containing (15% glycerol). The cell suspension was divided into 200 µl aliquots in microcentrifuge tubes and stored at -80 ºC for subsequently used.

2.5.6.2 Transformation

A volume of 200 µl of competent cells was thawed on ice for 5 min and 5 µl of ligated DNA product was added. The solution was gently mixed by pipetting and placed on ice for 30 min. The mixture was placed in a 42 ºC water bath for 45 sec and immediately removed and placed on ice for 5 min. The solution was added in a test tube containing 1 ml of SOC medium (2% Baco tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) The solution was incubated at 37 ºC with vigorous shaking for 1.5 h. The solution was transferred to a microcentrifuge tube and centrifuged at 8,000 rpm for 1 min at room temperature. The supernatant was discarded and the cell pellet was resuspended with 100 µl of SOC medium. The cell solution was spread on LB agar containing 50 µg/ml of ampicillin, 25 µg/ml of IPTG, and 20 µg/ml of X-gal. The plate was incubated at 37 ºC overnight. The recombinant clones containing inserted DNA were observed as white colony whereas the clones without inserted DNA were blue colony.

2.5.7 Detection of recombinant clone using Colony PCR

The recombinant colony was screened for the size of inserted DNA using colony PCR. The PCR was performed in a volume of 25 µl containing 1x buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.2 µM of pUC1 (5’-CCG GCT CGT ATG TTG TGT GGA-3’) and pUC2 (5’-GTG CTG CAA GGC GAT TAA GTT GG-3’) primers and 0.5 U of DyNAzyme^{TM} II DNA Polymerase (Finnzymes, Finland). Individual of recombinant colony was picked using micropipette tip and mixed in the amplification reaction. The PCR profiles was predenatured at 94 ºC for 3 min, followed by 35 cycles of 94 ºC for 30 sec, 55 ºC for 60 sec, and 72 ºC for 60 sec, and a final extension at 72 ºC for 7 min. The resulting PCR product was analysed using agarose gel electrophoresis.

2.5.8 Plasmid DNA extraction

Plasmid DNA was isolated using a HiYIeld ^{TM} Plasmid Mini Kit (RBC; Real Biotech Corporation). A recombinant clone was inoculated into 3 ml of LB broth (1% tryotine, 0.5% yeast extract, 1.0 % NaCl) containing 50 µg/ml of ampicillin and incubated at 37 ºC with constant shaking at 250 rpm overnight. The culture was transferred into 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 1 min.

The supernatant was discarded. The bacterial cell pellet was collected and resuspended with 200 µl of the PD1 buffer containing RNaseA and thoroughly mixed by vortex. The resuspended cell were lysed by the addition of 200 µl of the PD2 buffer and mixed gently by inverting the tube 10 times. The mixture was stood for 2 min at room temperature. After that, 300 µl of the buffer PD3 was added to neutralize the alkaline lysis step and mixed immediately by inverting the tube for 10 times. To separate the cell debris, the mixture was centrifuged at 14,000 rpm for 1 min. The supernatant was transferred into a new microcentrifuge tube and to the PD column and centrifuged at 6,000 xg (8,000 rpm) for 1 min. The flow-through was discarded. The PD column was placed back in the collection tube. The column was washed by adding 400 µl of the W1 buffer and centrifuged at 6,000 xg (8,000rpm) for 1 min. After discarding the flow-though, 600 µl of the ethano-added Wash buffer was added and centrifuged as above. The flow-though was discarded. The spin tube was centrifuged for an additional 2 min at 14,000 rpm to remove the residual Wash buffer. The dried DF column was placed in a new 1.5 ml microcentrifuge tube and 30-50 µl of the elute buffer or water was added at the center of the column to elute the extracted plasmid DNA. The column was left at room temperature for 2 min and centrifuged at 14,000 rpm for 2 min. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.5.9 Digestion of the amplified DNA insert from colony PCR

The insert size of recombinant plasmid was examined by digestion with *ECo*RI. The digestion was performed in a volume of 15 µl reaction containing 1x restriction buffer (90 mM Tris-HCl; pH 7.5, 10 mM NaCl and 50 mM $MgCl_2$), 1 µg of recombinant plasmid and 2-3 units of *ECo*RI and incubated at 37 ºC for 3-4 h. The resulting digestion was analysed using agarose gel electrophoresis.

2.5.10 DNA Sequencing and analysis

pGEM-T easy vector containing target DNA fragment was purified and subjected to sequencing analysis using automated DNA sequencer. M13 forward and/or M13 reverse primers were used as the sequencing primers. Sequencing analyses were carried out by MACROGEN (Korea). The obtained nucleotide sequences were compared with GenBank database using BLAST search (NCBI) to identify homologous nucleotide sequence.

2.6 Analyses of tissue distribution of target gene

Total RNA was extracted from gills, heat, hepatopancreas, lymphoid organs, intestine, stomach, epidermis, ovaries, testes, and muscle of *P. monodon* and subjected to the first strand cDNA synthesis. After that, the obtaining cDNA from each tissue was used as template and together with a pair of primers designed from specific target gene for PCR amplification. The expression of target gene in each tissue was indicated by the presence of DNA fragment from PCR product.

2.6.1 First strand cDNA synthesis using Reverse Transcription Polymerase Chain Reaction

Total RNA extracted from target tissues was used as template for synthesizing first strand cDNA in reverse transcription. The reaction is performed in the final volume of 20 µl, at 42 ºC, for 90 min using Improm II ™ reverse transcription kit condition (1 U of Improm II ™ reverse transcription, 2 µl of 1x Improm II ™ reaction buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTP mix, 0.5 µg Oligo dT, and 2 U of recombinant RNasin® Ribonuclease inhibitor). The obtained first strand cDNA template was kept at -20 ºC until use.

2.6.2 Tissues distribution analyses by RT-PCR

Designed primers for amplifying target genes using Primer Premier 5 program (Table 2.4) and were used to study the tissue mRNA expression by PCR reactions. The primers set elongation factor F 5'GGT TGT CAA CTT TGC CCC3' and elongation factor R 5'TTG ACC TCC TTG ATC ACA CC 3' were designed to amplify the elongation factor transcript from the same RNA for comparison. The reaction mixture of PCR contained 10X PCR buffer (10 mMTris-HCl pH 8.8, 50 mM KCl and 0.1% TritonX-100), 0.2 mM dNTPs, 2 mM $MgCl_2$, 1U of Taq DNA polymerase, 200 ng of cDNA template and 0.5 µM of forward and reverse primers. For target gene amplification, the reaction mixture was carried out in the following, thermal cycle; 1 cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 50 °C for 45 sec and 72 °C for 45 sec with elongation stop at 72 °C for 7 min. PCR products were analysed by electrophoresis using 1.2% agarose gels in TBE buffer.

Table 2.4 Sequence, length, and the melting temperature of primers designed for characterized genes.

Gene	Sequence	Length (bp)	Tm
1. Glucosamine-6-phosphate deaminase	F: 5' CAG CCA ATG CCC GTT TGT 3' R: 5' ACC ACC ACC ACT GCG ACA 3'	18 18	57.53 59.58
2. C type lectin like protein	F: 5' CCG CTG CTT ATC TGG TCT TG 3' R: 5' GCT TCG GTC TCC GCA CTT T 3'	20 19	59.89 57.72
3. Aquaporin	F: 5' CGG GCG AAC TCC TGA ACG A 3' R: 5' ATG CCT GGG CCG TGT CAA T 3'	19 19	61.88 59.72

2.7 Single strand conformational polymorphism (SSCP) analysis

The successful amplification products of various gene homologues were further characterized using single strand conformational polymorphism (SSCP) to examine whether the amplification product of the same genes in different shrimp individuals were polymorphic.

2.7.1 Preparation of Glass Plates

The long glass plate was thoroughly wiped with 1 ml of 95% commercial grade ethanol in one direction with an absorbent paper. This process was then repeated twice. Afterwards, the long glass plate was coated with 1 ml of freshly prepared Bind silane (10 µl of Bind silane, Amersaham Biosciences; 995 µl of 95% ethanol and 10 µl of 5% glacial acetic acid) and left for approximately 10-15 minutes. Excess binding solution was removed with a piece of absorbent paper. The long glass plate was further cleaned with 95% ethanol for 3 times. The short glass plate was treated as described above with the exception that the binding solution was replaced by the Repel silane (2% dimethyldichorosilane in octamethlylcyclotetrasitoxone). The cleaned glass plates were assembled with a pair of 0.4 mM spacer.

Different concentrations of low crosslink non-denaturing polyacrylamide gels (37.5:1 or 75:1 of acrylamide and bis-acrylamide) were prepared by dilution of a 40% stock solution to required gel concentration. The acrylamide gel solution (30 – 40 ml)

was mixed with 240 µl of 10% APS and 24 µl of TEMED. The analytical comb was inserted into the prepared gel and allow for polymerization for 4 h or overnight.

For SSCP analysis, 6 µl of the amplified PCR products were mixed with 24 µl of the SSCP loading dye (95% formamide, 0.23% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured in a boiling bath for 5 min and immediately cooled on ice for 3 min. The denatured products were electrophoretically analysed in native polyacrylamide gels at 250 – 300 volts for 14-18 h at 4 °C (see SSCP condition in Appendix A). Fractionated bands were visualized by silver staining.

2.7.2 Silver staining

The gel plates were carefully separated. The long glass plate with the gel was placed in a plastic tray containing 1.5 l of the fix/stop solution and agitated well for 25–30 min. The gel was soaked with shaking 3 times for 3 min with deionized water. The gel was lifted out from the tray between each wash and allowed the washed water draining out of the gel for 4 sec. The gel was transferred to 0.1% silver nitrate (1.5 l) and incubated with agitation at room temperature for 30 min. The gel was soaked in 1.5 l of deionized water with shaking (10 forward and 10 backward steps) and immediately placed in the tray containing 1.5 l of the chilled developing solution. This step is crucial and the time taken to soak the gel in the water and transfer it to chilled developing solution should be no longer than 5 – 10 sec. The gel was then transferred to another tray containing 1.5 l of chilled developer and shaked unit bands for every lane were observed (usually 2 – 3 min). One liter of the fix/stop solution was directly added to the developing solution and continued shaking for 3 min. The stained gel was soaked in deionized water twice for 3 min each. The gel was dried at 56 °C for 2-3 h.

2.8 Determination of stress tolerance in heat induced shrimp

Stress tolerances of the shrimp induced by heat were determined by comparing the survival rate between the shrimp earlier induced by heat and the normal shrimp after they were stressed. Stress treatments of the shrimp were conducted in 2 different conditions, pathogenic and environmental stresses. Each step of this experiment was conducted as follow.

2.8.1 Heat induction of the shrimp

Shrimp acclimated in rectangular tanks for 1 week in ambient condition (salinity: 10-12 ‰; temperature: 28 °C) were heat-induced by moving the shrimp to a tank containing 35°C water and maintained the temperature for 2 h. Then, the shrimp were back to their previous tank which contained water at ambient condition (salinity: 10-12 ‰; temperature: 28°C). These heat induced shrimp were then subjected to stress treatment.

2.8.2 Pathogenic stress

White spot syndrome virus (WSSV) was used as pathogenic stressor in this experiment. First, a batch of WSSV was prepared and an appropriate dose of WSSV for *P. monodon* was examined by challenging the shrimp with different levels. The obtaining dose of WSSV was then used for challenging the heat induced shrimp and normal shrimp. Mortalities of normal and heat induced shrimp were monitored.

2.8.2.1 WSSV preparation

WSSV extracted from *P. monodon* and used in this study was provided by Dr. Pikul Jiravanicphisal (National Center for Genetic Engineering and Biotechnology). Stock of WSSV was prepared as described by Xie, Xu, and Yang, (2006) with slight modification. Briefly, a batch of WSSV was prepared by injecting WSSV into ventral sinus cavity of the shrimp (20 to 25 g each, n = 50). Moribund shrimp were collected 3 days after infection. All tissues excluding hepatopancreas were homogenized in TNE buffer (50 mM Tris-HCl, 400 mM NaCl and 5 mM EDTA, pH 8.5) and then centrifuged at 3,500 x g for 5 min at 4 ºC. The supernatant was saved, and the pellet was re-homogenized in TEN buffers. The supernatant was filtered through membrane paper (Whatman filter paper no.1) and centrifuged at 7,500 x g for 30 min at 4 ºC. The supernatant was saved and filtered through 0.45 µm. Stock of WSSV solution was kept at -80 ºC.

2.8.2.2 Identification of WSSV in shrimp by PCR technique

Shrimp were checked for the presence of WSSV by using the DNA extracted from the body of shrimp and detection of WSSV was carried out using polymerase chain reaction.

Genomic DNA was extracted from a piece of tissue using a phenol-chloroform-proteinase K method (Klinbunga, et al., 1996). A piece of frozen or fresh tissues was placed in an ice-chilled microcentrifuge tube containing 500 μl of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA and 200 mM NaCl, pH 8). The tissue was briefly homogenized with a micropestel and added with SDS (10%) and RNase A (10 mg/ml) solutions to a final concentration of 1 % (w/v) and 100 μg/ml, respectively. The mixture was incubated at 37 ºC for 1 h. At the end of incubation period, proteinase K solution was added to the final concentration of 300 μg/ml and further incubated at 55 ºC for 3-4 h. An equal volume of buffer-equlibrated phenol: chloroform: isoamylalcohol (25:24:1) was added and gently mixed for 10 min. The solution was centrifuged at 10,000, rpm for 10 min at room temperature. The aqueous phase was transferred into a new microcentrifuge tube. The solvent exchange process was repeated once with phenol: chloroform: isoamylalcohol (25:24:1) and once with chloroform: isoamylalcohol (24:1). The aqueous phase was transferred into a new microcentrifuge tube. One-tenth volume of 3 M sodium acetate, pH 5.2 was added to the aqueous solution. Two volume of chilled absolute ethanol was added and gently mixed to precipitate genomic DNA. The mixture was kept at -80 ºC for 30 min. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 min at room temperature and washed twice with 1 ml of 70% ethanol. After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 50-80 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA). The DNA solution was incubated at 37 ºC for 1-2 h and kept at 4 ºC until use.

The PCR reaction was conducted by combining the following ingredients in a final volume of 50 μl reaction; 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 0.5 mM each of dNTPs, 1 unit of DyNAzyme™ II DNA Polymerase (Finnzymes), 0.5 μM of each primer (WSSV F 5'GGA GGG TCA TCA AAT TCA G 3' and WSSV R 5'GCT GCA ATG TAT TCT GGG TCG 3') and 100 ng of template DNA. PCR profiles consisted of predenaturing step at 94ºC for 3 min, followed by 20-35 cycles of 94ºC for 30 sec, 50ºC for 45 sec, and 72ºC for 45 sec, and a final extension at 72ºC for 7 min.

2.8.2.3 Quantification of WSSV particle in WSSV stock solution

2.8.2.3.1 Preparation of WSSV DNA as standard template

PCR product of WSSV gene fragment was amplified (as described in 2.8.2.2) and eluted (as described in 2.5.4). Then, the concentration of eluted DNA was spectrophotometrically determined by measuring the absorbance at the wavelength of 260 nm.

2.8.2.3.2 Optimization of quantitative PCR condition

The appropriate PCR condition used in quantitative analysis were determined based on the criteria that the PCR product must be on the log phase of amplification. Various concentrations of WSSV DNA amplified in 2.8.2.3.1 were used as standard templates (1:100, 1:500, 1:1000, 1:5000, and 1:10000). Various number of PCR cycles were also applied. PCR was performed in a PCR thermal cycle (Hybraid Limited, England). Primers of WSSV F 5'GGA GGG TCA TCA AAT TCA G 3' and WSSV R 5'GCT GCA ATG TAT TCT GGG TCG 3' were used. The PCR reaction was conducted based on the standard condition which consisted of 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 0.5 mM each of dNTPs, 0.5 µM of each primer and 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes) in a final volume of 50 µl reaction. The standard PCR profiles consisted of predenaturing step at 94ºC for 3 min, followed by 20-35 cycles of 94ºC for 30 sec, 50ºC for 45 sec, and 72ºC for 45 sec, and a final extension at 72ºC for 7 min.

2.8.2.3.3 Determination of the correlation between the concentrations and intensities of WSSV DNA

Different dilutions of WSSV DNA (1:100, 1:500, 1:1000, 1:5000 and 1:10000 dilutions) which was obtained from 2.8.2.3.1 were prepared and PCR amplifications were conducted using these various concentrations of WSSV DNA as templates. The PCR condition was conducted using the optimal condition obtained from 2.8.2.3.2. The resulting PCR products were separated in agarose gel and band intensities of the PCR products were measured. The intensity values were used for plotting standard graph against concentrations of WSSV DNA fragments.

2.8.2.3.4 Quantification of WSSV particle

WSSV from stock was diluted into various concentrations (1, 1:10, 1:25, 1: 50, and 1:100) and used as templates in quantitative PCR amplification using the same optimal condition obtained from 2.8.2.3.2. The same pair of primers (WSSV F 5'GGA GGG TCA TCA AAT TCA G 3' and WSSV R 5'GCT GCA ATG TAT TCT GGG TCG 3') was also used. The resulting PCR products were subjected to band intensity analysis. The obtaining intensity values of WSSV were then applied to standard graph for calculating the numbers of WSSV.

2.8.2.4 LC_{50} of WSSV on *P. monodon*

Shrimp were challenged by immersion with WSSV at 4 levels including 0, 1:100, 1:500, and 1: 1000 dilutions of the WSSV stock. This challenge test was conducted using static water system. Mortalities were recorded for 7 days after the challenge.

2.8.2.5 Challenge of WSSV on normal and heat induced shrimp

The experiment on WSSV infection was divided into 3 groups as follow. In group 1 (normal group), 10 individuals were challenged by immersion in an aerated tank containing WSSV solution at the dilution of 1:266. In group 2 (heat induced group), 10 individuals were induced by heat at 35°C for 2 h before challenged with WSSV with the same dilution as group 1. The last group was a control group which was not challenged by WSSV. Two replicates were applied in each experiment. Mortalities were recorded daily in all groups and tissues were collected and subjected to gene expression analysis.

2.8.3 Environmental stress

Ammonia is a nitrogenous waste commonly found in polluted water was used as environmental stressor in this experiment. Ammonium chloride dissolved in water providing ammonia was used for treating shrimp in different concentrations. First, LC_{50} of ammonia to *P. monodon* was determined. Then, the appropriate concentration of ammonia was applied to normal and heat induced shrimp. The tolerance level of the shrimp on ammonia was determined by comparing survival rate between experimental groups.

2.8.3.1 LC_{50} of ammonia on *P. monodon*

Ammonia toxicity test on shrimp was conducted using static water system. Shrimp were separated into 5 groups and each group was maintained in sea water containing ammonia at the concentration of 0, 0.01, 0.1, 1 and 10 mg-N/l, respectively. Mortalities were recorded for 7 days after ammonia treatment.

2.8.3.2 Treatment of ammonia on normal and heat induced shrimp

The environmental stress experiment was conducted in similar pattern of pathogenic stress but ammonia was used as stressor instead of WSSV. The level of ammonia used in the experiment was obtained from the 96 h, LC_{50} value of ammonia-N (NH_3-N) which was 0.69 mg-N/l. Ammonia was prepared by the dilution of ammonium chloride. Two replicates were applied in each experiment. Mortalities of shrimp were recorded daily in all experiment groups and tissues were collected and subjected to gene expression analysis.

2.8.3.3 Determination of ammonia and nitrite concentrations

The concentrations of ammonia and nitrite were analyses by the method of Srickland and Parsons (1972).

2.8.3.3.1 Determination of ammonia

Sample (1 ml) was added to a microcentrifuge tube, followed by 0.04 ml of phenol solution (20 g of crystalline analytical reagent grade phenol dissolved in 200 ml of 95% v/v ethyl alcohol), 0.04 ml of sodium nitroprusside solution (1.0 g of sodium nitroprusside (Na_2 Fe $(CN)_5NO.2H_2O$) dissolved in 200 ml of de-ionized water), and 0.1 ml of oxidizing solution (freshly prepared mixture of 100 ml of Alkaline reagent solution 100 g of sodium citrate and 5 g of sodium hydroxide dissolved in 500 ml of de-ionized water and 25 ml of Sodium hypochlorite). The solution was mixed after each addition. The mixture was allowed to stand at room temperature for 1 h. Then, the optical density of the solution was measured at the wave length of 640 nm using spectrophotometer. Analytical reagent quality of ammonium sulphate, (0.1 g) dissolved in 1000 ml of distilled water was prepared and used as standard ammonia solution.

2.8.3.3.2 Determination of nitrite

One milliliter of sample was added to microcentrifuge tube, followed by 0.02 ml of sulphanilamide solution (5 g of sulphanilamide in 50 ml of concentrated hydrochoric acid and diluted to final volume of 500 ml with distilled water), 0.02 ml of naphthylethylenediamine solution (0.50 g of N-(1-naphthyl)-Ethylenediamine dihydrochloride solution in 500 ml of distilled water. The mixture was allowed to react for 2-8 min. The optical density of the solution was measured at the wave length of 543 nm using spectrophotometer. Anhydrous analytical reagent quality of sodium nitrite (0.345 g) dissolved in 1000 ml of distilled water was prepared and used as standard nitrite solution.

2.9 Expression analysis of target genes in heat inducted shrimp

Expression levels of target genes, including aquaporin, glucosamine-6-phosphate deaminase and C-type lectin like protein genes in normal and heat induced shrimp were analyzed using semi-quantitative RT-PCR analysis. Elongation factor 1 alpha gene was used as internal control gene.

2.9.1 Experimental animal

Acclimated shrimp (weight 20-25 g) were induced by heat as described in 2.8.1 and were subjected to stress as described in 2.8.2.5 and 2.8.3.2. Shrimp were collected at 24, 48, 72 and 96 h after stress treatment. Tissues samples (gill and hepatopancreas) from each shrimp were dissected and subjected to RNA extraction and cDNA synthesis.

2.9.2 Total RNA extraction and the first strand cDNA synthesis

Total RNA was extracted from gill and hepatopancrease of juvenile *P. monodon* using TRI REAGENT®. The first strand cDNA synthesis was carried out as described previously.

2.9.3 Semi-quantitative analysis

2.9.3.1 Primers design

Primers designed specifically for each target gene for semi-quantitative RT-PCR are shown in Table 2.5.

Table 2.5 Sequence, length and the melting temperature of primers designed for characterized genes.

Gene	Sequence	Length (bp)	Tm
1. Glucosamine-6-phosphate deaminase	F: 5' CAG CCA ATG CCC GTT TGT 3' R: 5' ACC ACC ACC ACT GCG ACA 3'	18 18	57.53 59.58
2. C type lectin like protein	F: 5' CCG CTG CTT ATC TGG TCT TG 3' R: 5' GCT TCG GTC TCC GCA CTT T 3'	20 19	59.89 57.72
3. Aquaporin	F: 5' CGG GCG AAC TCC TGA ACG A 3' R: 5' ATG CCT GGG CCG TGT CAA T 3'	19 19	61.88 59.72
4. Elongation factor - 1 alpha	F: 5' ATGGTTGTCAACTTTGCCCC 3' R: 5' TTGACCTCCTTGATCACACC 3'	20 20	 57.58

2.9.3.2 Optimization of PCR condition

Prior to the quantitative analysis, the appropriate PCR conditions including temperature, template concentration, number of cycles, and $MgCl_2$ concentration for each of target genes and reference gene were verified based on the criteria that the PCR product must be on the log phase of amplification.

PCR was performed in a PCR thermal cycle (Hybraid Limited, England). The PCR reaction was based on the standard condition consisted of 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1% Triton X-100), 0.2 mM each of dNTPs, and 1 unit of DyNAzyme^{TM} II DNA Polymerase (Finnzymes) in a final volume of 25 µl reaction.The standard PCR profiles consisted of predenaturing step at 94 ºC for 3 min, followed by 35 cycles of 94 ºC for 30 sec, 50-65 ºC for 45 sec (depending on the melting temperature of the primers), and 72 ºC for 45 sec, and a final extension at 72 ºC for 7 min. The condition was optimized as follows.

First, the annealing temperature for each target gene was adjusted within several degrees to obtain the best intensity and specificity of the target band. Then, PCR reactions with various concentrations of DNA templates (between 50 to 1000 ng) and amplified in different numbers of PCR cycles (20, 25, 30 and 35 cycles) were

carried out. The condition that amplified the PCR product in the exponential range and did not reach a plateau level was chosen. Also, the applications of $MgCl_2$ and primer concentration, ranged from 0.5, 1 and 1.5 mM for $MgCl_2$ and 0.05, 0.1, 0.15 and 0.2 μM for primers, were determined. The concentrations that gave the highest yield and specificity were chosen.

2.9.3.3 Semi-quantitative RT-PCR gel electrophoresis and data analysis

Semi-quantitative RT-PCR for each target gene was conducted using the optimized condition as shown in Table 3.10. The PCR product was analysed using agarose gel electrophoresis. The gel was stained with EtBr for 5 min and destained in thee running tap water for 15 min. The intensity of target band was examined using the Quantity I Program (BioRad). The expression ratio of target gene and elongation factor 1 alpha gene was analysed using statistical package in SPSS Version 11.5 for Window. The difference in expression ratio among groups of treatment was tested for normality and variance homogeneity using Shapiro-Wilk and Levene's test. Significant different among group of treatments was examined using Duncan test at $P<0.05$.

2.10 *In vitro* expression of the full length cDNA using the bacterial expression system

2.10.1 Designation of primers

A primer pair was designed to amplify the mature full length cDNA of aquaporin. The forward and reverse primers containing a *Nde* I and an *Eco*RI site encoded nucleotides are shown in Table 2.6.

2.10.2 Construction of recombinant plasmid in cloning and expression vectors

The mature full length cDNA of aquaporin was amplified, ligated, cloned into pGEM-T easy vector and transformed in to *E. coli* JM 109. Plasmid DNA of the positive clones was sequenced to confirm the orientation of recombinant clones and used as the template for amplification using the forward and reverse primer for cloning into the pET17b expression vector.

Table 2.6 Nucleotide sequences of primers used for *in vitro* expression of aquaporin.

Primer	Sequence
ORF	
AQ-ORF	F: 5' ACA TAT GGG CAA AAT CAA GGA CAT 3'
	R: 5' TTA AGC ATT GTT GGT TCT CTT GTT A 3'

The amplification product was digested with *Nde* I and *Eco*RI and analysed by agarose gel electrophoresis. The gel-eluted product was ligated into pET17b and transformed into *E. coli* JM 109. Plasmid DNA of the positive clones was subsequently transformed into *E. coli* BL21(DE3) *pLyss*

2.10.3 Expression of recombinant protein

A single colony of recombinant *E. coli* BL21(DE3) *pLyss* carrying desired recombinant plasmid was inoculated in 3 ml of LB medium, containing 50 µ g/ml ampicillin and 34 µg/ml chloramphinical at 37 °C overnight culture was transferred to 50 ml of LB medium containing g 50 µg/ml ampicillin and 34 µg/ml chloramphinical and further incubated to an OD600 of 0.4-0.6. After IPTG induction (1.0 mM final concentration), appropriate volume of the culture corresponding to the OD of 1.0 was taken with time-interval of 1, 2, 3, 6, 12 and 24 h and centrifuged at 12000 g for 1 min. The pellet was resuspended and examined by 15% SDS-PAGE. In addition, aliquots of 1 ml of the IPTG induced-culture (3 or 6 h) were centrifuged, resuspended in 1X PBS, and repeatedly freeze-thawed 4 times in liquid nitrogen. The protein concentration of both soluble and insoluble portions was measured using a dye-binding assay (Bradford, 1972). Overexpression of the recombinant protein was analysed by 15% SDS-PAGE.

CHAPTER III

RESULTS

3.1 Complete sequence of aquaporin, glucosamine-6-phosphate deaminase and C type lectin like protein genes

Full length sequences of aquaporin, glucosamine-6-phosphate deaminase and C type lectin-like protein genes were identified using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR) technique. Partial sequences of these target genes, which were used for designing RACE-PCR primers, were obtained from EST transcripts of heat-induced haemocyte and gill libraries. Sequences of EST transcripts and 5' and 3' RACE-PCR primers of these genes are shown in appendix B).

5' and 3' RACE-PCR of aquaporin gene generated fragments of 1,500 and 1,900 bp, respectively (Fig 3.1). The full length cDNA of aquaporin of *P. monodon* was obtained at 2,943 bp in length with an open reading frame (ORF) of 786 bp corresponding to 261 amino acids (Fig. 3.3). This transcript significantly matched with aquaporin1 from many organisms including *Coptotermes formosanus* (E-value = 8e-69), *Aedes aegypti* (E-value = 5e-65), and *Danio rerio* (E-value = 2e-57) (BLAST result in appendix C). A deduced aquaporin protein contained a mammalian major intrinsic protein (MIP) located at position 16-234 of the deduced protein sequence (E-value = 3.10e-96) (result of *domain prediction* in appendix D). Calculated molecular weight (MW) and isoelectric point (p*I*) of the deduced aquaporin were 27.77 kDa and 7.57, respectively.

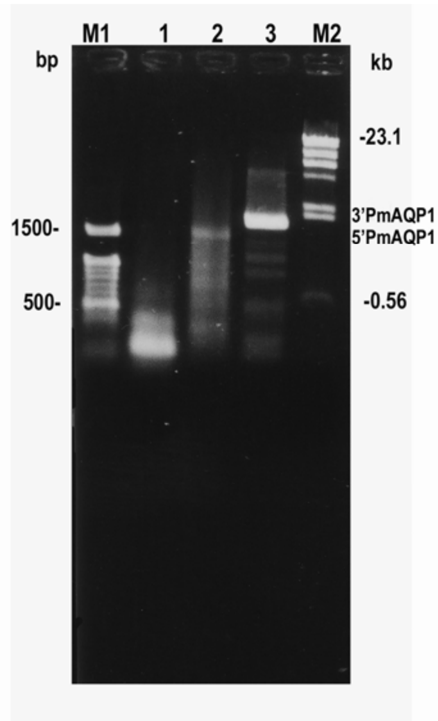

Figure 3.1 5' and 3' RACE-PCR of Aquaporin (Lane 2-3). Lane M1 is 100 bp DNA ladder. Lane M2 is λ Hind III DNA ladder.

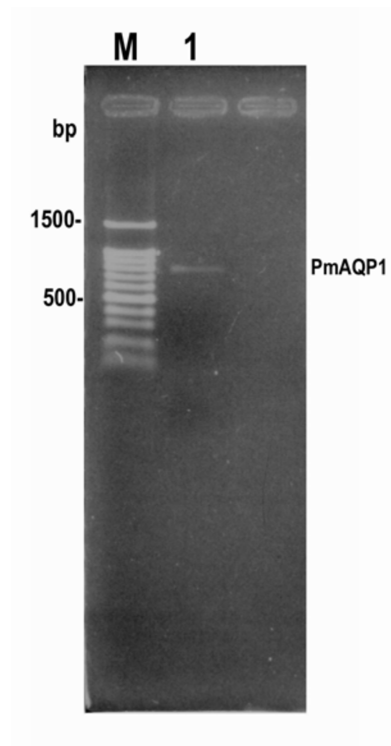

Figure 3.2 PCR product of full length cDNA sequence of aquaporin gene (Lane 1). Lane M is 100 bp DNA ladder.

```
   1 ACGCGGGGGGGGGGGCGAGTCTTCTCGCGACTGTCAAGCTGAGAACGTCGGCCCAGGATTCCTTCCGCGA 70
  71 CGCACGTGCATATATATATCTCTCTCTCTCGCTCTCTTTCTCTCTCATACTCACAAGTCTTTTTTTTACG 140
 141 TCTATTCCCTCTCGAGTTTTATTCGATCGCGTTTAATTCCCGCATCTGAGAAGGGAAGCGGAAAGAAAGG 210
 211 ACACTTAGAAATATAAAAAAAAAGACAGAAAAAAAAAAACACACCTGCATTAGGAAGAACAAAACGAGAGA 280
 281 GAGAGAGAGATTAACAAAAGACCAAAAAAAAACAAAACCCCACACACGCCCGAGAAAGGCCACAAGAGCGC 350
           M  G  K  I  K  D  M  K  E  Y  I  G  T  G  E  L  L  N  D  R  R  21
 351 CGTCGACCATGGGCAAAATCAAGGACATGAAGGAGTACATCGGCACGGGCGAACTCCTGAACGACCGCCG 420
      V  W  K  A  F  L  A  E  F  L  G  T  M  F  L  V  F  I  G  C  G  S  C  44
 421 CGTGTGGAAAGCCTTCCTGGCGGAGTTCCTGGGCACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGC 490
     I  G  S  W  S  E  G  Y  A  P  S  V  V  Q  I  S  L  A  F  G  V  T  V  A 68
 491 ATCGGCTCCTGGAGCGAAGGCTACGCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGG 560
       S  I  A  Q  A  V  G  H  V  S  G  C  H  I  N  P  A  V  T  C  A  M  L  91
 561 CGTCCATCGCGCAGGCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCGCCATGCT 630
      V  A  R  H  V  S  V  I  R  A  L  I  Y  I  V  C  Q  C  L  G  A  I  V  114
 631 TGTTGCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCCAGTGCCTTGGTGCCATCGTA 700
     G  A  A  I  L  K  G  V  T  P  A  D  I  Q  G  S  L  G  M  T  L  R  N  E 138
 701 GGGGCTGCCATTCTGAAGGGAGTGACCCCCGCAGACATCCAGGGGTCCCTCGGCATGACCCTGAGAAATG 770
       K  I  D  T  A  Q  A  L  G  I  E  L  L  I  T  F  V  L  V  I  T  V  F  161
 771 AGAAGATTGACACGGCCCAGGCATTGGGCATCGAGCTCCTCATCACTTTCGTCCTAGTCATCACCGTCTT 840
      G  A  C  D  E  R  R  N  D  V  K  G  S  A  P  L  A  I  G  L  S  I  T  184
 841 CGGGGCGTGTGATGAGCGAAGGAATGACGTGAAGGGGTCAGCTCCTCTCGCCATTGGCCTGTCGATCACC 910
     T  C  H  L  F  A  V  P  I  T  G  S  S  M  N  P  A  R  S  F  G  P  A  V 208
 911 ACTTGTCATCTCTTCGCCGTCCCCATCACCGGATCCTCGATGAACCCGGCAAGGAGCTTCGGTCCAGCGG 980
       I  S  G  L  W  Q  D  H  W  V  Y  W  A  G  P  I  L  G  G  L  A  A  A  231
 981 TCATTTCCGGTCTCTGGCAGGACCACTGGGTGTACTGGGCTGGTCCTATTCTTGGTGGTCTCGCAGCCGC 1050
      L  I  Y  S  Y  V  F  R  A  P  K  D  A  A  A  Y  D  V  E  M  D  N  Y  254
1051 TCTAATCTACTCCTACGTTTTCCGAGCTCCCAAGGACGCCGCTGCTTATGACGTGGAGATGGACAACTAT 1120
     N  K  R  T  N  N  A  *                                                261
1121 AACAAGAGAACCAACAATGCTTAAATGGAAAAGATCTACTGGCTCGGCCCTATCATGGGCGGTCTCCTCG 1190
1191 CGGGCCGTATTTACAAAATAACCTGGCCAGGAGATGACGAAGCCGAAGAGGAAAACAACCACGGGAAGAA 1260
1261 CAAGAAGGAGAGCGTAAAGAACGGCGAGAAAGAGAGGAACGGCGAGAGAGAGAGAAGAGTGACTGTAGGTAC 1330
1331 GAGCTGACCATCCCCATCGACGACCTCACTGGGCATCAGTTCCAAAAAGATCAGTATAGTTGCCGCTTGA 1400
1401 GTGAGAAGGACGACCCACACCATCTCATGGAAGAGGAAGAGGAATACGATATGAATGAAGAGGAAGAGGA 1470
1471 GAAGAAGAAGTTGGTCGATGTGGAGAAGGTGGTTATTGTTAAGTCTCAGAAAAAGAATGAAGAGGAGGAG 1540
1541 ATACAAGAGAAGGAAAACGACTGTGTGATATCGGAGAGGACGACCGTTATATAAGTGTCATTTCGGGTCT 1610
1611 TTAGTCCTTCATTAGCTACAATAAGACTAAACCATTTTTTTCCACCAATTTTGAAAAAGAATGAAGAGGA 1680
1681 GGAGATACAAGAGAAGGAAAACGACTGTGTGATATCGGAGAGGACGACCGTTATATAAGTGTCATTTCGG 1750
1751 GTCTTTAGTCCTTCATTAGCTACAATAAGACTAAACCATTTTTTTTCACAAGTTTTGCTCTTGTTCAGTC 1820
1821 TATTTATGATCTTTTATTCATAGATTATCATTAGCTTTCATATCAGTATTTGTATTTTTATTTGGGTATC 1890
1891 CCCCATGTATTCGTTTTCTTTCTCTTGCAACGCTTGTAATGTAAGGGAAACTAAAACAAAAATGCTGCTC 1960
1961 GTGATATTCCTCATTTCTGGTTATATTGTGTGCATTGCTTGATAAAAACTTACCAAAAAGGATATGCAAA 2030
2031 GCAGATTAAACTTCTTTTTTGCTTAAAGCTTTAAGTAAATAAGCAGAATCTGCAACATAATGTAAAGTAG 2100
2101 TAAAGTTACATCATATTGATGATGATTATTTTTCATGTCTCTAATCATTTATTTGTAATCTTTAATTCA 2170
2171 TGTAAAGTAAGATATGGCTTTCAAGTATACTGTCATCACAAAATTACATACATTATTCATTTAAACCATT 2240
2241 TGTGTTCACGAGGTTAGGAATTCTTCTCATTAACTTTCCCTTAAATGTGTTAGTTAACACTTTTTTTTCG 2310
2311 CTCAGATAGTGATTTCAGTGTATTCTGTTCTGACTAGTTTTTAGATCTTTTTCTCTCTCCATAACTGCCT 2380
2381 TGTCTATATAGAAAGAATATATGTATAAAAATAAGAATGAATAACAAAGCGAATGATGTCGAGTCATTTT 2450
2451 ACAACGCAACAGAAATAACAGAATAAGGAATAAACTCCCATGAAATACTCTATTGCAATTTTGATTAAAA 2520
2521 TTTAATCGAAACGCTGCCTGTGTTATCATGTTATTCATTCTGTTTTTGTCATGATAAAGTCCATTCATAT 2590
2591 ATTTTAATGATACTACGGTAATTTTCGTGTTGATTCGATGCAGAGACAGATAGGCACAGTTGTTCCGTAT 2660
2661 TTTAAGACTATCATAGATATAGCAAGTATTTTTATATAGACAATTAACTAATTCCTGCCAGAGCAAAACA 2730
2731 CTTCAGATGGCAGTTTTAAACTACACCTTTCTAGTAAACAATACAAGCAGACAAATTCTATTGCAGTTGT 2800
2801 GTTTGTTATATATTTTTTTGGAATAAGCGTGTTTCTTGCAACTCGGATTTAGAGTTAGCCAAAGAAGCCC 2870
2871 CTAGTTTTAATGTGATTGAATAATAAAGAAATTAAGATAAGAACGAAAAAAAAAAAAAAAAAAAAAAAAA 2940
2941 AAG 2943
```

Figure 3.3 Full length cDNA and deduced amino acid sequences of *P .monodon* aquaporin (PmAQP1). ORF consists of 786 bp, corresponding to 261 amino acid residues. Start and stop codons are illustrated in bold and underlined. A mammalian major intrinsic protein (position 16-234, E-value =3.70e-96) is highlighted.

```
               ....|....| ....|....| ....|....| ....|....| ....|....|
                   5         15        25        35        45
A.aegypti      MTESAGVK-Q LVGVADITEN RNIWRMLVAE FLGTFFLVSI G--IGSTMG-
P. monodon     MGKIKDMK-E YIGTGELLND RRVWKAFLAE FLGTMFLVFI G--CGSCIGS
A.anguilla     -----MMK-E LK-------S KAFWRAVLAE LLGMTLFIFL S--IAAAIGN
D. rerio       MRVVLLMQ-Q YHGSL-RCVD SGILAGGFRR VFGHDNIRAA QSWIHHQLGS
Clustal Co           :: :          .    .     . . .:*   :           :*

               ....|....| ....|....| ....|....| ....|....| ....|....|
                   55        65        75        85        95
A.aegypti      WGGDYAPTMT QIAFTFGLVV ATLAQAFGHV SGCHIN-PAV TIGLMITADI
P. monodon     WSEGYAPSVV QISLAFGVTV ASIAQAVGHV SGCHIN-PAV TCAMLVARHV
A.anguilla     PNNSNPDQEV KVSLAFGLSI ATLAQSLGHI SGAHLN-PAV TLGMLASCQI
D. rerio       KTGEPSTRRP CPHLPLLWLV HRNPRPVFRA HQRRPHQPGR YCGNGCYTEA
Clustal Co           .          :.:   :     .:.. :      : : *.     .     .

               ....|....| ....|....| ....|....| ....|....| ....|....|
                  105       115       125       135       145
A.aegypti      SILKGAFYIV SQCVGAIAG- AALIKAATPS -DVIGGLGVT GIDPRLTAGQ
P. monodon     SVIRALIYIV CQCLGAIVG- AAILKGVTPA TDIQGSLGMT LRNEKIDTAQ
A.anguilla     SMLKAVMYIV AQMLGASVA- SGIVYGVRPE --GVTALGLN SLN-KITPSQ
D. rerio       ESGEGCVLFV GTVFGGSSGS SHLVWSDTGV SERRNGSDVC TRDLRWSCYC
Clustal Co     .  .. . :*     .*.  .  : :: .             . .:     : :

               ....|....| ....|....| ....|....| ....|....| ....|....|
                  155       165       175       185       195
A.aegypti      GVMIEAL-IT FILVFVVHGV CDNRRSDIKG SAPLAIGLS- ----ITAGHL
P. monodon     ALGIELL-IT FVLVITVFGA CDERRNDVKG SAPLAIGLS- ----ITTCHL
A.anguilla     GVGVELL-AT FQLVLCVIAT TDKRRRDVTG SAPLAIGLS- ----VALGHL
D. rerio       DRAHNHF-AC FHCLCYL-PQ ASSQRFGSTG YWSVCVHRPS LCDPVHWGQY
Clustal Co         : :     *  :  :      ..:* . .*    .:.:  .        :   :

               ....|....| ....|....| ....|....| ....|....| ....|....|
                  205       215       225       235       245
A.aegypti      SAIKYTGASM NPARSFGPAV VMG---NWTD QWVYWV---- ----------
P. monodon     FAVPITGSSM NPARSFGPAV ISG---LWQD HWVYWA---- ----------
A.anguilla     TAISFTGCGI NPARSFGPAV ILG---DFSD HWVYWV---- ----------
D. rerio       ESSSLFWSRS HHGKMAGPLG VLGGPFNRRN PCCSCI-IPL LS-P-PEAPL
Clustal Co      :      .   : .:  **    : *       :

               ....|....| ....|....| ....|....| ....|....| ....|....|
                  255       265       275       285       295
A.aegypti      --GPIVGGIL AGAVYRLFFK VRKD------ -------EH- ----------
P. monodon     --GPILGGLA AALIYSYVFR APKD------ -------AAA YDVEMDNYNK
A.anguilla     --GPMCGGVA AALVYDFLLH PKFDDFPERM KVLVSGPDGD YDVNGPDDVP
D. rerio       C-RPLQEPLP DGAISSGGHR LVPQRSGSAH GQAGGTQSAG FGEEGARVHR
Clustal Co        *:   :    . :      :     :

               ....|...
                  305
A.aegypti      --------
P. monodon     RTNNA---
A.anguilla     AVEMSSK-
D. rerio       RGAVIRM-
Clustal Co
```

Figure 3.4 Multiple alignments of deduced amino acid sequence of *P. monodon* aquaporin gene and the sequences of *A. aegypti, A. anguilla*, and *D. rerio*.

Transcript containing complete cDNA sequence of GluN6P-deaminase was obtained from heat-induced gill EST library of *P. monodon*. Figure 3.5 shows PCR product covering full length of GluN6P-deaminase gene. Complete cDNA sequence of GluN6P-deaminase consisted of 1,058 bp with an ORF of 948 bp corresponding to 315 amino acids (Fig. 3.6). This transcript significantly matched with GluN6P deaminase reported in many organisms including *Culex guinquefasctus* (E-value =

3e-122), *Aedes aegypti* (E-value = 3e-122) and *Danio rerio* (E-value = 4e-118) (BLAST result in appendix C). A deduced GluN6P-deaminase protein contained a Glucosamine_iso domain located at position 15-250 of the deduced protein (E-value = 2.10e-171) (result of *domain prediction* in appendix D). Calculated MW and p*I* of the deduced GluN6P-deaminase from its ORF were 35.92 kDa and 8.95, respectively.

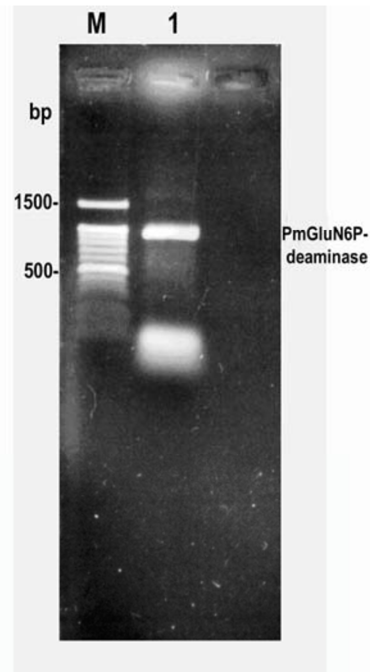

Figure 3.5 PCR product of full length cDNA sequence of Glucosamine-6-phosphate deaminase gene (Lane 1). Lane M is 100 bp DNA ladder.

```
                                             M  R  L  V  I  L  D  D  A 9
   1 GGAGAAGGACATTCGTTGGCAATTGCAAGAAGAAGATGCGTCTGGTTATCTTAGACGATG 60
       G  R  V  A  E  W  A  A  R  Y  I  I  K  R  I  N  D  F  K  P 29
  61 CGGGCAGAGTGGCCGAGTGGGCAGCGAGATATATCATCAAACGGATCAACGACTTTAAAC 120
       N  P  E  K  F  F  V  L  G  L  P  T  G  S  T  P  L  G  T  Y 49
 121 CAAACCCAGAGAAGTTCTTTGTCCTTGGCTTGCCCACAGGAAGCACTCCATTGGGCACTT 180
       Q  R  L  V  Q  Y  H  K  A  G  K  I  S  F  K  Y  V  K  T  F 69
 181 ACCAAAGACTAGTGCAATATCATAAAGCAGGAAAGATTTCATTCAAATATGTCAAGACGT 240
       N  M  D  E  Y  V  G  I  P  R  D  H  S  Q  S  Y  H  T  F  M 89
 241 TTAACATGGACGAATATGTTGGTATCCCAAGGGACCACTCCCAGAGCTATCACACCTTCA 300
       W  E  N  L  F  K  H  I  D  I  D  P  A  N  A  H  I  L  D  G 109
 301 TGTGGGAGAATCTCTTCAAGCACATAGACATTGACCCTGCTAATGCACACATTCTGGACG 360
       N  A  Q  D  L  E  K  E  C  Q  L  Y  E  Q  K  I  K  E  A  G 129
 361 GCAATGCACAGGACCTGGAGAAAGAGTGCCAGTTGTATGAGCAGAAGATTAAGGAAGCCG 420
       G  I  E  L  F  M  G  G  I  G  P  D  G  H  I  A  F  N  E  P 149
 421 GTGGGATCGAGTTATTTATGGGAGGTATAGGCCCAGATGGCCACATTGCTTTCAATGAGC 480
       G  S  S  L  V  S  R  T  R  V  K  T  L  N  Q  E  T  I  T  A 169
 481 CAGGCTCTAGCCTTGTTTCGCGAACCCGTGTCAAGACACTCAACCAAGAGACCATCACAG 540
       N  A  R  F  F  N  N  D  M  S  K  V  P  K  Q  S  L  T  V  G 189
 541 CCAATGCCCGTTTCTTTAACAATGACATGTCTAAAGTTCCAAAGCAGTCACTTACTGTGG 600
       V  G  T  V  M  D  A  R  E  V  M  I  L  I  T  G  S  H  K  A 209
 601 GCGTTGGGACTGTTATGGATGCCAGAGAGGTAATGATCCTTATCACGGGATCACACAAGG 660
       Y  A  L  H  M  A  I  E  E  G  V  N  H  M  W  T  V  S  A  F 229
 661 CCTATGCCCTTCACATGGCGATTGAGGAAGGGGTCAACCACATGTGGACTGTATCAGCAT 720
       Q  Q  H  P  R  T  L  M  L  C  D  E  D  A  T  L  E  L  K  V 249
 721 TCCAGCAACACCCCCGCACCCTTATGCTCTGTGATGAGGATGCGACTCTTGAGCTGAAGG 780
       K  T  V  K  Y  F  K  D  L  W  S  V  H  S  K  L  I  D  D  P 269
 781 TCAAGACTGTCAAGTACTTTAAGGATTTGTGGTCGGTGCACAGCAAACTCATTGACGACC 840
       M  M  A  S  F  Q  E  S  R  M  K  P  F  R  A  F  H  R  N  C 289
 841 CAATGATGGCATCGTTTCAAGAGTCAAGGATGAAGCCGTTTCGTGCTTTTCACAGAAATT 900
       R  S  G  G  G  H  S  F  L  Y  G  G  G  L  M  I  K  D  F  E 309
 901 GTCGCAGTGGTGGTGGTCATTCATTTCTTTACGGTGGAGGCTTAATGATAAAAGATTTTG 960
       I  I  F  R  I  R  *                                         315
 961 AAATCATATTCCGAATACGGTAATGGTGCTATTGATCATTGAGTAAGATTGAGTTTTTAT 1020
1021 TTTTGGTGAATTATTTATAAAAAAAAAAAAAAAAAAA 1058
```

Figure 3.6 Full length cDNA and deduced amino acid sequences of *P. monodon* Glucosamine-6-phosphate deaminase (PmGluN6P-deaminase). ORF consists of 948 bp, corresponding to 315 amino acid residues. Start and stop codons are illustrated in bold and underlined. A Glucosamine_iso domain (position 15-250, E-value =2.10e-171) is highlighted.

```
                        ....|....| ....|....| ....|....| ....|....| ....|....|
                            5         15         25         35         45
C. intestinalis         MRLVILDDYD KVSLWAAKYI RNRINEFNPG PEKFFVLGLP TGSTPLGTYK
N. vitripenis           MRLVICDDSA NVAEWSAKYV LKRIHDFKPN ENKYFVLGLP TGGTPLGMYK
C. quinquefasciatus     MRLIILDTAA YVGEWSAKYV MKRINDFKPG PDKYFTLGLP TGSTPLGLYK
A. aegypti              MRLIILDTSD YVGEWSAKYV MKRINDFKPG PSRFFTLGLP TGSTPLGLYR
P. monodon              MRLVILDDAG RVAEWAARYI IKRINDFKPN PEKFFVLGLP TGSTPLGTYQ
Clustal Co              ***:* *     *. *:*:*:  :**::*:*.  .::*.**** **.**** *:

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                           55         65         75         85         95
C. intestinalis         KLIEFYKSGA LSFKYVKTFN MDEYVNLPRD HPESYHSFMW ENLFKHIDID
N. vitripenis           KLIEYYKAGK ISFKYVKTFN MDEYVDLPRD HPESYHYYMY NNFFKHIDID
C. quinquefasciatus     NLIKFHKEGK ISFKYVKTFN MDEYVDLARD HPESYHYFMW HNFFKHIDID
A. aegypti              NLIKFHQEGK ISFKYVKTFN MDEYVDLPRD HPESYHYFMW HNFFKHIDID
P. monodon              RLVQYHKAGK ISFKYVKTFN MDEYVGIPRD HSQSYHTFMW ENLFKHIDID
Clustal Co              .*::::: *  :********* *****.:.** *.:*** :*: .*:*******

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                          105        115        125        135        145
C. intestinalis         PKNAHILDGN AEDVIKECQS FEDKIKEAGG VNLFVGGIGP DGHIAFNEPG
N. vitripenis           PENVHILDGN AKDLEHECNE FERKMKEAGG VELFIGGIGP DGHIAFNEPG
C. quinquefasciatus     PQNVHILDGN APDLVAECDA FEEKIKAAGG IELFIGGIGP DGHIAFNEPG
A. aegypti              PVNVHILDGN APDLVAECNA FEDKIKAAGG IELFIGGIGP DGHIAFNEPG
P. monodon              PANAHILDGN AQDLEKECQL YEQKIKEAGG IELFMGGIGP DGHIAFNEPG
Clustal Co              * *.****** * *:  **:  :* *:* *** ::**:***** **********

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                          155        165        175        185        195
C. intestinalis         SSLVSRTRVK TLAQDTILAN ARFFDNDLSQ VPRQALTVGV GTVMDAEEVM
N. vitripenis           SSLASRTRVK TLAQDTLEAN ARFFGNDVNK VPKQALTVGV GTVMDAKEVM
C. quinquefasciatus     SSLVSRTRVK TLAQDTLEAN ARFFGNDISK VPKQALTVGV GTVMDAREVM
A. aegypti              SSLVSRTRVK TLAQDTLEAN ARFFGNDISK VPKQALTVGV GTVMDAREVM
P. monodon              SSLVSRTRVK TLNQETITAN ARFFNNDMSK VPKQSLTVGV GTVMDAREVM
Clustal Co              ***.****** ** *:*: ** ****.**:.: **:*:***** ******.***

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                          205        215        225        235        245
C. intestinalis         ILITGAHKSY ALHKAIEGGV SHMWTVSAFQ QHPRTIFICD EDATLELKVK
N. vitripenis           ILITGSHKAF ALYKAIEEGI NHMWTVSAFQ QHPRTLIICD EDATLELRVK
C. quinquefasciatus     ILITGAHKAF ALYKAIEEGV NHMWTVSAFQ QHQHTIMVCD EDATLELRVK
A. aegypti              ILIIGAHKAF ALYKAIEEGV NHMWTVSAFQ QHPHTIMICD EDATLELRVK
P. monodon              ILITGSHKAY ALHMAIEEGV NHMWTVSAFQ QHPRTLMLCD EDATLELKVK
Clustal Co              *** *:**:: **: *** *: .********* ** :*:::** *******:**

                        ....|....| ....|....| ....|....| ....|....| ....|....|
                          255        265        275        285        295
C. intestinalis         TVKYFKGLME IHNKLVEERV PTAQLTES-- ---------- ----------
N. vitripenis           TVKYFKALSA VHHKLIEEDG EAFPRRPQNN A--------- ----------
C. quinquefasciatus     TVKYFKSLYD VHSKLIEGA- ---------- ---------- ----------
A. aegypti              TVKYFKSLYD VHSKLIEGT- ---------- ---------- ----------
P. monodon              TVKYFKDLWS VHSKLIDDPM MASFQESRMK PFRAFHRNCR SGGGHSFLYG
Clustal Co              ****** *    :* **::

                        ....|....| ....|
                          305        315
C. intestinalis         ---------- -----
N. vitripenis           ---------- -----
C. quinquefasciatus     ---------- -----
A. aegypti              ---------- -----
P. monodon              GGLMIKDFEI IFRIR
Clustal Co
```

Figure 3.7 Multiple alignments of deduced amino acid sequence of *P. monodon* GluN6P-deaminase and the sequences of *C. intestinalis*, *N. vitripenis*, *C. quinquefasciatus*, and *A. aegypti*.

Partial cDNA sequence of C-type lectin like protein gene was obtained from the transcript of heat-induced gill EST library. RACE primers specific to the sequence of this transcript were created. 5’ and 3’ RACE–PCR were carried out and the results generated fragments of 900 and 800 bp, respectively (Fig 3.8 and 3.9). After sequencing analysis, complete sequence of the gene was obtained by overlapping the 2 fragments. Full length cDNA of *P. monodon* C type lectin like protein gene consisted of 1,274 bp with an ORF of 678 bp corresponding to 225 amino acids (Fig.3.11). BLAST result (Appendix C) indicated that *P. monodon* C type lectin like protein gene was similar to lectin of *Dicentrarchus labrax* (E-value = 6e-8), *Danio rerio* (E-value = 6e-8) and *Ctenopharyngodon idella* (E-value = 5e-7). This gene contained a CLECT domain, a C-type lectin (CTL) or carbohydrate-recognition domain (CRD), located at position 37-157 of the deduced protein (E-value = 1.18e-12) (result of *domain prediction* in appendix D), confirming the presence of unique identity of C type lectin. Calculated MW and p*I* of the deduced protein were 24.75 kDa and 4.60, respectively.

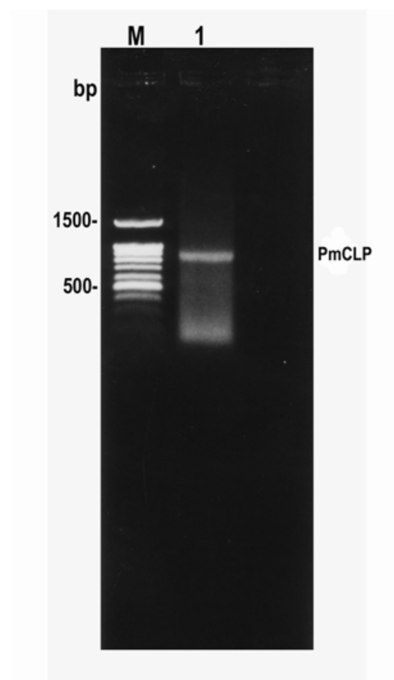

Figure 3.8 5’ RACE-PCR product of C type lectin like protein gene (lane 1). Lane is 100 bp DNA ladder.

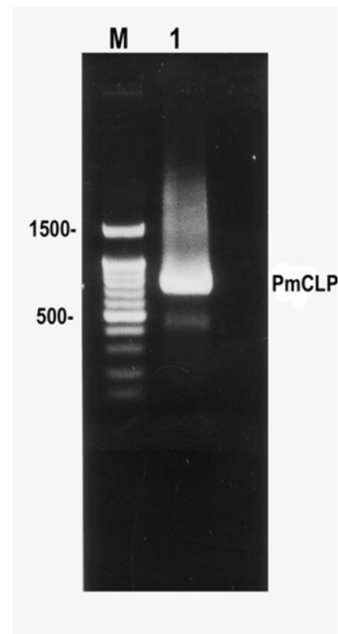

Figure 3.9 3' RACE-PCR product of C type lectin like protein gene (lane 1). Lane is 100 bp DNA ladder.

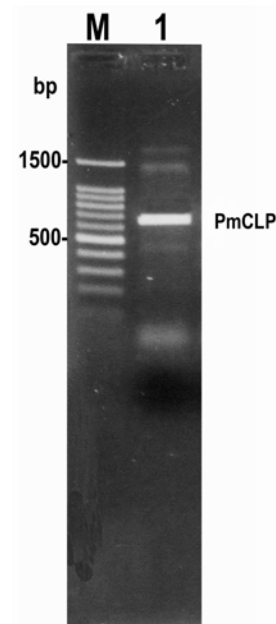

Figure 3.10 PCR product of full length cDNA sequence of C type lectin like protein gene (Lane 1). Lane M is 100 bp DNA ladder.

```
   1 ACGCGGGGGTTAGTTGTCATCAGAAGACCGCGTCAGTATCTCGTGTGCTCGCTGGCTGCT 60
               M  T  S  L  T  T  S  L  L  F  T  A  T  S  L  L  F  T 18
  61 GTTGCAAGATGACTTCATTGACAACAAGTCTTTTATTCACCGCAACAAGTCTTTTATTCA 120
       A  A  Y  L  V  L  V  P  S  L  A  V  S  Q  L  Q  R  N  C  P 38
 121 CCGCTGCTTATCTGGTCTTGGTGCCCAGCCTTGCGGTCTCACAACTACAGAGGAATTGCC 180
       D  N  Y  V  L  L  A  D  K  C  Y  G  F  R  R  T  V  T  D  W 58
 181 CAGACAACTATGTGCTCTTGGCTGACAAATGCTATGGCTTTCGGCGCACAGTGACAGACT 240
       N  N  A  R  A  T  C  L  N  E  N  A  D  L  T  S  V  L  T  T 78
 241 GGAATAACGCCCGTGCAACTTGTCTGAACGAGAATGCTGACCTGACCTCCGTTCTCACGA 300
       Q  E  Y  T  E  I  L  A  H  L  A  A  N  Y  P  G  V  Y  W  V 98
 301 CTCAAGAGTACACGGAGATTCTGGCGCATCTGGCTGCTAACTACCCCGGCGTGTACTGGG 360
       G  G  A  T  S  N  Q  G  A  W  R  W  V  A  S  G  A  P  M  N 118
 361 TTGGAGGCGCCACGAGTAACCAGGGCGCGTGGAGGTGGGTGGCTTCGGGCGCGCCCATGA 420
       E  Q  W  W  G  G  D  H  T  P  T  S  Q  R  C  A  Y  F  C  S 138
 421 ATGAGCAGTGGTGGGGCGGCGACCACACCCCCACTTCACAACGCTGCGCCTACTTCTGCT 480
       H  T  R  K  Y  W  S  S  T  C  G  V  S  K  N  F  I  C  E  K 158
 481 CCCATACTCGCAAATACTGGAGCAGCACATGCGGGGTGTCCAAGAACTTCATCTGCGAAA 540
       S  A  E  T  E  A  E  S  G  E  E  F  E  P  E  G  D  T  D  P 178
 541 AAAGTGCGGAGACCGAAGCCGAATCAGGAGAAGAATTTGAACCAGAGGGGGACACCGATC 600
       E  N  E  S  D  E  A  D  A  E  R  I  P  S  L  P  S  V  R  A 198
 601 CGGAAAATGAATCGGACGAAGCAGACGCTGAGAGGATACCTTCCCTTCCCTCTGTAAGGG 660
       E  L  R  S  G  V  S  S  T  T  Q  N  M  A  C  L  V  Y  L  L 218
 661 CGGAGCTGCGGTCTGGAGTTTCGTCCACCACACAAAACATGGCCTGTCTCGTTTATCTGT 720
       S  L  C  A  V  M  V  *                                     225
 721 TGAGTCTTTGTGCAGTCATGGTCTAATACAGATATATGGGACTTGTGAGCCAGTGACTAA 780
 781 AATGCCTTTTCAAACCCCATGTGAAGCTCTACGTAACCATATTTGTACAGTAGATTCTTA 840
 841 GCTTACATGAGATTGACTGATCAGTGTGTTTAAGGATTTGTAATATCTATGAATTTATCA 900
 901 ATAAGTAAAAACTGGAGCCAACAGAAGAGACTCAGTTCGTGTGATTTACGTATCCAGAAA 960
 961 TGTAATGACATCAATTTCACTCATGAAATTTACATTTTATCTACCTTTTTGTTATTGATG 1020
1021 CTCTTATTTTTCTTAGTGGGTAGTTAATAATTCATTGATGTTTCGCTAACGGGCAACTTT 1080
1081 ACCGTACTGTGGGGTCGTAACTTTCAATATCTTTTTAAATCTCAGCAGTGTGGAGTTCGA 1140
1141 CCGTCACTAATTCACTTTGTTTGGTCACCATACATATGTAATATGTATATAAGTAAGACT 1200
1201 GTATCTGTCATTGATTTAGTACAAATGAAACAAATAAAGAATCCCCGCAAAAAAAAAAAA 1260
1261 AAAAAAAAAAAAGT 1274
```

Figure 3.11 Full length cDNA and deduced amino acid sequences of *P. monodon* C type lectin like protein gene (PmCLP). ORF consists of 678 bp, corresponding to 225 amino acid residues. Start and stop codons are illustrated in bold and underlined. A CLECT domain (position 37-157, E-value =1.18e-12) is highlighted.

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                     10         20         30         40         50
D. rerio     MEDIENYTSL QEFTEDISHC GNRPILSSQS KQGVHKGVKC LRGQVSLVLL
O. latipes   MSHSEAFSKL VDDEGNQDTL SSRADVRHSD KNRPQRGD-- -EGTTSMQTD
S. salar     -MEVENYTSL HEFTEDISSR GNKPILNNHS AQGLKRGSEC LRGQTALFLL
P. monodon   -MTSLTTSLL FTATSLLFTA AYLVLVPSLA VSQLQRNCP- --------DN
Clustal Co          : *            .    :      .  ::.

             ....|....| ....|....| ....|....| ....|....| ....|....|
                     60         70         80         90        100
D. rerio     IALLTSVCAN IGLGVLLVNS RRSSISAEPV NSESEAATLS LKLTATQ--E
O. latipes   VSALTALISK LEHENKQLQE EKGAVL---- ------AKLD LKLTTKA--P
S. salar     IGLLASICAN IAVSVLLFNR PLPSVP---- ---LETAALT LKLNSVR--G
P. monodon   YVLLADKCYG FRRTVTDWNN ARATCLN--- ----ENADLT SVLTTQEYTE
Clustal Co      *:      :       :     :             * *    *.:

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    110        120        130        140        150
D. rerio     RFSRLCSEYT NLGQACSKSV IKCRPCPEDW MHLSEKCYYF SDDKLDWQHS
O. latipes   PVVSLMPSPT GVP------- ---VECPTNW HLFNSNCYFI STQMKPWRDS
S. salar     RYVRLCDDYS KLGQSCSKTV RKCRECPEGW IHVDEKCYSF SNDKMDWPSS
P. monodon   ILAHLAANYP GVYWVGG--- --ATSNQGAW RWVASGAPMN ----EQWWGG
Clustal Co       *  . .  :                  *   . . .          *  .

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    160        170        180        190        200
D. rerio     KESCASMGGH LTILHSHEQH HTLEAVARNH GGMDYHFWIG LSDTETEGVW
O. latipes   QTYCQSQGAH LAIIHTAEEQ TFLWDLLP-- RAYWNAYWFG ISDRQKEDEW
S. salar     RDSCTSLGSH LTILHSKEQH DALEKEARRI GGFDYHFWIG LSDIEKEGDW
P. monodon   DHTPTSQRCA YFCSHTRKYW SSTCGVSKN- -------FIC EKSAETEAES
Clustal Co        *         *: :                     ::   .. :.*

             ....|....| ....|....| ....|....| ....|....| ....|....|
                    210        220        230        240        250
D. rerio     KWVDNTVVNK TYWNEWEKEP NNHRSGGVHG EDCAVLDSRS KTWFDVPCDF
O. latipes   KWVDGTSLGK SFWEEGEPNN HINEDCGYIV KTQVLERVAI RSWYDAPCDM
S. salar     RWVDNTTLTN KYWDEYSSEP DNHHSGGSHG EDCATLDSHS QTWFDVPCDH
P. monodon   -GEEFEPEGD TDPENESDEA DAERIPSLPS VRAELRSGVS STTQNMACLV
Clustal Co      :     . .  :: . :  . ..  .                :  : .*

             ....|....| ....|
                    260
D. rerio     HYKRICEMDP IAFSV
O. latipes   SIKFICEKEM KTQ--
S. salar     IYKRICQMDA IRLD-
P. monodon   YLLSLCAVMV -----
Clustal Co       :*
```

Figure 3.12 Multiple alignments of deduced amino acid sequence of *P. monodon* C type lectin like protein and the sequences of *D. rerio*, *O. latipes*, and *S. salar*.

3.2 Tissues distribution of the target genes expressions.

Expression levels of the target genes (PmAQP1, PmGluN6P-deaminase, and PmCLP) in various tissues of *P. monodon* were determined by RT-PCR. The results were indicated by the appearance and intensity of PCR products of the genes in each tissue.

PmAQP1 was expressed in 7 tissues including hepatopancreas, gill, ovaries, testes, intestine, stomach and heart. The expression levels of PmAQP1 in gill appeared to be greater than that of other tissues. PmAQP1 was not detected in haemocyte, epidermis, lymphoid organs and muscle. (Fig.3.13).

PmGluN6P-deaminase was expressed in 9 tissues including haemocyte, hepatopancreas, epidermis, gill, ovaries, testes, intestine, lymphoid organs and stomach. The levels of Glucosamine-6-phosphate deaminase present in gill, ovaries and testes appeared to be greater than that of other tissues. PmGluN6P-deaminase was not found in heart and muscle (Fig. 3.14).

PmCLP was highly expressed in all examined tissues (Fig. 3.15).

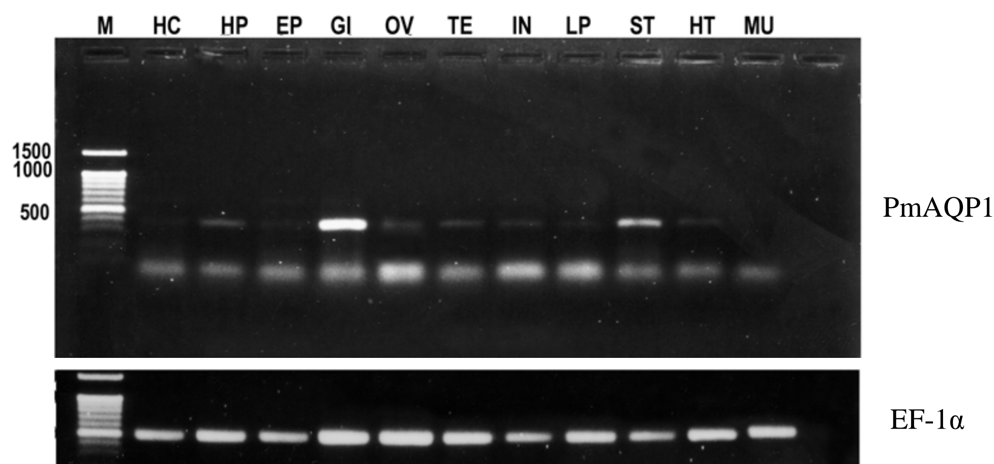

Figure 3.13 Tissues distribution analysis of PmAQP1 gene in *P. monodon*. Expression of the gene was detected in haemocyte (HC), hepatopancreas (HP), epidermis (EP) gill (GI), ovaries (OV), testes (TE), intestine (IN), lymphoid organs (LP), stomach (ST), heart (HT) and muscle (MU) tissue. A 100 bp DNA standard was in lane M. *EF* 1- α gene was used as control.

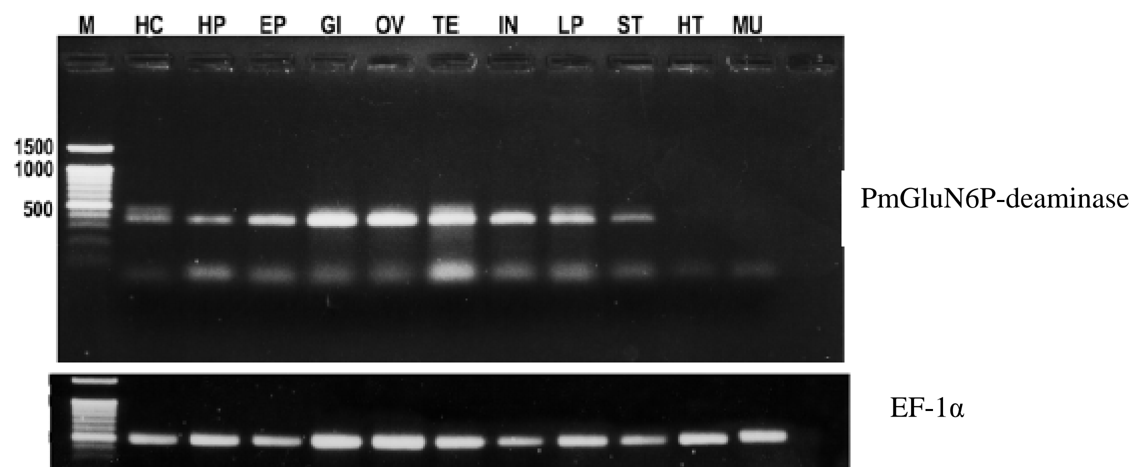

Figure 3.14 Tissues distribution analysis of PmGluN6P-deaminase gene in *P. monodon*. Expression of the gene was detected in haemocyte (HC), hepatopancreas (HP), epidermis (EP) gill (GI), ovaries (OV), testes (TE), intestine (IN), lymphoid organs (LP), stomach (ST), heart (HT) and muscle (MU) tissue. A 100 bp DNA standard was in lane M. *EF* 1- α gene was used as control.

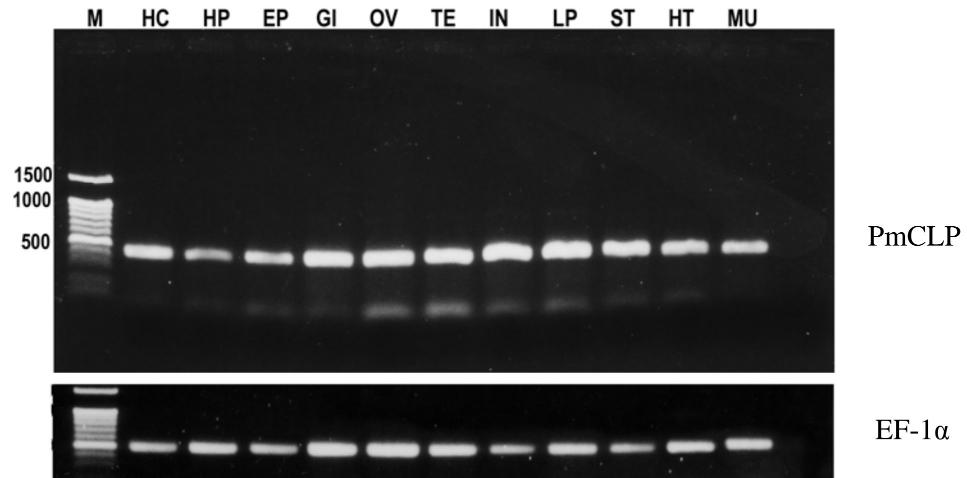

Figure 3.15 Tissues distribution analysis of PmCLP gene in *P. monodon*. Expression of the gene was detected in haemocyte (HC), hepatopancreas (HP), epidermis (EP), gill (GI), ovaries (OV), testes (TE), intestine (IN), lymphoid organs (LP), stomach (ST), heart (HT) and muscle (MU) tissue. A 100 bp DNA standard was in lane M. *EF* 1- α gene was used as control.

Table 3.1 Expression levels of target genes in various tissues of *P. monodon*

Gene	Expression level										
	HC	**HP**	**EP**	**GI**	**OV**	**TE**	**IN**	**LP**	**ST**	**HT**	**MU**
1. QP1		+		+++	+	+	+		++	+	
2. PmGluN6P-deaminase	++	++	+++	+++	+++	+++	+++	+++	++		
3. PmCLP	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

+++: high expression level, ++: moderate expression level, +: low expression level, -: not expression level

3.3 Single strand conformational polymorphism analysis

Nucleotide sequence diversity of PmAQP1 was analysed by PCR-SSCP. The analysis was conducted on 9 first strand cDNA samples of gills from normal shrimp, no heat induced, and preheat-induced shrimp exposed to ammonia stress (3 replications). Polymorphic sites were determined within the MIP domain of PmAQP1 gene. Multiple bands of cDNA fragments (5-9 bands) were observed in each individual (Fig 3.16). Nine SSCP patterns were observed from 9 individuals and none of these patterns can be clarified as specific polymorphic marker for shrimp from each treatment, indicating that the presence of different patterns from samples do not correlate with the stress conditions that the shrimp exposed. Six different DNA fragments of SSCP products (b1-b6) were subjected to sequencing analysis. Result of sequencing analysis revealed 2 identical sequences (b4 and b5), indicating that these 2 sequences were complementary. Deletion and insertion was not detected within the fragments between samples. Alignments of nucleotide sequence of these 6 fragments (result show in Fig. 3.17) revealed 5 variable sites in the amplified region of PmAQP1 genes. This included 439A>C, 451G>A, 562T>C, and 721A>C. One non-synonymous SNP was detected (579T>G) (result show in Fig. 3.18).

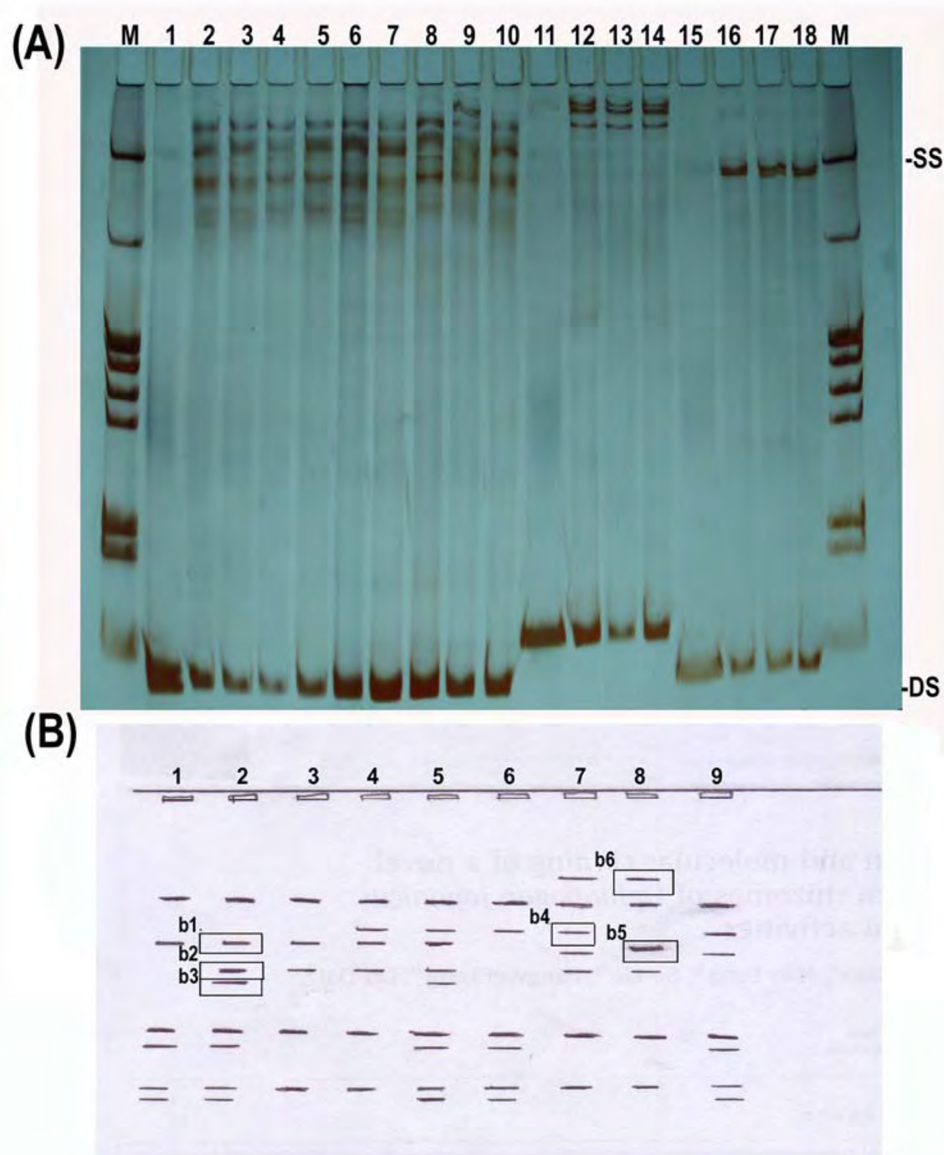

Figure 3.16 PCR-SSCP analysis of PmAQP1 gene obtained from normal, heat-induced, and un-induced shrimp. Samples were analysed by 12.5 % polyacrylamide gel electrophoresis. (A). Diagram of PCR-SSCP products subjected to sequencing analysis (b1-b6) are shown in B.

Lane M ; DNA ladder

Lane 1 ; double stand of PmAQP1 gene in normal gill

Lane 2-4 ; single stand of PmAQP1 gene in normal gill

Lane 5-7 ; single stand of PmAQP1 gene in thermal heat gill

Lane 8-10 ; single stand of PmAQP1 gene in thermal heat gill and teat with ammonia

Lane 11 ; double stand of PmCLP gene in normal hepatopancreas

Lane12-14; single stand of PmCLP gene in normal hepatopancreas

Lane 15 ; double stand of GluN6P-deaminase gene in normal hepatopancreas

Land16-18; single stand of GluN6P-deaminase gene in normal hepatopancreas

```
b1    CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTGGGC 59
b3    CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTAGGC 59
b4    CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTGGGC 59
b5    CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTGGGC 60
b2    CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTGGGC 59
b6    CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAGGCCTTCCTGGCGGAGTTCCTGGGC 59
      ********************************** ********************* ***

b1    ACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAGGCTAC 119
b3    ACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAGGCTAC 119
b4    ACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAGGCTAC 119
b5    ACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAGGCTAC 120
b2    ACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAGGCTAC 119
b6    ACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAGGCTAC 119
      ************************************************************

b1    GCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCGTCCATCGCGCAG 179
b3    GCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCGTCCATCGCGCAG 179
b4    GCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCATCCATCGCGCAG 179
b5    GCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCATCCATCGCGCAG 180
b2    GCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCGTCCATCGCGCAG 179
b6    GCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCGTCCATCGCGCAG 179
      *********************************************** ************

b1    GCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCGCCATGCTTGTT 239
b3    GCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCGCCATGCTTGTT 239
b4    GCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCGCCATGCTTGTT 239
b5    GCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCGCCATGCTTGTT 240
b2    GCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCGCCATGCTTGTT 239
b6    GCCGCCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCGCCATGCTTGTT 239
      **** *******************************************************

b1    GCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCCAGTGCCTTGGTGCC 299
b3    GCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCCAGTGCCTTGGTGCC 299
b4    GCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCCAGTGCCTTGGTGCC 299
b5    GCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCCAGTGCCTTGGTGCC 300
b2    GCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCCAGTGCCTTGGTGCC 299
b6    GCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCCAGTGCCTTGGTGCC 299
      ************************************************************

b1    ATCGTAGGGGCTGCCATTCTGAAGGGCGTGACCCCCGCAGACATCCAGGGGTCCCTCGGC 359
b3    ATCGTAGGGGCTGCCATTCTGAAGGGCGTGACCCCCGCAGACATCCAGGGGTCCCTCGGC 359
b4    ATCGTAGGGGCTGCCATTCTGAAGGGAGTGACCCCCGCAGACATCCAGGGGTCCCTCGGC 359
b5    ATCGTAGGGGCTGCCATTCTGAAGGGAGTGACCCCCGCAGACATCCAGGGGTCCCTCGGC 360
b2    ATCGTAGGGGCTGCCATTCTGAAGGGAGTGACCCCCGCAGACATCCAGGGGTCCCTCGGC 359
b6    ATCGTAGGGGCTGCCATTCTGAAGGGAGTGACCCCCGCAGACATCCAGGGGTCCCTCGGC 359
      ************************** *********************************

b1    ATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT 399
b3    ATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT 399
b4    ATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT 399
b5    ATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT 399
b2    ATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT 398
b6    ATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT 398
      ****************************************
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Figure 3.17 Multiple sequence alignment of partial nucleotides sequences of aquaporin gene obtained from PCR-SSCP analysis. Various sequences (b1-b6) are aligned using multiple alignment methods.

```
b1              GELLNDRRVWKAFLAEFLGTMFLVFIGCGSCIGSWSEGYAPSVVQISLAFGVTVASIAQA 60
b2              GELLNDRRVWKAFLAEFLGTMFLVFIGCGSCIGSWSEGYAPSVVQISLAFGVTVASIAQA 60
b3              GELLNDRRVWKAFLAEFLGTMFLVFIGCGSCIGSWSEGYAPSVVQISLAFGVTVASIAQA 60
b4              GELLNDRRVWKAFLAEFLGTMFLVFIGCGSCIGSWSEGYAPSVVQISLAFGVTVASIAQA 60
b5              GELLNDRRVWKAFLAEFLGTMFLVFIGCGSCIGSWSEGYAPSVVQISLAFGVTVASIAQA 60
b6              GELLNDRRVWKAFLAEFLGTMFLVFIGCGSCIGSWSEGYAPSVVQISLAFGVTVASIAQA 60
                ************************************************************

b1              VGHVSGCHINPAVTCAMLVARHVSVIRALIYIVCQCLGAIVGAAILKGVTPADIQGSLGM 120
b2              VGHVSGCHINPAVTCAMLVARHVSVIRALIYIVCQCLGAIVGAAILKGVTPADIQGSLGM 120
b3              VGHVSGCHINPAVTCAMLVARHVSVIRALIYIVCQCLGAIVGAAILKGVTPADIQGSLGM 120
b4              VGHVSGCHINPAVTCAMLVARHVSVIRALIYIVCQCLGAIVGAAILKGVTPADIQGSLGM 120
b5              VGHVSGCHINPAVTCAMLVARHVSVIRALIYIVCQCLGAIVGAAILKGVTPADIQGSLGM 120
b6              AGHVSGCHINPAVTCAMLVARHVSVIRALIYIVCQCLGAIVGAAILKGVTPADIQGSLGM 120
                .***********************************************************

b1              TLRNEKIDTAQA 132
b2              TLRNEKIDTAQA 132
b3              TLRNEKIDTAQA 132
b4              TLRNEKIDTAQA 132
b5              TLRNEKIDTAQA 132
b6              TLRNEKIDTAQA 132
                ************
```

Figure 3.18 Multiple sequence alignment of partial amino acids sequences of aquaporin gene obtained from PCR-SSCP analysis. Various sequences (b1-b6) are aligned using multiple alignment methods.

3.4 Stress tolerance in heat induced shrimp

Shrimp induced with no lethal level of heat (35 ºC for 2 h) were challenged with various levels of virus and ammonia. Mortality of the experimented shrimp was monitored in comparison with normal shrimp and the shrimp with no challenge.

3.4.1 Pathogenic stress

3.4.1.1 Acute toxicity test of *P. monodon* challenged with WSSV

The result of range finding test revealed that complete mortality was obtained in shrimp exposed to virus between 0 and 1:1000 dilution of WSSV stock in seawater. As the result, definitive test for LC_{50} was conducted with 4 serial concentrations ranging from 0 to 1:1000 dilutions. For the result of definitive test, mortality of shrimp exposed to virus at the concentration of 1:100, 1:500 and 1:1000 dilution of the WSSV stock after 6 days of exposure was 100, 70, and 60, respectively. The calculated 24, 48, 72, and 96 h LC_{50} values of virus dilution for *P. monodon* were 48.52, 66.19, 131.06, and 266.71 virus dilution, respectively (Table 3.3).

Table 3.2 Accumulative mortality of shrimp from the range finding test.

Dilution of the stock WSSV in seawater	Accumulative mortality of shrimp (%) (N=20)						
	24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**
0	0	0	0	0	5	10	40
1:100	0	20	60	75	75	100	100
1:500	0	0	10	40	50	70	100
1:1000	5	5	10	20	50	60	100

Table 3.3 The LC_{50} and 95% confidence interval at various exposure periods of challenge with WSSV to juvenile *P. monodon*.

Time (h)	Dilution of the stock WSSV in seawater	95% Confidence Limits	Standard Error Log10[LD50]
24	48.52	1.70 – 4.20	1.20
48	66.19	2.61 – 15.11	0.59
72	131.06	2.34 – 42.34	0.35
96	266.71	0.03 – 67.46	0.46

3.4.1.2 Quantification of WSSV

3.4.1.2.1 Preparation of standard WSSV DNA stock solution

Method was conduced by amplifying DNA fragment of WSSV. The obtaining PCR product of WSSV was purified and quantified by measuring the absorbance at A260. This DNA was used as standard WSSV DNA stock.

The result of A260 measurement revealed that the concentration of standard WSSV DNA stock was 159.34 µg DNA /ml. According to the size of PCR product (402 bp, Fig. 3.19), a single fragment (double strand) of standard WSSV DNA weighed 248,250.3 Da which equals to 4.12×10^{-13} µg (Da = $1/N_A$ (grams) where N_A = 6.02×10^{23}, therefore, 1 Da = 1.661×10^{-18} µg). From equation: MW=gram/mol where 1 mol represents 1 WSSV particle, 1 ml of WSSV DNA stock solution contains 3.86×10^{14} WSSV particles (Table 3.4).

The optimization condition of quantitative PCR of standard WSSV dilutions (1:100, 1:500, 1:1000, 1:5000 and 1:10000) is shown in Fig 3.20. Intensity of PCR product from each dilution was measured and plotted against the dilution of standard WSSV DNA. The result and equation are shown in Fig 3.21.

GGAGGGTCATCAAATTCAGCATCTTCTACATCCTGGAAGTAATAGTCTTCCAGTTGT
TCGGGAGGAGGGTACGGCAATACTGGAGGAGGTACATCCACTGTTACAATGTCTTCC
ATCTCATTAGGCTGGTCACATACATTGGGTAGTAAACACTGGGTACAGATCAGGGAA
CATTTGCTATCACCAGTTTCTCCCTCCACCATCTTAAAGAGTTTAACGGGCGGTCTG
AATCTATTATCCACCACAGTAAATTTTGAATCCTTGTCTAGTGCAAAGTCCTTGTCC
ATCTTACACATCTCCATGAATTCGGCCTGAAGAAGTTCCACAGTATAGAAGCATTTC
TCTGCTCGCTTGATGAGTTCGGTGATGAGAAGTTTGTTGGCTGCAATGTATTCTGGG
TCG

Figure 3.19 Partial sequence of WSSV gene. WSSV F and WSSV R primers were illustrated in underline letter. Expect size of PCR products 402 bp. (Yang, et al., 2001)

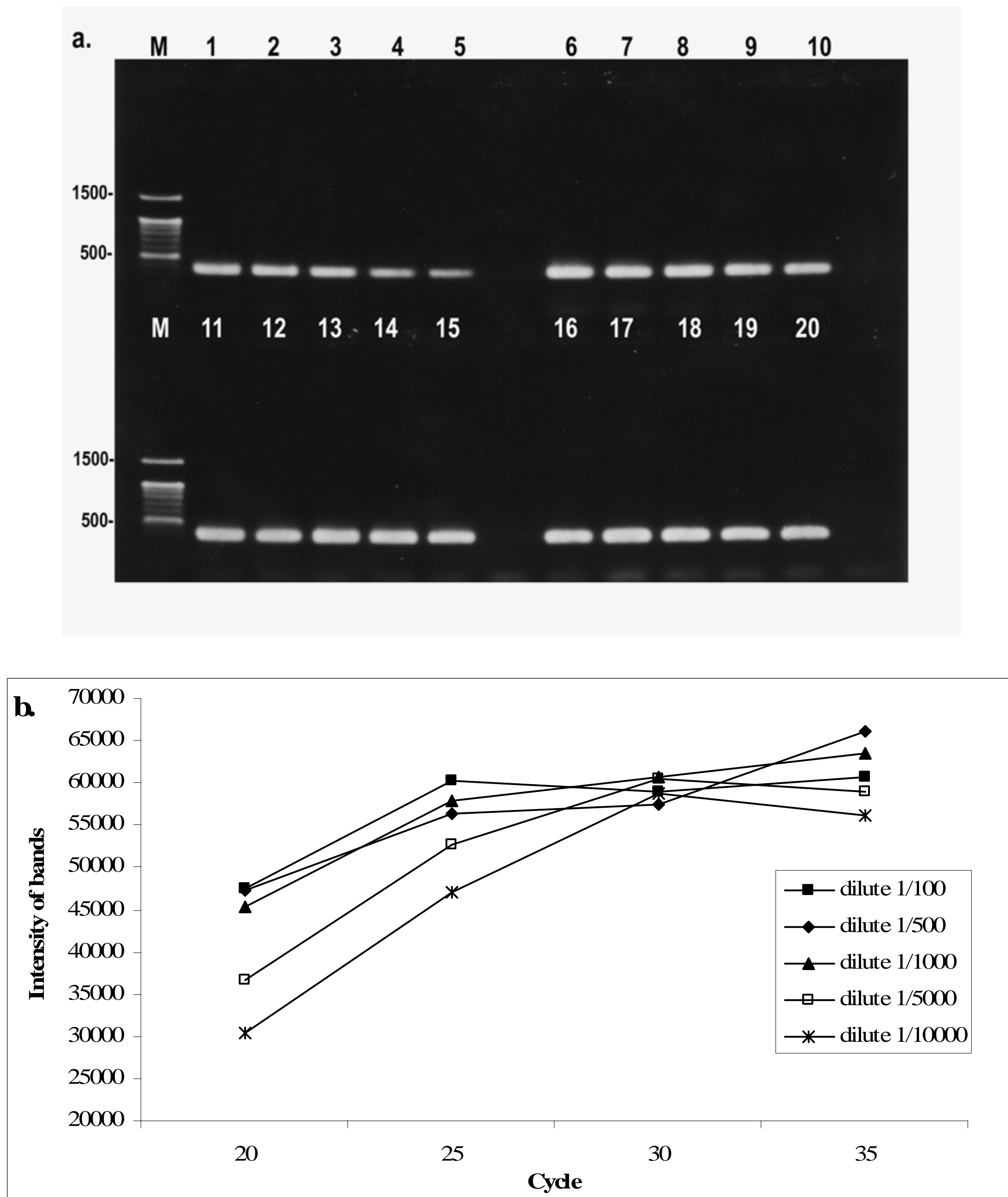

Figure 3.20 Optimization of PCR condition of white spot syndrome virus (WSSV). Number of cycle was examined from the varied numbers of 20 (Lane a1-a5), 25 (Lane a6-a10), 30(Lane a11-a15), and 35 (Lane a16-a20) cycles for standard DNA dilution of WSSV 1:100, 1:500, 1:1000, 1:5000 and 1:10000. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (b.).

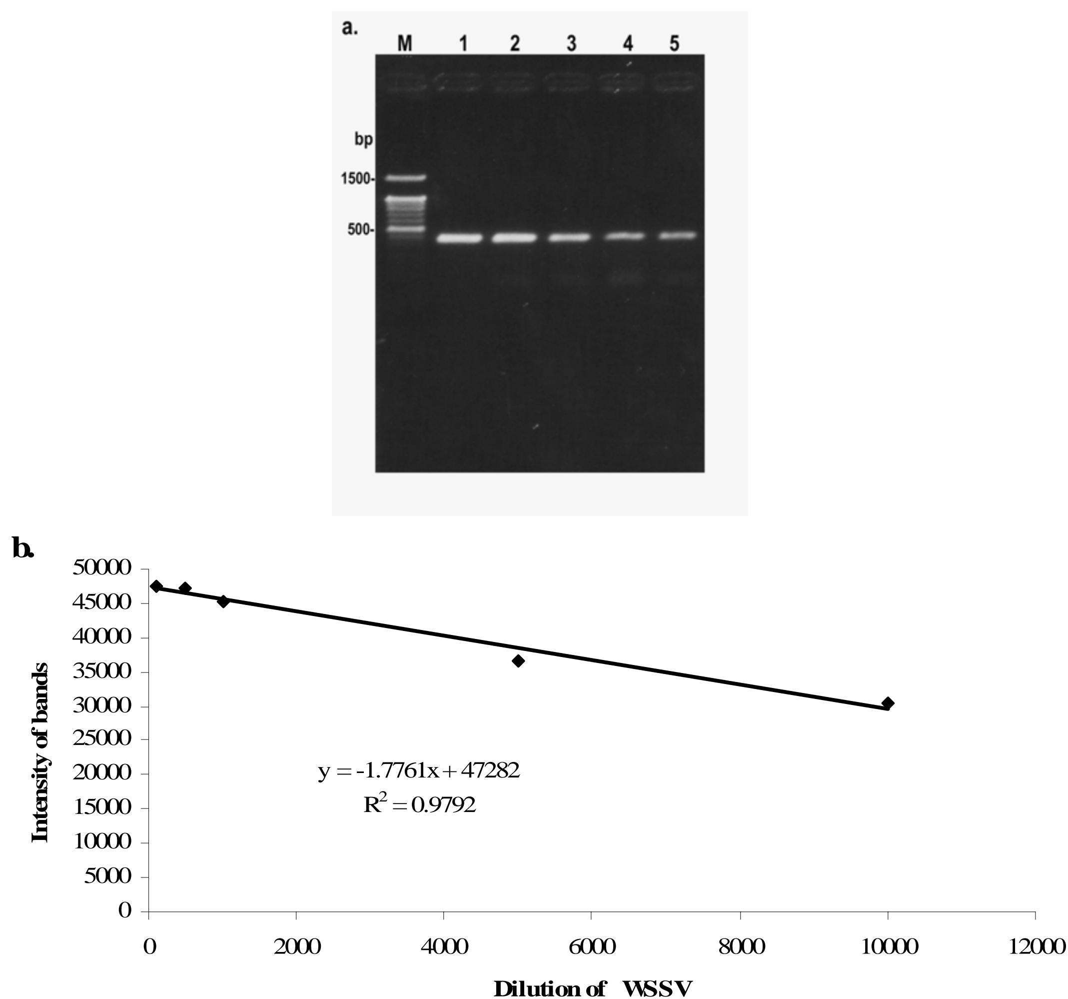

Figure 3.21 Correlation between the number of WSSV particles and the Intensity of PCR product amplified from various dilutions of WSSV stock. Number of cycle was examined from 23 cycles for standard DNA dilution of WSSV; 1:100, 1:500, 1:1000, 1:5000 and 1:10000 (Lane a1-a5). Lane M is DNA ladder. The intensity of amplified product was plotted against the dilution of WSSV (b).

3.4.1.2.2 Determination of WSSV concentration in WSSV stock solution

Numbers of WSSV particles in WSSV stock solution, formerly prepared from WSSV infected shrimp, were determined by semi-quantitative PCR using DNA extracted from WSSV stock and diluted into 5 solutions (1;1, 1:10, 1:25, 1:50 and 1:100 dilution) as templates (Fig. 3.22). After amplification, the results of DNA band intensities were measured and the number of WSSV particles in WSSV stock was calculated as described above. The result showed in Table 3.4.

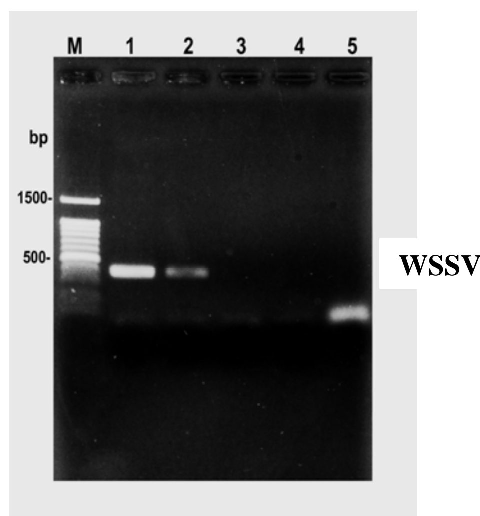

Figure 3.22 PCR products of WSSV gene in stocks of white spot syndrome virus (WSSV). Dilute 1, 1: 10, 1:25, 1:50 and 1:100 (Lane 1-5) dilution of stock WSSV in PCR water. Lane M is DNA ladder.

Table 3.4 Particles of WSSV determined in the experiment.

Stock	Virus particle/ml
Standard WSSV DNA	3.86×10^{14}
WSSV stock	4.15×10^{11}
Challenge concentration of WSSV (1:266 dilution)	1.24×10^{9}

3.4.1.3 Quantitative analysis of WSSV in challenged shrimp

Quantitative analysis of WSSV was conducted intestinal tract of normal and heat-induced shrimp challenged by WSSV. As shown in figure 3.23 and table 3.5, numbers of WSSV particles detected from both normal and heat-induced shrimp were increased significantly after 72 h of challenge and decreased to almost the same level as the early hours of post challenge. However, at the peak level (72 h), the number of WSSV in heat induced shrimp was significantly lower than that of un-induced shrimp. This indicated that heat induction influenced the increasing numbers of WSSV in the shrimp.

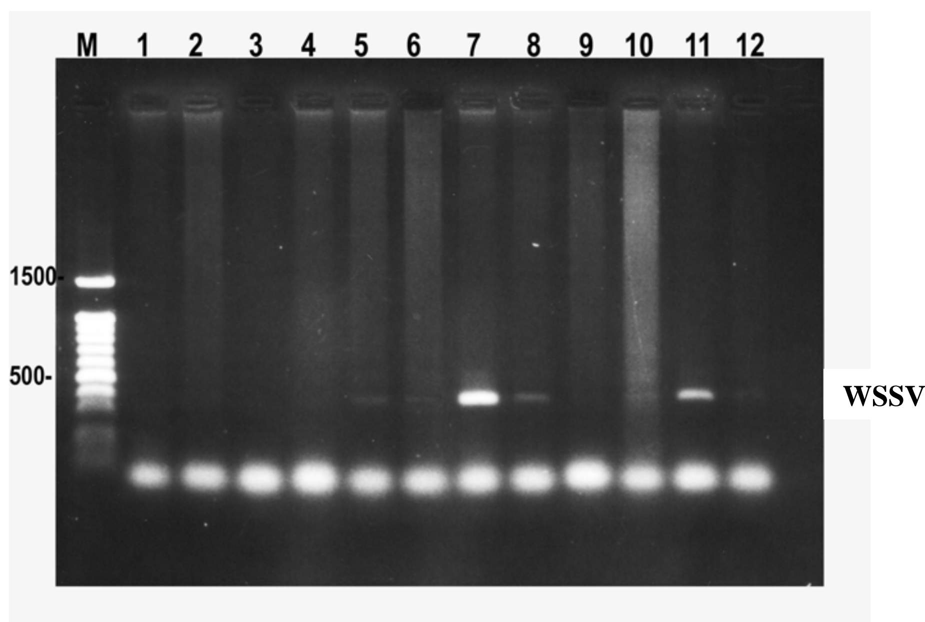

Figure 3.23 PCR products of WSSV gene in intestinal tract of *P. monodon* after challenge white spot syndrome virus. The control group is showing land 1-4, un-induced group; land 5-8 and heat induced group; land 9-12. Time-course 24, 48, 72 and 96 hours post challenge WSSV respectively. Lane M is DNA ladder.

Table 3.5 Virus particles calculated from intensity value of PCR products.

Treatment	Hours	Intensity of bands	Particle of virus
Un-induced group	24	5023	$1.62x10^{10}$
	48	3675	$1.57x10^{10}$
	72	68570	$1.69x10^{11}$
	96	22529	$2.76x10^{10}$
Heat induced group	24	3198	$1.55x10^{10}$
	48	7280	$1.71x10^{10}$
	72	38112	$7.47x10^{10}$
	96	4568	$1.60x10^{10}$

3.4.1.4 WSSV tolerance in *P. monodon*

Tolerance of the shrimp to WSSV stress was determined in normal and heat-induced shrimp. As shown in Fig. 3.24 and Table 3.6, significant difference between survival rates of the shrimp from different treatments was observed after 72 h of

WSSV challenge. Survival rate of un-induced shrimp was significantly lower than that of heat-induced and control shrimp (*P*<0.05). The survival rate of heat induced shrimp began to be significantly lower than that of control shrimp after 96 h of post challenge but still significantly higher than that of un-induced shrimp. Similar result was also obtained at 120 h of post challenge. Complete mortality was observed from heat-induced and un-induced shrimp after 144 h of post challenge while almost 80% of control shrimp (no challenge) survived after 7 days of post challenge. This result indicated that heat induction enhanced WSSV tolerance in *P. monodon*.

Table 3.6 Accumulative mortality of shrimp challenged with 1: 266 dilution of the stock WSSV.

Treatment	Accumulative mortality of shrimps (%) N=40						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Control	0	0	5	5	10	10	22.5
Heat-induced	0	0	7.5	25	60	100	100
Un-induced	0	0	25	40	87.5	100	100

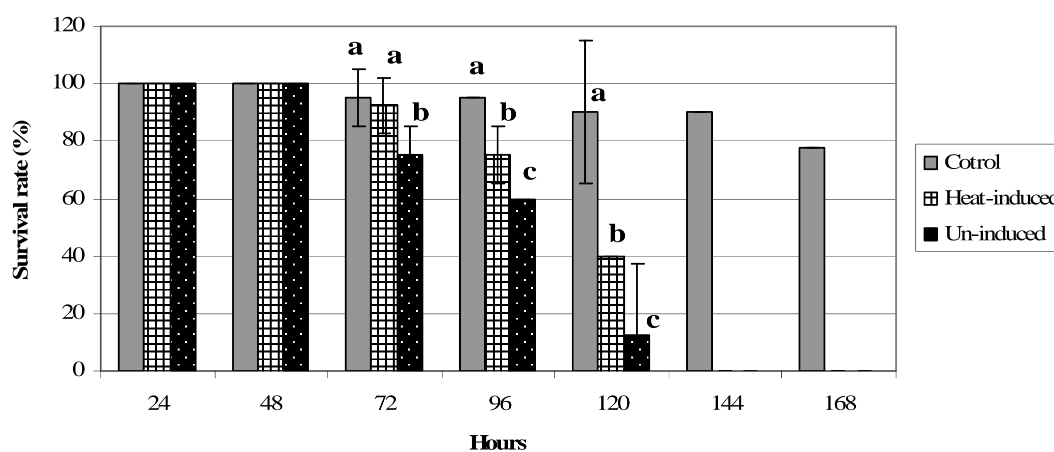

Figure 3.24 Survival rate of *P. monodon* challenged with 1: 266 dilution of the stock WSSV in comparison with control group (no challenge).

3.4.2 Environmental stress

Similar to pathogenic stress, acute toxicity test of ammonia to normal shrimp was firstly conducted. Then, LC_{50} at 96 h of ammonia was used for challenging heat-induced shrimp. Comparison of survival rates between normal and heat induced shrimp exposed to ammonia stress was conducted.

3.4.2.1 Acute toxicity test of ammonia

The result of range finding test and literature review indicated that complete mortality should be obtained in shrimp exposed between 0.01 and 10 mg-N/l of ammonia. As the result, definitive test for LC_{50} was conducted with 5 serial concentrations ranging from 0.01 to 10 mg-N/l of ammonia. For the result of definitive test, mortality of shrimp exposed to ammonia at the concentration of 0.01, 0.1, 1, and 10 mg-N/l after 96 h exposure was 25, 30, 15, and 100, respectively, while mortality of control shrimp was not found throughout the experiment. The calculated 24, 48, 72, and 96 h LC_{50} value of ammonia for *P. monodon* were 1.40, 0.99, 0.92, and 0.69 mg-N/l, respectively (Table 3.8).

Table 3.7 Accumulative mortality of shrimp from the range finding test.

Ammonia Concentration (mg-N/l)	**Accumulative mortality of shrimp (%) (N=20)**							
	1 h	**3 h**	**6 h**	**12 h**	**24 h**	**48 h**	**72 h**	**96 h**
0	0	0	0	0	0	0	0	0
0.01	0	0	0	0	0	10	15	25
0.1	0	0	0	0	15	25	25	30
1	0	0	0	10	15	15	15	15
10	0	100	100	100	100	100	100	100

Table 3.8 The LC_{50} and 95% confidence interval at various exposure periods of ammonia to juvenile *P. monodon*.

Time (h)	**Concentration mg-N/l** (NH_4-N)	**Concentration (mg-N/l)** (NH_3-N)	**95% Confidence Limits**	**Standard Error Log10[LD50]**
24	45.33	1.40	2.45 - 54.45	0.35
48	31.98	0.99	2.60 - 57.40	0.57
72	29.75	0.92	4.14 - 57.86	0.65
96	22.57	0.69	18.52 - 42.00	0.84

Note: NH_3-N calculated based on a temperature of 30 °C, salinity of 11 ‰ and pH of 7.6

3.4.2.2 Ammonia tolerance in *P. monodon*

Tolerance of the shrimp to ammonia stress was determined in normal and heat-induced shrimp. As shown in Fig. 3.25 and Table 3.9, significant difference of survival rates of the shrimp from different treatments were observed after 72 h of ammonia exposure. Survival rates of un-induced and heat-induced shrimp were significantly lower than that of control shrimp (no ammonia exposure) and there was no significant difference between the survival rates of un-induced and heat-induced shrimp. The survival rate of un-induced shrimp was significantly lower than that of heat induced shrimp at 96 h of post exposure ($P<0.05$). At 120 h of post exposure, no significant difference between the survival rates of un-induced and heat-induced shrimp, however, their survival rates were much lower than that of control shrimp and there was no significant difference between their survival rates throughout the experiment. No mortality was observed in control shrimp while 50 and 60 % mortality were obtained from heat-induced and un-induced shrimp, respectively, after 7 days of post exposure. This result indicated that heat induction enhanced ammonia tolerance in *P. monodon* (result see in Fig. 3.25)

Table 3.9 Accumulative mortality of shrimp treated NH_3-N at the concentration of 0.69 mg-N/l.

Treatment	Accumulative mortality of shrimps (%) N=40						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Control	0	0	0	0	0	0	0
Heat-induced	0	10	20	32.5	50	50	50
Un-induced	0	10	35	50	60	60	60

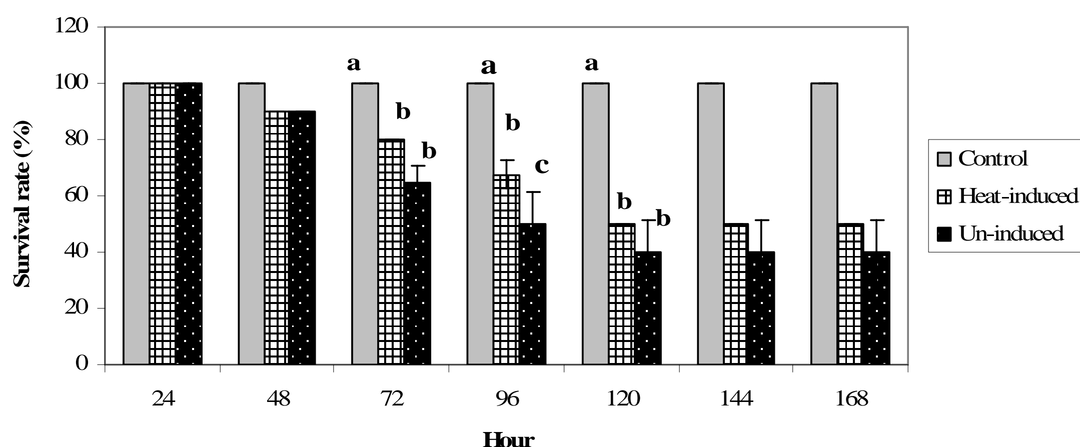

Figure 3.25 Survival rate of *P. monodon* treated with NH_3-N at the concentration of 0.69 mg-N/l in comparison with control group (no ammonia treatment).

3.5 Expression analysis of the genes in heat inducted shrimp

Expression levels of target genes, including PmAQP1, PmGluN6P-deaminase, and PmCLP, in heat induced shrimp were analysed using semi-quantitative RT-PCR analysis. Elongation factor 1 alpha was used as internal control gene.

3.5.1 Optimization of PCR condition

Prior to the quantitative analysis, the appropriate PCR conditions including temperature, template concentration, number of cycles, and $MgCl_2$ concentration for each of target genes and reference gene were verified based on the criteria that the PCR product must be on the log phase of amplification. Variations of annealing temperature, $MgCl_2$ concentration, primers concentration, Cycle number, and DNA

template concentration were tried out in PCR amplification of each target genes. The optimal condition of PCR for each target gene is shown in Fig. 3.26 - 3.31 and Table 3.10.

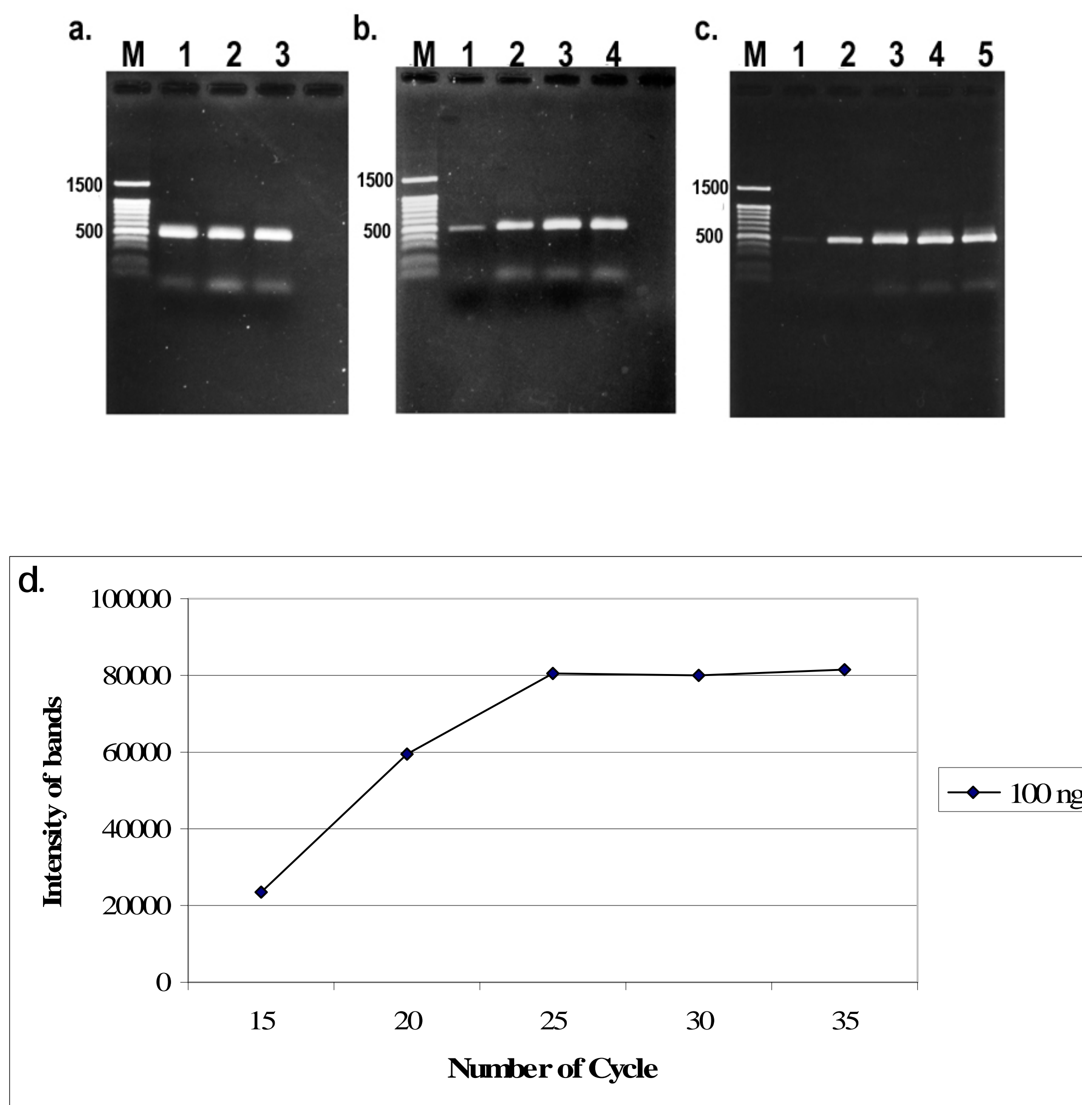

Figure 3.26 Optimization of PCR condition for quantitative analysis of elongation factor 1 alpha gene. Various concentrations of $MgCl_2$ (0.5, 1.0, and 1.5 mM) (Lane a1-a3), primers (0.05, 0.10, 0.15 and 0.2 μM) (Lane b1-b4), numbers of cycles (15, 20, 25, 30, and 35 cycles) (Lane c1-c5), and 100 ng of gill first strand cDNA template from shrimp treated with ammonia were carried out. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

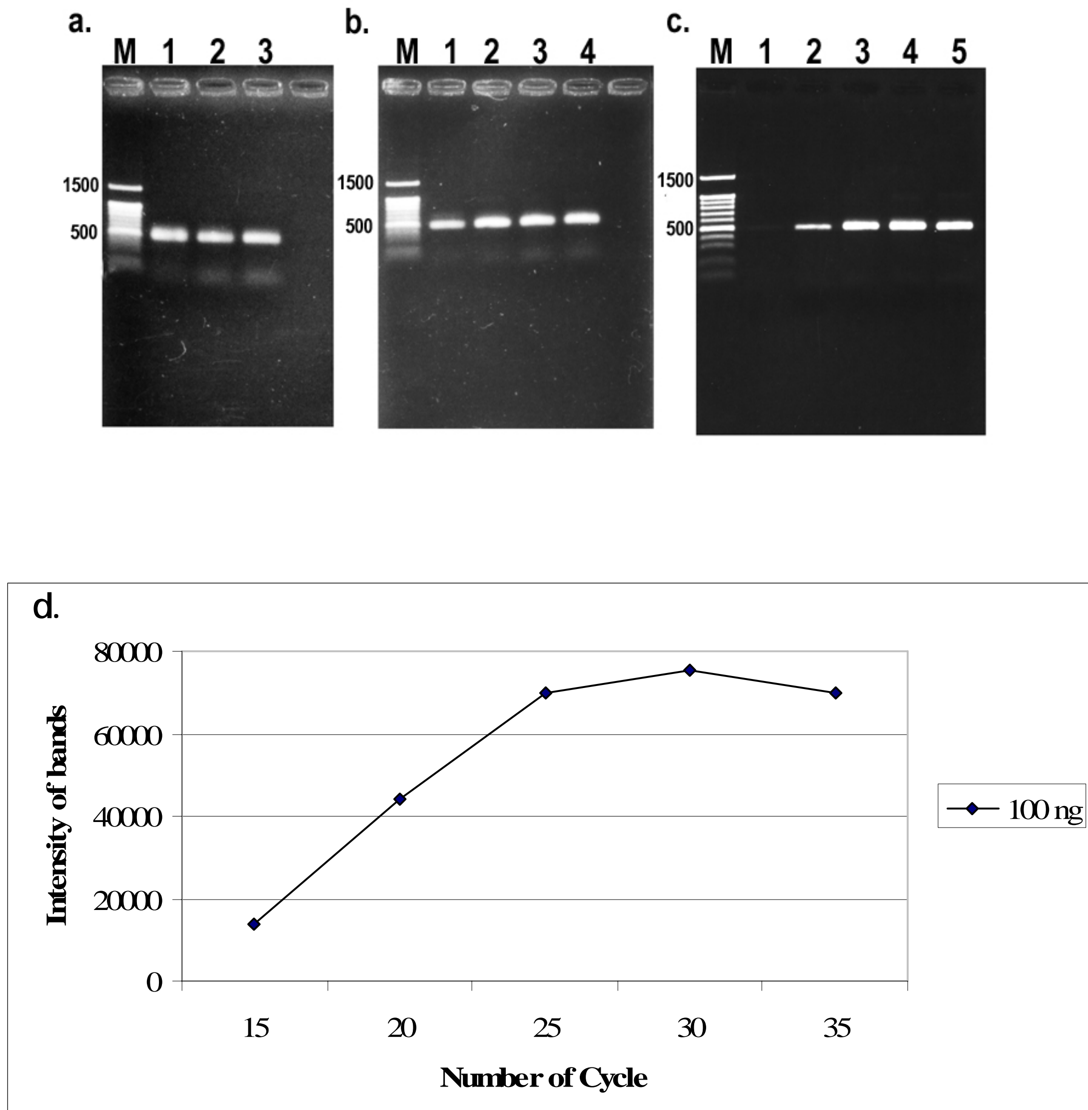

Figure 3.27 Optimization of PCR condition for quantitative analysis of elongation factor 1 alpha gene. Various concentrations of $MgCl_2$ (0.5, 1.0, and 1.5 mM) (Lane a1-a3), primers (0.05, 0.10, 0.15 and 0.2 μM) (Lane b1-b4), numbers of cycle (15, 20, 25, 30, and 35 cycles) (Lane c1-c5), and 100 ng of hepatopancreas first strand cDNA template from shrimp treated with ammonia were carried out. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

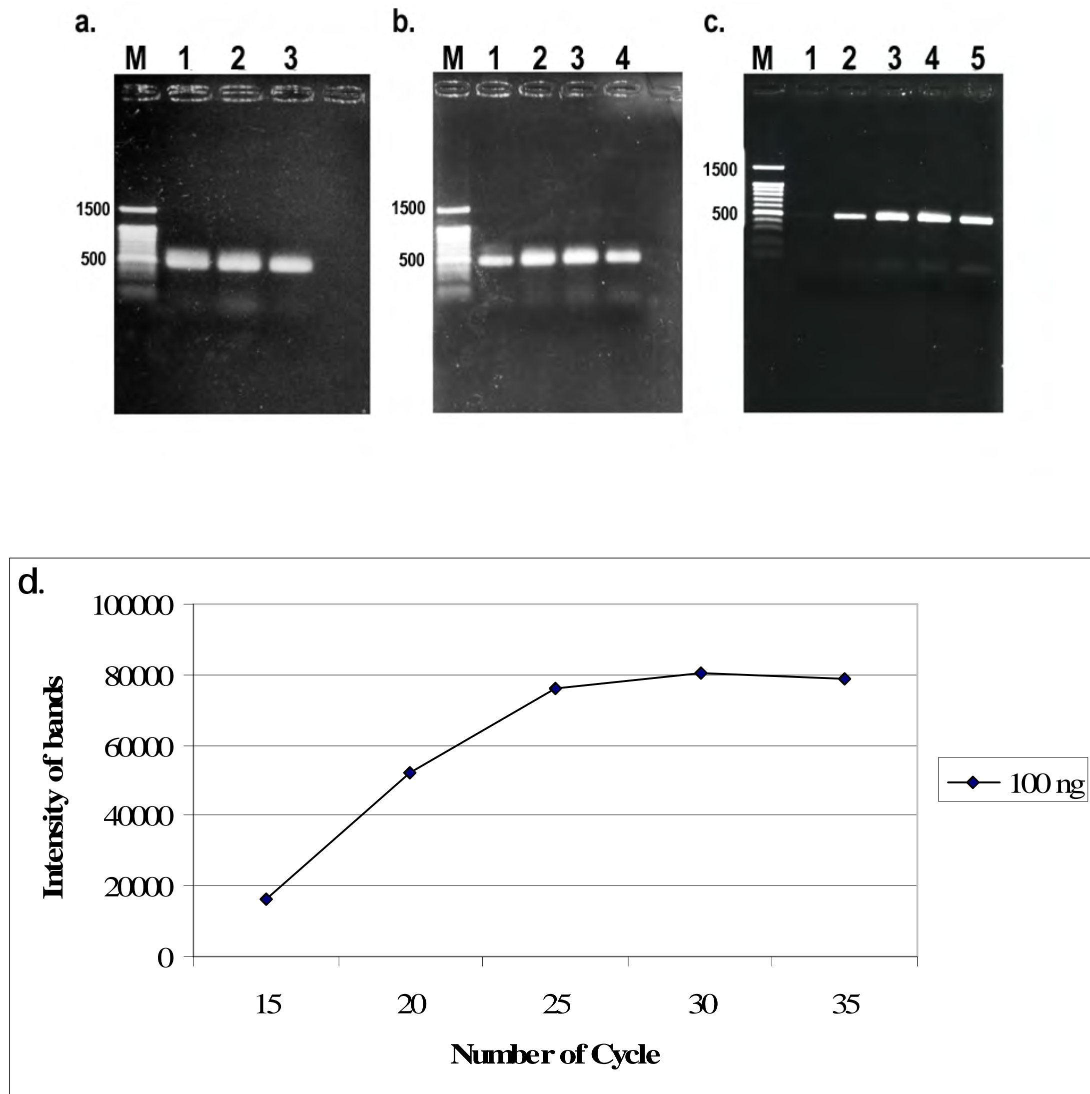

Figure 3.28 Optimization of PCR condition for quantitative analysis of elongation factor 1 alpha gene. Various concentrations of $MgCl_2$ (0.5, 1.0, and 1.5 mM) (Lane a1-a3), primers (0.05, 0.10, 0.15 and 0.2 μM) (Lane b1-b4), numbers of cycle (15, 20, 25, 30, and 35 cycles) (Lane c1-c5), and 100 ng of hepatopancreas first strand cDNA template from shrimp treated with WSSV were carried out. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

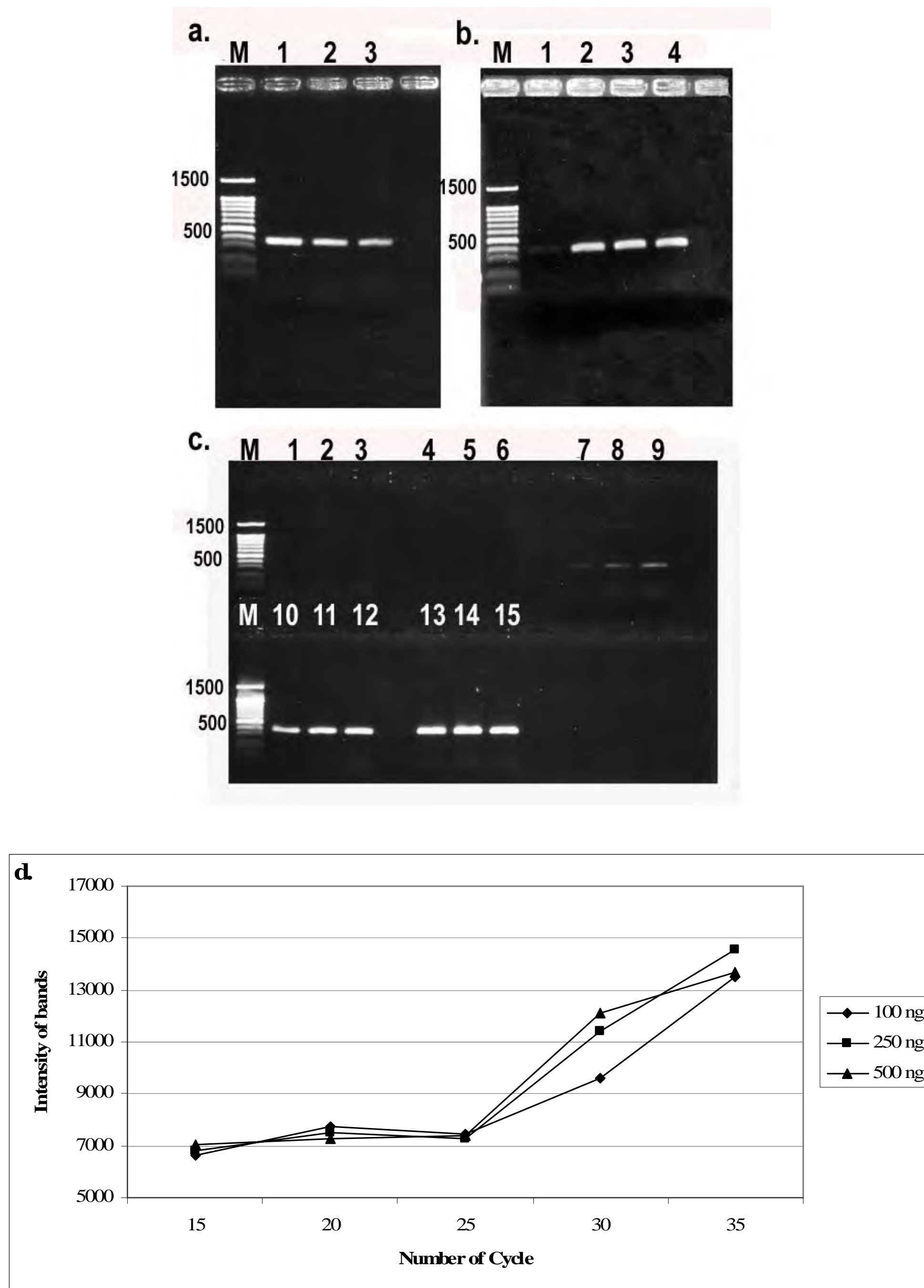

Figure 3.29 Optimization of PCR condition for quantitative analysis of PmAQP1 gene. Various concentrations of $MgCl_2$ (0.5, 1.0, and 1.5 mM) (Lane a1-a3), primers (0.05, 0.10, 0.15 and 0.2 μM) (Lane b1-b4), numbers of cycle (15 (Lane c1-c3), 20 (Lane c4-c6), 25 (Lane c7-c9), 30 (Lane c10-c12), and 35 cycles) (Lane c13-c15), template (100, 250, and 500 ng of gill first strand cDNA from shrimp treated with ammonia) were carried out. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

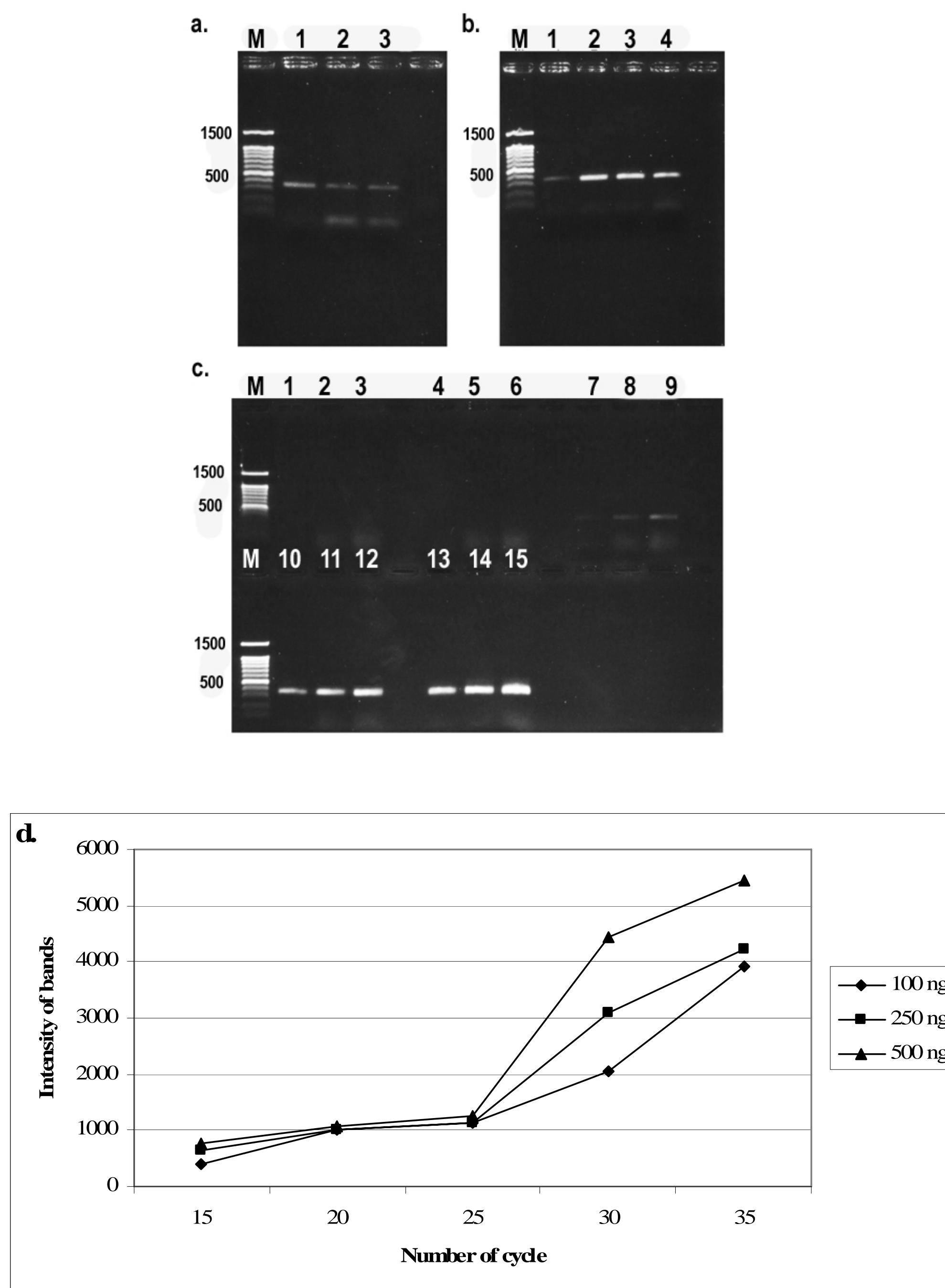

Figure 3.30 Optimization of PCR condition for quantitative analysis of PmGluN6P-deaminase gene. Various concentrations of $MgCl_2$ (0.5, 1.0, and 1.5 mM) (Lane a1-a3), primers (0.05, 0.10, 0.15 and 0.2 μM) (Lane b1-a4), number of cycle (15 (Lane c1-c3), 20 (Lane c4-c6), 25 (Lane c7-c9), 30 (Lane c10-c12), and 35 cycles) (Lane c13-c15), template (100, 250, and 500 ng of hepatopancreas first strand cDNA from shrimp treated with ammonia) were carried out. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

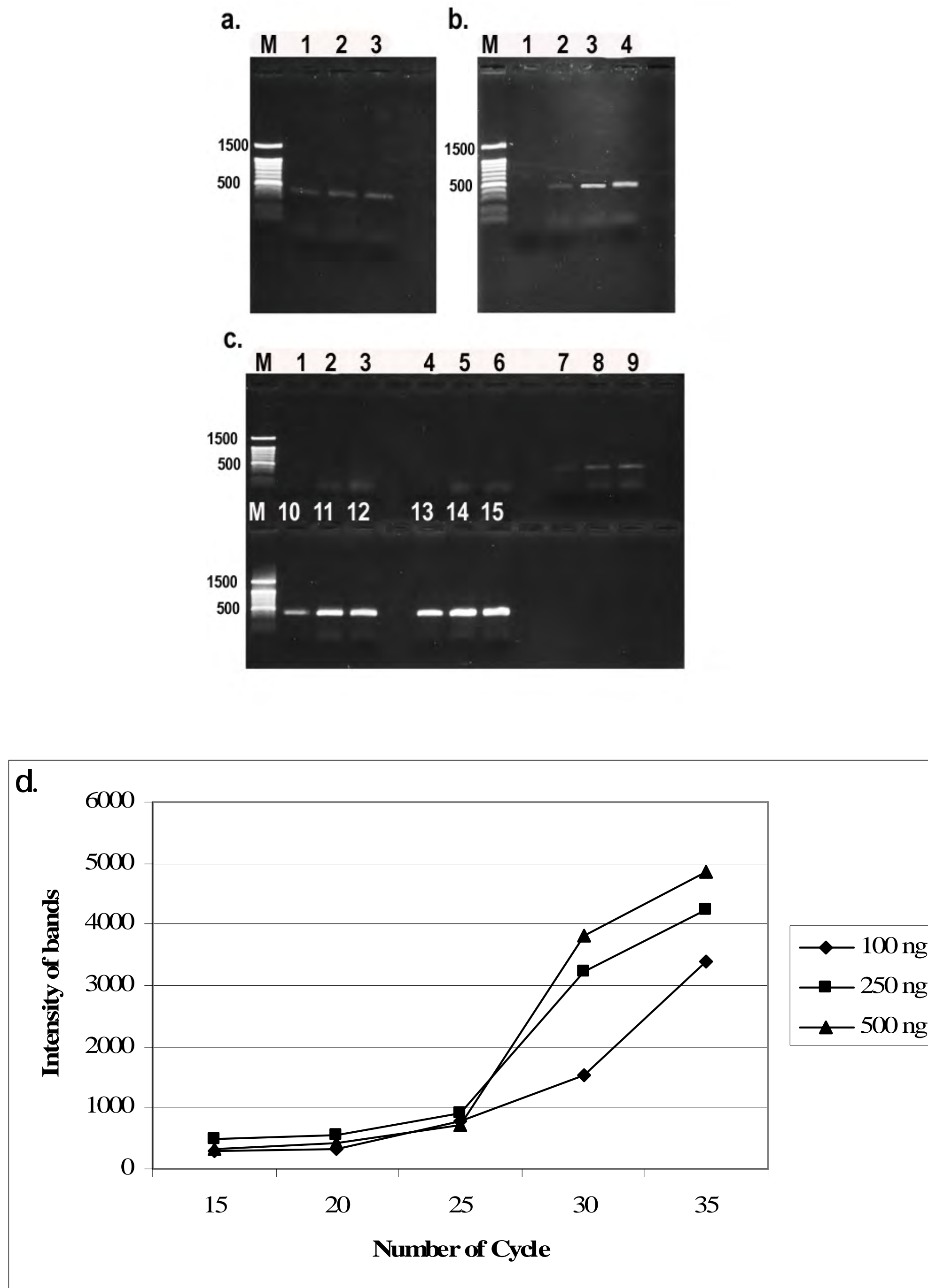

Figure 3.31 Optimization of PCR condition for quantitative analysis of PmCLP gene. Various concentrations of $MgCl_2$ (0.5, 1.0, and 1.5 mM) (Lane a1-a3), primers (0.05, 0.10, 0.15 and 0.2 µM) (Lane b1-a4), numbers of cycle (15 (Lane c1-c3), 20 (Lane c4-c6), 25 (Lane c7-c9), 30 (Lane c10-c12), and 35 cycles) (Lane c13-c15), template (100, 250, and 500 ng of hepatopancreas first strand cDNA from shrimp challenged with WSSV) were carried out. Lane M is DNA ladder. The intensity of amplified product was plotted against the number of amplification cycle (d.).

Table 3.10 Optimal condition for Semi-quantitative RT-PCR of target genes.

Gene		Template (ng)	$MgCl_2$ (mM)	Primer (µM)	Annealing Temperature (°C)	PCR Cycle Number	PCR Product (bp)
1.PmAQP1	Gi-NH_3	500	0.1	1	50	28	403
2. PmCLP	Hep-WSSV	250	0.2	1	50	28	439
3. PmGluN6P-deaminase	Hep-NH_3	500	0.1	1	50	28	380
4. Elongation factor -1 alpha	Gi-NH_3	100	0.1	1	55	23	500
5. Elongation factor -1 alpha	Hep-WSSV	100	0.1	1.5	55	23	500
6. Elongation factor -1 alpha	Hep-NH_3	100	0.1	1	55	23	500

Remark: Gi- NH_3; gill first strand cDNA template from shrimp treated with ammonia.

Hep- NH_3; hepatopancreas first strand cDNA template from shrimp treated with ammonia

Hep-WSSV; hepatopancreas first strand cDNA template from shrimp challenged with WSSV.

3.5.2 Transcriptional analysis of target genes by semi-quantitative RT-PCR analysis

3.5.2.1 Transcription levels of target genes in heat-induced shrimp

Shrimp (n=5) induced by heat at 35°C for 2 h were randomly collected at 6 and 12 h of post induction. Gill and hepatopancreas were dissected and subjected to semi-quantitative RT-PCR analysis.

For PmAQP1 gene analysis, the result indicated that the transcription level of PmAQP1 gene in juvenile *P. monodon* was increased significantly after 6 h and remained high at 12 h of post induction when compared to control ($P<0.05$). Details were shown in Table 3.11 and Fig 3.32 and 3.33.

For PmGluN6P-deaminase gene analysis, the result indicated that the transcription level of PmGluN6P-deaminase gene in juvenile *P. monodon* was increased significantly after 12 h of post induction when compared to control ($P<0.05$). Details were show in Table 3.12 and Fig. 3.34 and 3.35.

For PmCLP gene analysis, no change in the transcription level of PmCLP gene in juvenile *P. monodon* was detected during heat treatment at 35°C for 2 h ($P>0.05$). The result is shown in Table 3.13 and Fig. 3.36 and 3.37.

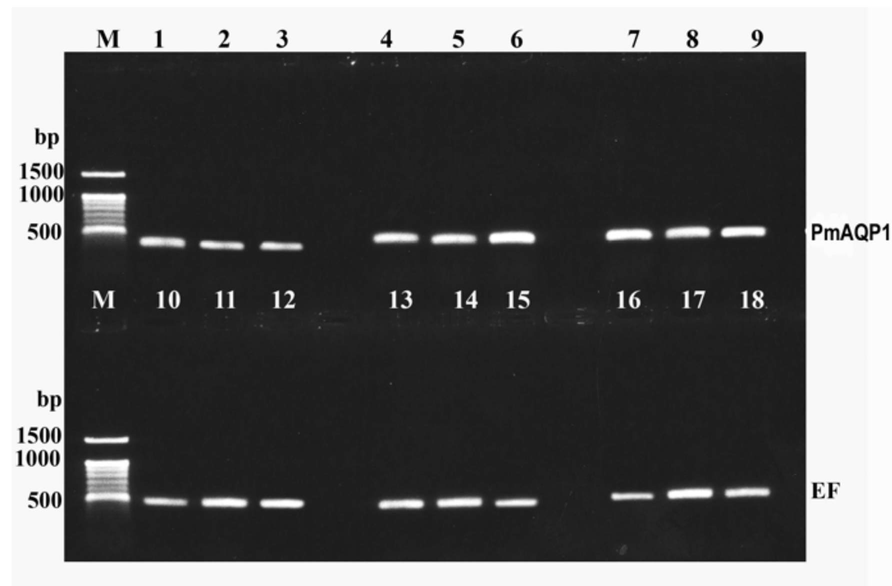

Figure 3.32 RT-PCR products of PmAQP1 gene in gill after heat treatment (35 °C for 2 h). Elongation factor 1 alpha gene from the same template was used as internal control.

Lane M = DNA ladder

For PmAQP1 gene

Lane 1, 2, 3, = Control

Lane 4, 5, 6 = 6 h after heat treatment

Lane 7, 8, 9 = 12 h after heat treatment

For Elongation factor gene

Lane 10, 11, 12, = Control

Lane 13, 14, 15 = 6 h after heat treatment

Lane 16, 17, 18 = 12 h after heat treatment

Table 3.11 The transcription level of PmAQP1 gene in gill of *P. monodon* after heat treatment. Transcriptional level was obtained from the ratio of DNA intensities between PmAQP1 and elongation factor 1 alpha genes.

Gene	Expression level of PmAQP1 after heat treatment		
	Control	**6 h post treatment**	**12 h post treatment**
PmAQP1	47725.9698	62455.2435	63139.76741
EF	52825.56034	52825.56034	52825.56034
PmAQP1/EF	0.90 ± 0.11^{a}	1.18 ± 0.15^{b}	1.20 ± 0.07^{b}

Note: Different superscripts indicate significant difference between treatments ($p \leq 0.05$)

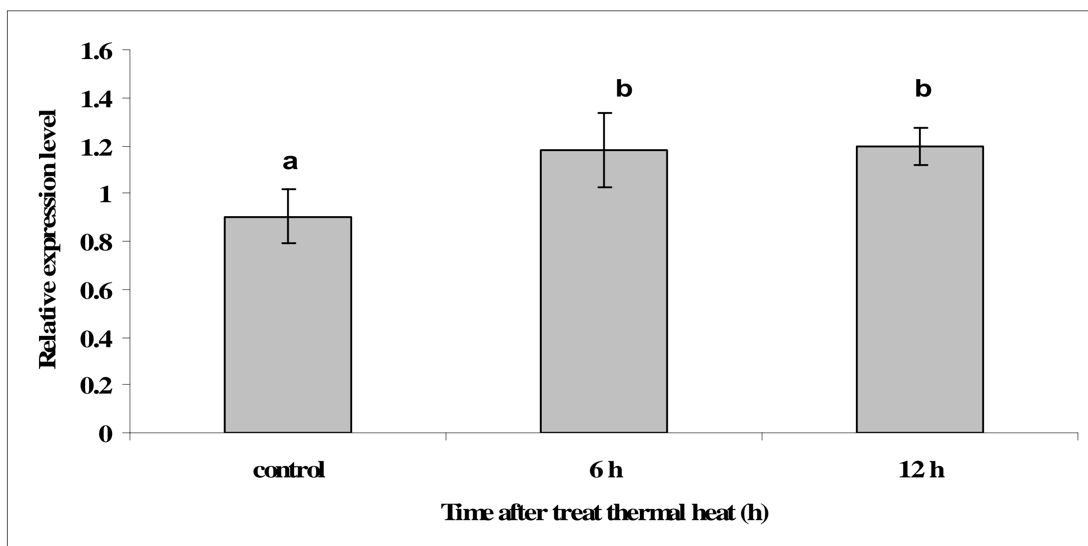

Figure 3.33 Relative transcription levels of PmAQP1 gene and elongation factor 1-alpha genes from control shrimp and shrimp after 6 and 12 h of heat treatment.

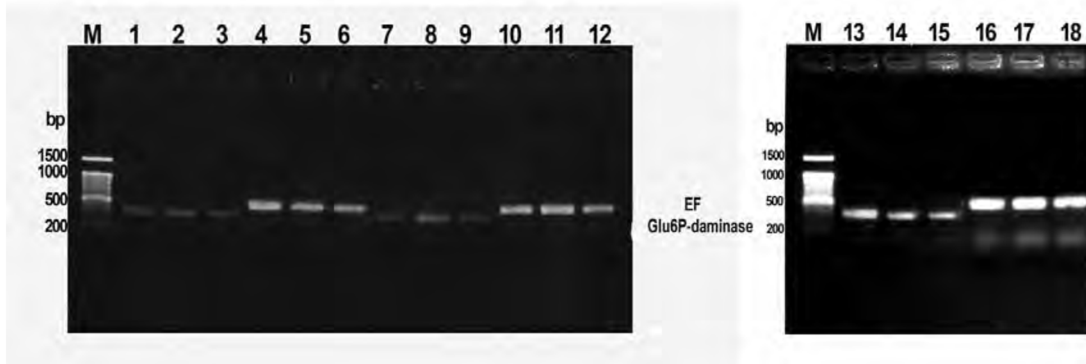

Figure 3.34 RT-PCR products of PmGluN6P-deaminase gene in hepatopancreas after heat treatment (35 °C for 2 h). Elongation factor 1 alpha gene from the same template was used as internal control.

Lane M = DNA ladder

For PmGlcN6Pdeaminase gene

Lane 1, 2, 3, = Control

Lane 7, 8, 9 = 6 h after heat treatment

Lane 13, 14, 15 = 12 h after heat treatment

For Elongation factor

Lane 4, 5, 6, = Control

Lane 10, 11, 12 = 6 h after heat treatment

Lane 16, 17, 18 = 12 h after heat treatment

Table 3.12 The transcription level of PmGluN6P-deaminase gene in hepatopancreas of *P. monodon* after heat treatment. Transcriptional level was obtained from the ratio of DNA intensities between PmGluN6P-deaminase and elongation factor 1 alpha genes.

Gene	Expression level of PmGlcN6Pdeaminase after heat treatment		
	control	6 h post treatment	12 h post treatment
PmGluN6P-deaminase	255.31	279.56	1152.62
EF	1387.13	1387.13	1387.13
PmGluN6P-deaminase /EF	0.18±0.02[a]	0.20±0.06[a]	0.83±0.21[b]

Note: Different superscripts indicate significant difference between treatments ($p \leq 0.05$)

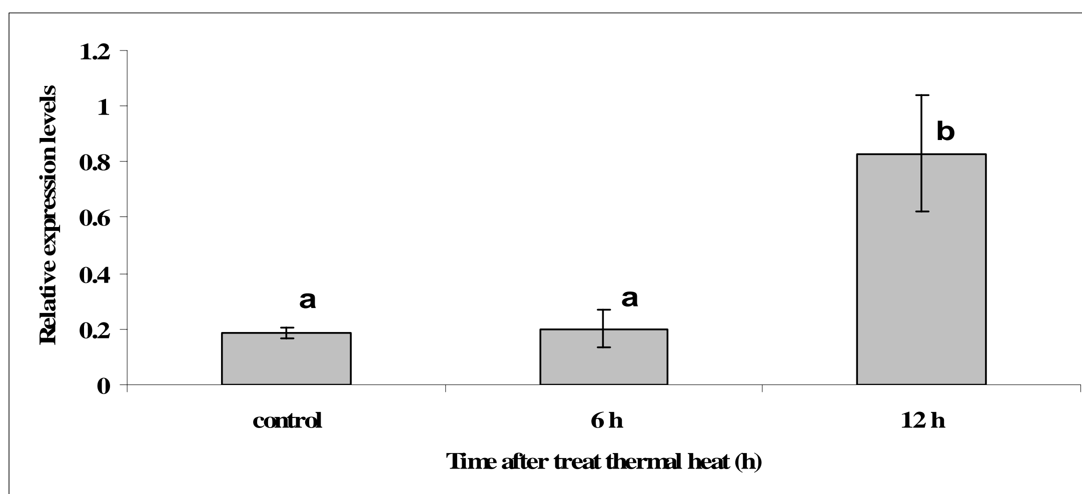

Figure 3.35 Relative transcription levels of PmGluN6P-deaminase gene and elongation factor 1- alpha gene from control shrimp and shrimp after 6 and 12 h of heat treatment.

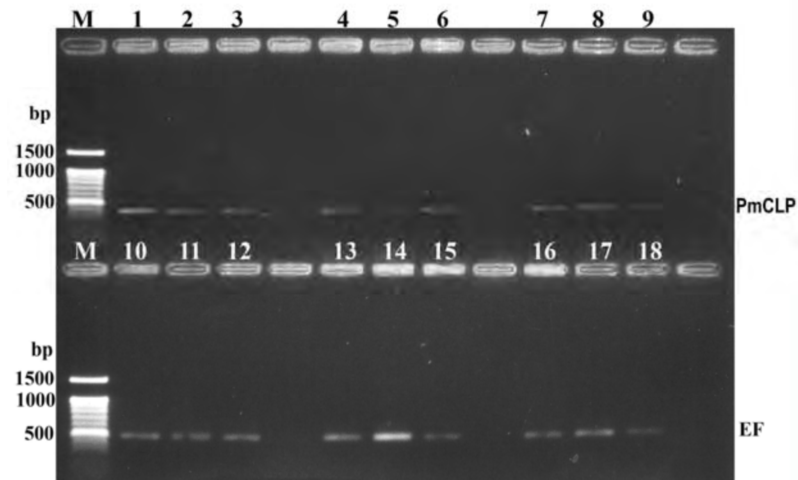

Figure 3.36 RT-PCR products of PmCLP gene in hepatopancreas after heat treatment (35 °C for 2 h). Elongation factor 1 alpha gene from the same template was used as internal control.

Lane M = DNA ladder

For PmCLP gene

Lane 1, 2, 3, = Control

Lane 4, 5, 6 = 6 h after heat treatment

Lane 7, 8, 9 = 12 h after heat treatment

For Elongation factor gene

Lane 10, 11, 12, = Control

Lane 13, 14, 15 = 6 h after heat treatment

Lane 16, 17, 18 = 12 h after heat treatment

Table 3.13 The transcription level of PmCLP gene in hepatopancreas of *P. monodon* after heat treatment. Transcriptional level was obtained from the ratio of DNA intensities between PmCLP and elongation factor 1 alpha genes.

Gene	Expression level of PmCLP after heat treatment		
	Control	6 h post treatment	12 h post treatment
PmCLP	28424.7308	30967.8502	27294.827
EF	38913.13	38913.13	38913.13
PmCLP /EF	0.73±0.06	0.79±0.06	0.70±0.00

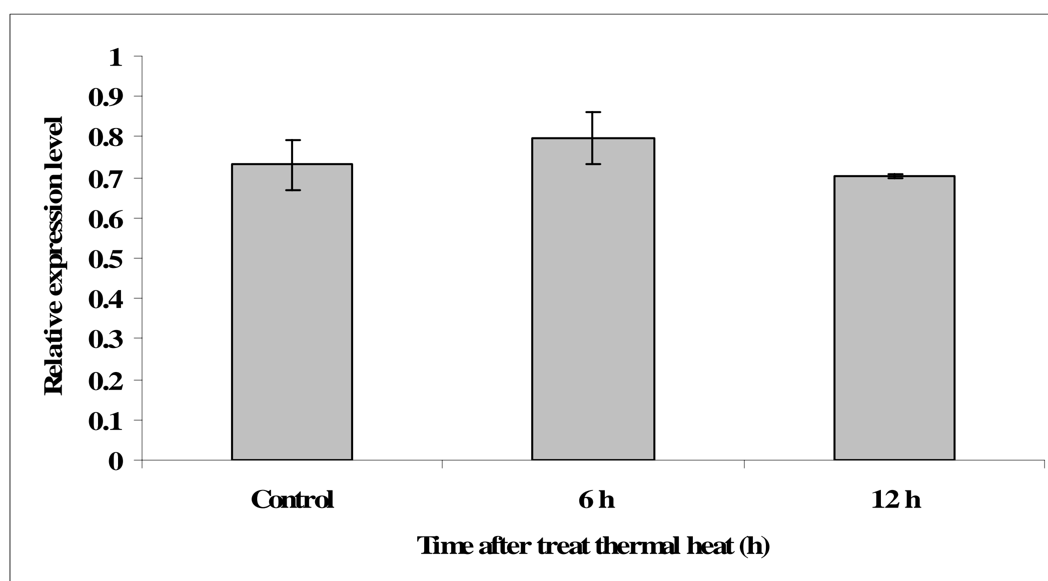

Figure 3.37 Relative transcription levels of PmCLP gene and elongation factor 1-alpha gene from control shrimp and shrimp after 6 and 12 h of heat treatment.

3.5.2.2 Transcription levels of target genes in heat-induced shrimp after stress treatment

PmAQP1

The transcription levels of PmAQP1 gene were determined in shrimp induced by heat (35°C for 2 h) and normal (un-induced) shrimp after ammonia treatment. The results (Fig.3.38, 3.39) showed that within 24 h, transcription level of PmAQP1 gene from heat induced, un-induced, and control (no stress) shrimp were significantly

different. PmAQP1 was expressed higher in the gill of heat-induced shrimp than that of un-induced shrimp and control (no stress) shrimp (Table 3.14). Transcription level of PmAQP1 gene from both induced and un-induced shrimp after ammonia treatment were significantly higher than that of no ammonia treatment shrimp. After 48 h, transcription level of aquaporin gene from un-induced shrimp was significantly increased to the same level as that of heat induced shrimp. Within the heat induced shrimp, transcription level of PmAQP1 gene was initially up-regulated at 24 h post treatment (0.91 ± 0.12, $P<0.05$) which was the highest level. It was slightly decreased but remained significantly higher than that of control treatment after 48 h of post treatment while the transcription level of PmAQP1 gene from un-induced shrimp reached its peak after 48 h of post treatment. After 72 h of post ammonia treatment, transcription levels of PmAQP1 gene from heat induced and un-induced shrimp were decreased and the levels in the shrimp from all treatments were not significantly different.

PmGluN6P-deaminase

The significant difference of the expression level of GluN6P-deaminase gene was not detected. (Fig.3.40, 3.41, Table 3.15). The expression levels of GluN6P-deaminase in shrimp from all treatments appeared to be decreasing during the experiment. However, they were no significant different in corresponding to time.

PmCLP

The transcription levels of PmCLP gene in hepatopancreas of heat induced and un-induced shrimp were determined after challenged with WSSV. Between 24 and 48 h of post WSSV challenge, the transcription levels of PmCLP gene from the shrimp in all treatments were not significantly different. After 72 h, the level in heat induced shrimp was significantly higher than that of un-induced and the control shrimp (no WSSV challenge) ($P< 0.05$) (Fig.3.42, 3.43, Table 3.16). After 96 h, the transcription levels of PmCLP gene from all shrimp were no longer different.

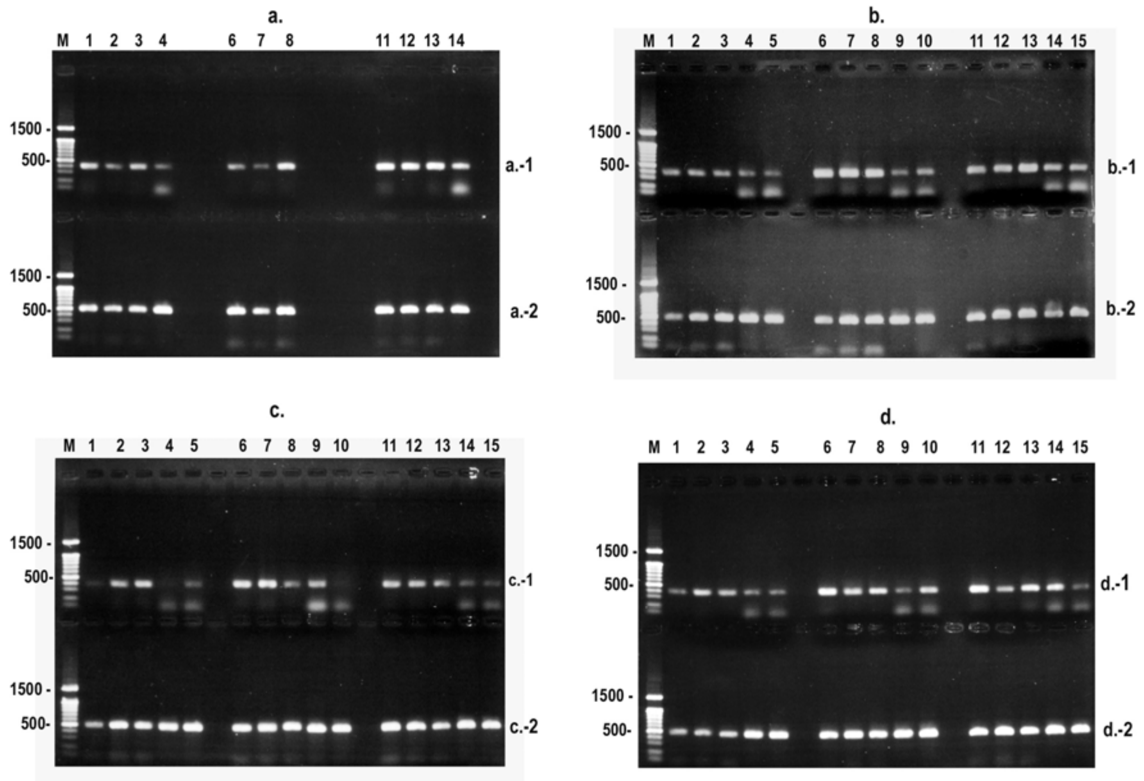

Figure 3.38 Semi-quantitative RT-PCR of PmAQP1 gene in gill of *P. monodon* treated with ammonia at 24, 48, 72 and, 96 h (a-1- d-1). Elongation factor 1 alpha from the same template was used as internal control (a-2 – d-2).

Lane M = DNA ladder

For a: 24 h

Lane 1, 2, 3, 4 = Control

Lane 6, 7, 8 = Un-induced shrimp

Lane 11, 12, 13, 14 = Heat-induced shrimp

For b: 48 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For c: 72 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For d: 96 h

Lane 1, 2, 3 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

Table 3.14 The transcription level of PmAQP1 gene in gill of *P. monodon* after ammonia treatment. Transcriptional level was obtained from the ratio of DNA intensities between PmAQP1 and elongation factor 1-alpha genes.

Time of Exposure (h)	Transcription level after ammonia treatment		
	Control	**Un-induced shrimp**	**Heat-induced shrimp**
24	$0.51\underline{+}0.15^{a}$	$0.57\underline{+}0.26^{a}$	$0.91\underline{+}0.12^{b}$
48	$0.46\underline{+}0.02^{a}$	$0.81\underline{+}0.26^{b}$	$0.73\underline{+}0.10^{b}$
72	$0.42\underline{+}0.19$	$0.64\underline{+}0.27$	$0.48\underline{+}0.18$
96	$0.50\underline{+}0.12$	$0.76\underline{+}0.18$	$0.72\underline{+}0.24$

Note: Different superscripts indicate significant difference between treatments ($p \leq 0.05$)

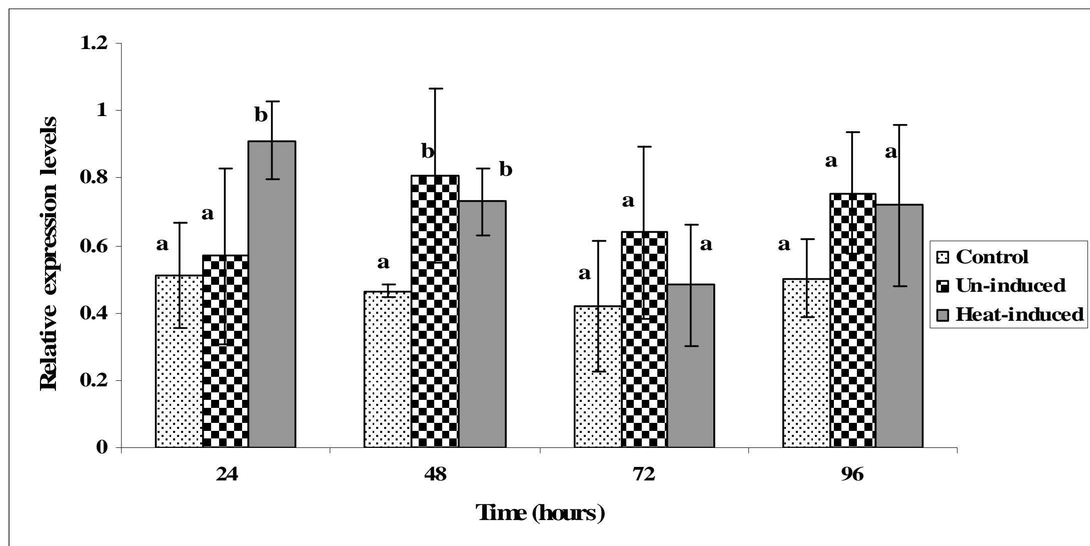

Figure 3.39 The time-course relative expression level of PmAQP1 gene and elongation factor 1- alpha gene for 24, 48, 72 and 96 h after ammonia treatment.

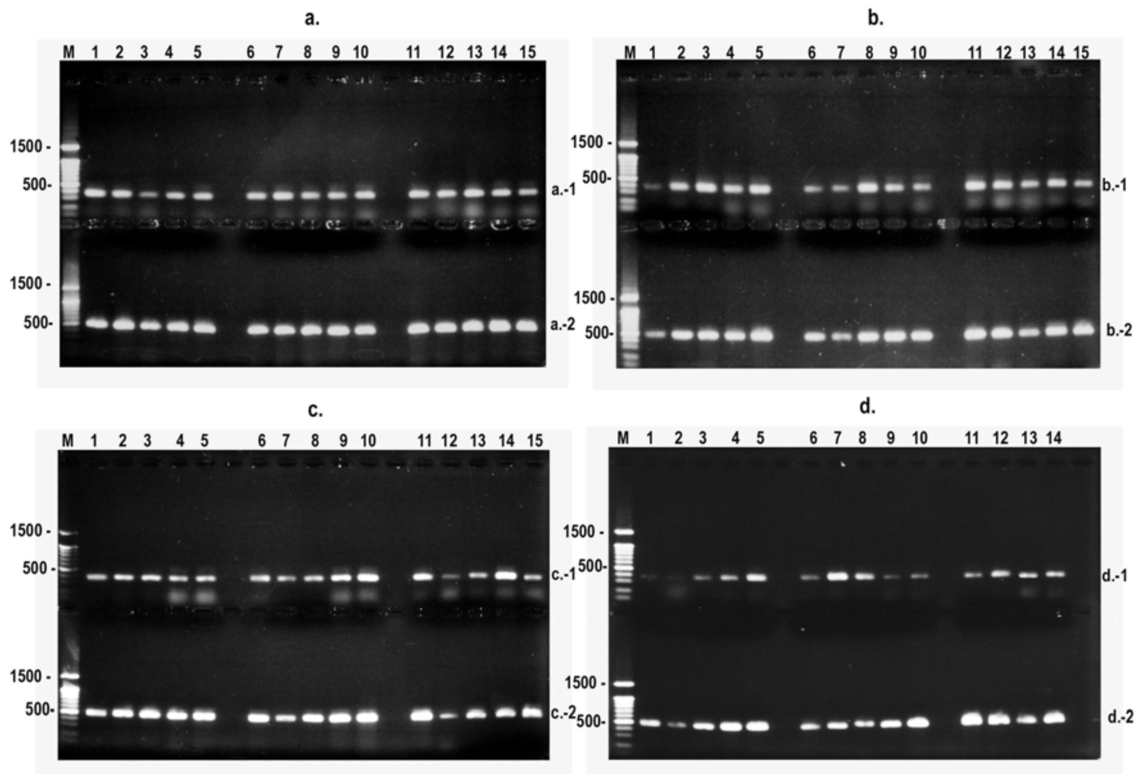

Figure 3.40 Semi-quantitative RT-PCR of PmGluN6P-deaminase in hepatopancreas of *P. monodon* treated with ammonia at 24, 48, 72 and, 96 h (a-1- d-1). Elongation factor 1 alpha from the same template was used as internal control (a-2 – d-2).

Lane M = DNA ladder

For a: 24 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For b: 48 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For c: 72 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For d: 96 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14 = Heat-induced shrimp

Table 3.15 The transcription level of PmGluN6P-deaminase gene in hepatopancreas of *P. monodon* after ammonia treatment. Transcriptional level was obtained from the ratio of DNA intensities between PmGluN6P-deaminase and elongation factor 1 alpha genes.

Time of Exposure (h)	Transcription level after ammonia treatment		
	Control	Un-induced shrimp	Heat-induced shrimp
24	0.64±0.15	0.76±0.07	0.72±0.13
48	0.73±0.20	0.69±0.17	0.75±0.13
72	0.60±0.05	0.575±0.18	0.65±0.27
96	0.38±0.21	0.47±0.25	0.50±0.08

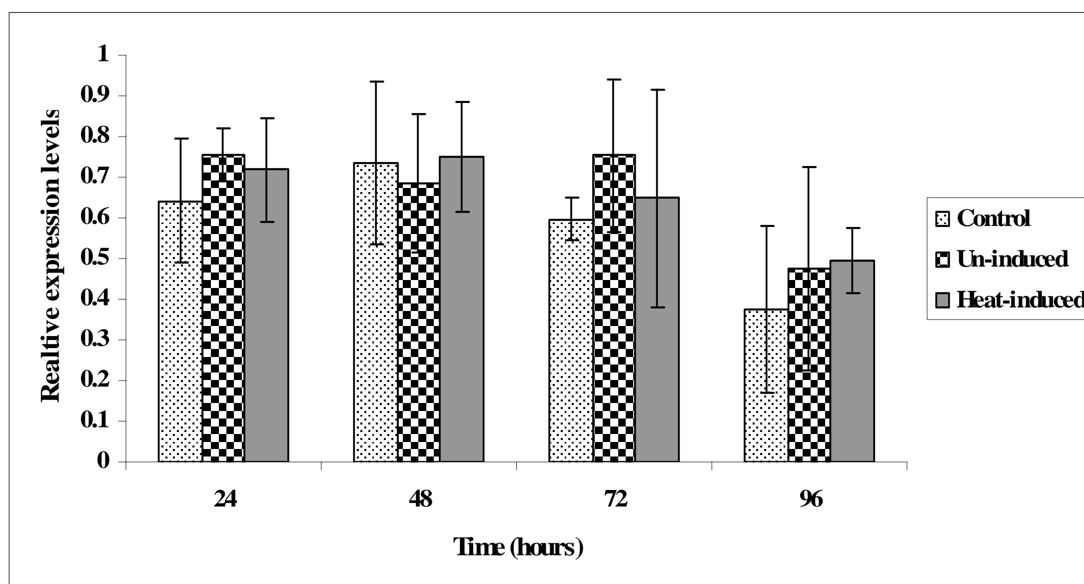

Figure 3.41 The time-course relative expression level of PmGluN6P-deaminase gene and elongation factor 1- alpha gene for 24, 48, 72 and 96 h after ammonia treatment.

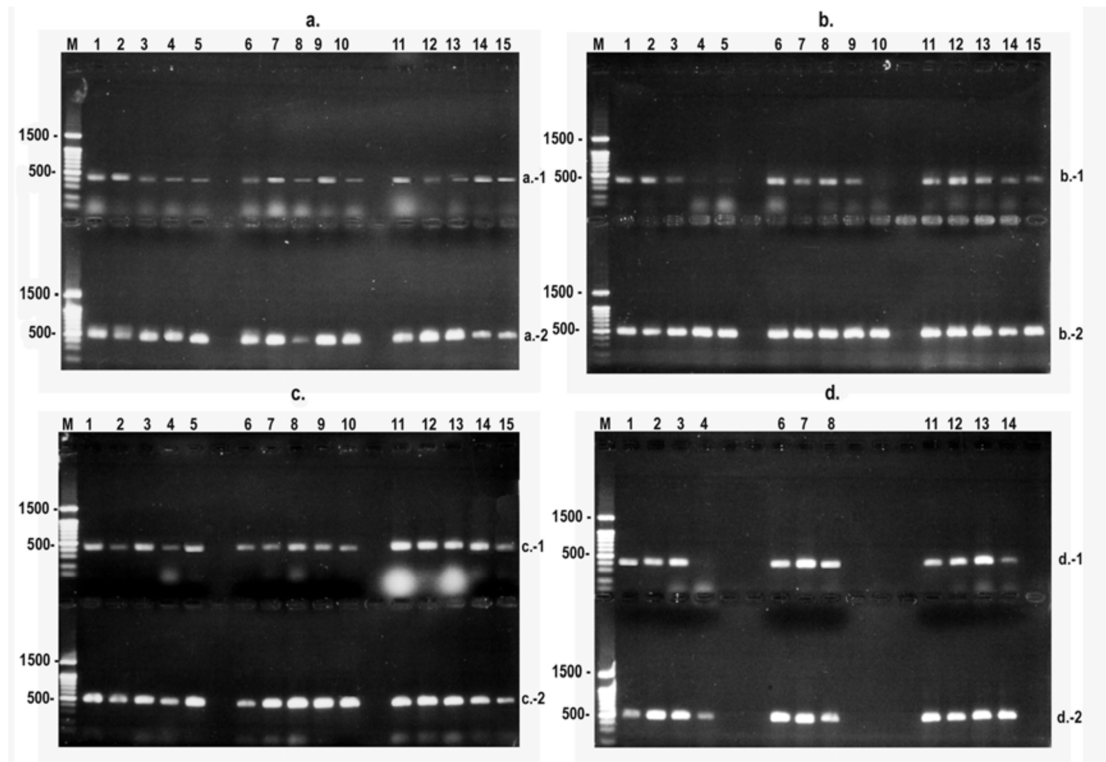

Figure 3.42 Semi-quantitative RT-PCR of PmCLP gene in hepatopancreas of *P. monodon* challenged with WSSV at 24, 48, 72 and, 96 h (a-1- d-1). Elongation factor 1 alpha from the same template was used as internal control (a-2 – d-2).

Lane M = DNA ladder

For a: 24 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For b: 48 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For c: 72 h

Lane 1, 2, 3, 4, 5 = Control

Lane 6, 7, 8, 9, 10 = Un-induced shrimp

Lane 11, 12, 13, 14, 15 = Heat-induced shrimp

For d: 96 h

Lane 1, 2, 3, 4 = Control

Lane 6, 7, 8 = Un-induced shrimp

Lane 11, 12, 13, 14 = Heat-induced shrimp

Table 3.16 The transcription level of PmCLP gene in hepatopancreas of *P. monodon* after WSSV challenge. Transcriptional level was obtained from the ratio of DNA intensities between PmCLP and elongation factor 1 alpha genes.

Time of Exposure (h)	Transcription level after WSSV treatment		
	Control	**Un-induced shrimp**	**Heat-induced shrimp**
24	0.50$\pm$0.11	0.52$\pm$0.10	0.53$\pm$0.08
48	0.36$\pm$0.17	0.49$\pm$0.16	0.51$\pm$0.14
72	0.56$\pm$0.22^{a}	0.58$\pm$0.11^{a}	0.90$\pm$0.24^{b}
96	0.66$\pm$0.27	0.87$\pm$0.12	0.73$\pm$0.25

Note: Different superscripts indicate significant difference between treatments ($p \leq 0.05$)

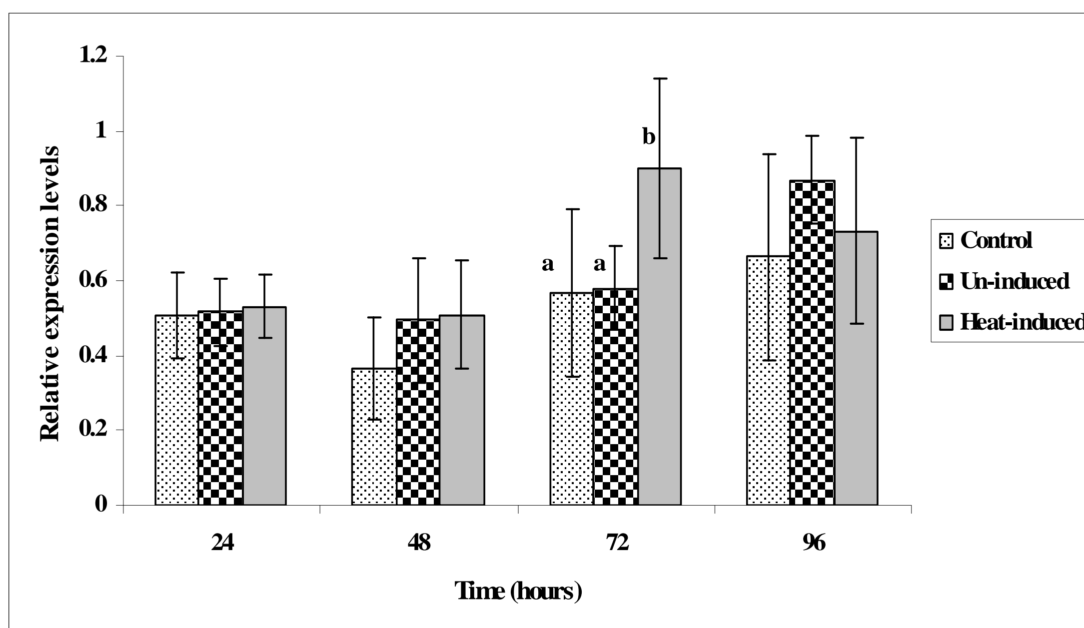

Figure 3.43 The time-course relative expression level of PmCLP gene and elongation factor 1- alpha gene for 24, 48, 72 and 96 h post challenge with WSSV.

3.6 In vitro expression of recombinant PmAQP1

The full length cDNA of PmAQP1 gene of *P. monodon* was obtained at 2,943 bp in length with ORF of 783 bp corresponding to 261 amino acids. Calculated molecular weight of the deduced PmAQP1 was 27.77 kDa. Expression of recombinant clone of PmAQP1 (27.77 kDa) after induced by IPTG at 37 °C was examined. The protein found at the expected size after induced by IPTG for 6 h. The recombinant protein was stably expressed at 12, 24 h post IPTG induction (Fig.3.44).

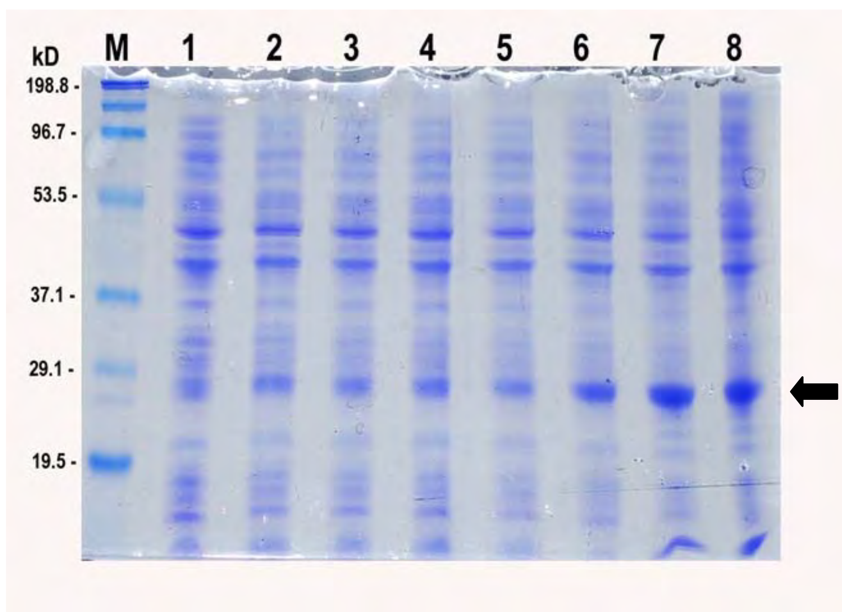

Figure 3.44 *In vitro* expression of recombinant PmAQP1 of *P. monodon* at 0, 1, 3, 6, 12, 24 h after induced by 1 mM IPTG (lanes 3-8) using SDS-PAGE. pET17b vector not insert in *E. coli* BL21(DE3) *pLyss* (lanes1) and pET17b vector in *E. coli* BL21(DE3) *pLyss* cell not induced by IPTG (lanes 2) were included as the control.

CHAPTER IV

DISCUSSION

4.1 Determination of WSSV and ammonia stresses in *P. monodon*

WSSV has been reported to cause severe mortalities to various species of penaeid shrimp as well as to other aquatic invertebrates. Hameed, Charles, and Anilkumur, (2000) compared the susceptibilities between 3 species of freshwater prawn and 2 species of marine shrimp to WSSV and the results indicated that the freshwater prawns were more tolerant than the marine shrimp. The WSSV caused 43.3% and 53.3% mortality in *Macrobrachium lamerrae* and *M. idella* respectively when the animals were exposed to pathogen by immersion method whereas the same virus caused 53.3% and 66.7% mortality in *M. lamerrae* and *M. idella* respectively when the pathogen was administered orally after 15 days. The WSSV caused 100% mortality in *P. indicus, P. monodon, M. lamerrae* and *M. idella* at the end of 72 h, 48 h, 5 days and 8 days post-inoculum respectively when the animals were injected with the viral inoculum intramuscularly. These studies confirm the specific action of WSSV as a pathogen on marine shrimp and freshwater prawns.

Although, a number of WSSV challenge tests have been conducted in various species of shrimp including *P. monodon*, these challenge conditions are not easily reconstructed due to the inconsistency of virus stock and its virulence. In this study, acute toxicity of WSSV was carried out with the addition of the method for determining the quantity of WSSV used in the test. Number of WSSV particles from the stock was first quantified using comparative PCR. The result revealed that the WSSV stock was 1.45 x 10^{11} viral particles/ml. Therefore, the result of 96 h LC_{50} value of WSSV for *P. monodon* at 266.71 dilutions can be calculated as 1.24x 10^9 viral particles/ml. With this technique, the value of LC_{50} obtained in this study can be adopted for the future WSSV challenge test and the problem with various concentrations of virus dilution between different virus stocks will be minimized, resulting in the possibility for comparing the results between different challenge tests.

In ammonia stress, toxic effects of ammonia on the juveniles of different species of penaeid shrimps are given in Table 4.1. LC_{50} is commonly used as measurable value of toxic effect of most toxicants to aquatic animals. Toxicants of ammonia are separated into 2 types, total ammonia-N and NH_3–N. The values of 96 h LC_{50} of NH_3-N ranged

between 0.87 mg/l in *M. ensis* (Nan and Chen, 1991) to 2.47 mg/l in *P. chinensis* (Chen and Lin, 1992). The result of LC_{50} value at 96 h of ammonia to *P. monodon* obtained from this study is 0.69 mg/l. It appears that 96 h LC_{50} in this study is considerably lower than that of other studies. Previous documents on toxicity of ammonia to *P. monodon* reported that 24-h LC_{50} on larvae, nauplius, zoea, mysis and postlarvae, were 0.54, 0.76, 2.17 and 4.70 mg/l, respectively (Chin and Chen, 1987) while 24-h LC_{50} on *P. monodon* juveniles were 2.68 mg/l for NH_3 –N (Chen and Lin, 1991). Acute toxicity tests in most of previous studies were conducted using static renewal condition which water exchange was included while in this study static condition (no water exchange) was used. Hence, *P. monodon* used in this experiment appears to be more sensitive to ammonia than other shrimp and *P. monodon* earlier reported. Salinity is proved to be one of the major factors that influence stress tolerance in shrimp. Chen and Lin (1992) reported that toxicity of ammonia increased as salinity decreased. Inconsistency of LC_{50} values can be seen between studies indicating the significant effect of various experimental conditions and different shrimp status. Therefore, it is necessary to perform acute toxicity test for different experiment conditions.

Table 4.1 The 96h LC_{50} of NH_4-N and NH_3-N on several species of penaeid shrimp.

Species	Salinity (‰)	NH_4-N (mg l^{-1})	NH_3-N (mg l^{-1})	References
P. chinesis (36.1 mm)	10 20 30	28.18 38.87 42.44	1.94 2.46 2.47	Chen and Lin (1992)
P. monodon (35.4 mm)	20	45.58	1.29	Chen and Lei (1990)
P. paulenesis (5.45 g)	28	38.72	1.10	Ostrensky and Wasielesky (1995)
P. penicillatus (35.8 mm)	25 34	24.88 29.77	0.99 1.11	Chen and Lin (1991)
L. vannamei	15 25 35	24.39 35.40 39.54	1.20 1.57 1.60	Lin and Chen (2001)
M. ensis (10.7 mm)	25	35.59	0.87	Nan and Chen (1991)
P. monodon	11	22.57	0.69	present study

4.2 Induction of stress tolerance in *P. monodon*

It has been demonstrated that heat induction or heat acclimation induces adaptive changes that improve the ability to cope with extreme environmental heat (Horowitz and Meiri, 1985; Claussen, 1979; Horowitz, 2007). The extra outcome of this acclamatory process is that an adjustment to one stressor can intensify the degree of adjustment to another stressor. This cross reinforcement raises the possibility of inducing adaptation to a given stressor without prior exposure to it. This process is defined as cross-tolerance. Heat induction in this study was conducted by shocking the experiment shrimp in water at 35ºC for 2 h and brought back to ambient temperature (28 ºC). The condition was based on the thermal tolerance limit of *P. monodon* (Kanchana Doungpunta, 2004).

It should be noted that caution must be paid to the range of appropriate temperature used for heat acclimation in each species. Basically, the inducing temperature should be high enough to stimulate the expression of the genes crucial for tolerance

mechanisms but it also has to be lower than lethal temperature. Narrow window between heat acclimation and lethal heat treatment could cause failure on tolerance induction. As demonstrated in freshwater prawn, *Mcrobrachium rosenbergii*, heat shock at 38ºC (10ºC higher than ambient and 1ºC lower that lethal temperature) for 30 min did not induce thermotolerance or hypersalinity and ammonia stresses (Rahman, et al, 2004). The results from the present study provide more evidences on suitable heat induction in *P. monodon* that enhances cross tolerance during pathogenic and environmental stresses.

4.2.1 Cross tolerance to pathogenic stress

Survival rate of heat-induced shrimp after WSSV challenge was 15% higher than that of normal shrimp. Additionally, the number of virus particles detected in heat-induced shrimp after 72 h of post challenge was significantly lower than that of un-induced shrimp, indicating that heat induction might be involved in controlling virus replication in *P. monodon*. These results confirmed that heat induction has caused a better tolerance to WSSV infection in *P. monodon*. Granja, et al., (2003) studied on the enhancement of pathogenic tolerance by hyperthermia. It has been suggested that hyperthermia could facilitate apoptosis in WSSV-infected *L. vannamei* and might be one of the mechanisms responsive for increased survival rate of infected shrimp maintained at relatively high temperature (32°C). The result from previous study also confirmed that pre-heat at 35°C for 2-6 h can enhance the *V. harveyi* (concentration at 10^{8} CFU/ml) tolerance in *P. monodon* (Kanchana Doungpunta, 2004). There are numerous cases regarding cross tolerances induced by heat. For example, DuBeau, et al., (1998) studied the involvement of heat induction that can enhance osmotic tolerance (salinity stress) in Juvenile salmon, Clegg, et al., (1998) studied thermotolerance in the pacific oyster by heat and the result indicated that heat induced oyster exhibited 20% less mortality when compared to un-induced oyster.

4.2.2 Cross tolerance to environmental stress

Ammonia tolerance of *P. monodon* was determined between normal shrimp and shrimp induced by heat (35ºC, 2 h). The result revealed that the survival rate of heat induced shrimp after ammonia exposure was at least 20% higher than that of un-induced shrimp. This indicates that heat induction can enhance ammonia tolerance in *P. monodon*. Similar results can be obtained from the study of rainbow trout. Linton, et al. (1998) reported the experiment on the long term acclimation of the fish to small temperature

increase (+2°C above the natural fluctuating water temperature) prior to exposure to lethal temperature and ammonia stress. The result indicated that the acclimated fish were more resistant to the stresses.

4.3 Molecular characterization of the stress responsive genes in *P. monodon* during pathogenic and environmental stresses

Complete cDNA sequences, tissue distribution, and expression patterns of three potentially heat sensitive genes from *P. monodon* have been studied and reported in this study. These include aquaporin, Glucosamine-6-phosphate deaminase, and C-type lectin-like protein genes. These genes were preliminarily recognized as heat inducible genes due to their presences in heat-induced haemocyte and gill EST libraries.

4.3.1 Aquaporin

4.3.1.1 Molecular characterization

Aquaporins are a large family of integral membrane proteins that facilitate rapid transport of water across cell membranes in all organisms. The physiological role of these water channel proteins is particularly important in plants, because of their continuous water recruitment (Chrispeels and Maurel, 1994; Kjellbom, et al., 1999), and in specialized fluid-conducting organs of animals, such as, e.g., kidney, lacrimal glands, and lungs, because of their active control of water homeostasis (Echevarria and Ilunda´in, 1998).

Partial sequence of aquaporin obtained from heat-induced gill EST transcript was used for further determining the full length cDNA sequence. *P. monodon* AQP was cloned from gill and a deduced protein contained 261 amino acid residues containing major intrinsic protein domain (MIP). This confirms the identity of AQP. MIP was first reported in eye lens fiber cells (Gorin, et al. 1984) but AQP1 was the first shown to be a highly specific water channel (Preston, et al., 1992). The MIP domain is now known as a main character in proteins of aquaporin family (Heymann and Engel, 1999). All major intrinsic proteins can be divided into two large groups: water-selective aquaporins (AQP-Z) that regulates water exchange and aquaglyceroporins (GlpF) that allow passage of small solutes, such as glycerol and urea.

The number of characterized AQPs is still growing in various species of organisms. So far, 13 members of the AQP family in animals have been reported. These include AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 (water-selective

aquaporins) (King, et al., 2004), AQP3, AQP7, AQP9 and AQP10 (aquaglyceroporins), AQP11 (cannot be classified as either, because no transport function was shown) (Gorelick, et al., 2006) and, AQP12 (intracellular and no tested function) (Ishibashi, 2006). AQP8 is more similar to the AQP-Z group from its primary sequence, but the genome structure is unique and can be independently grouped, although it has only one member. In bacteria, a third subgroup has been proposed that permeated both water and small molecules (Froger, et al., 2001). They are name superaquaporins as they belong to the aquaporin superfamily with very low homology with conventional AQPs.

There are reports that AQP1 transport cations (Anthony, et al., 2002) and gases such as carbon dioxide (Cooper and Boron, 1998) and ammonia (Nakhoul, et al., 2001). Among highly conserved amino acids in AQP family, two repetitions of asparagines-proline-alanine residues (NPA box) are present (Park and Saier, 1996). Two NPA repeats that are critical for the water and substrate permeation function were also located in AQP of *P. monodon*. The second NPA box is the clue for the distinction between GlpF and AQP-Z groups. In GlpF group, NPA box contains NPARD while NPARS/A is present in NPA box of AQP-Z group. The result in present study confirms the identity of AQP in *P. monodon* and categorizes it into AQP-Z group. (Table 4.2, Table 4.3)

P. monodon AQP shares high homology to AQP1 genes from several species including *Coptotermes formosanus* (BAG72254.1, 55% identity), *Aedes aegypti* (EAT45185.1, 53% identity), *Lutzomyia longipalpis* (ABV60346.1, 51% identity), and *Bemisia tabaci* (ABW96354.1, 49% identity). Therefore, *P. monodon* AQP gene in this study is identified as AQP1 and designated as PmAQP1

Phylogenic analysis of PmAQP1 with other AQP1 from different species were divided into two clusters: AQP1s from cow, sheep, amphibians, insects, and shrimp (*P. monodon*) in one large cluster, and AQP1s from human, rat, and mouse in another group (Fig 4.1).

AQPs are commonly found in all tissues. They are generally expressed in both fluid-transporting tissue (such as kidney tubules and glandular epithelia) and non-fluid-transporting tissue (such as epidermis, adipose tissue). Although in mammals, ten MIPs have been cloned and functionally characterized. Some of them, such as AQP1, AQP3, and AQP4, are widely distributed in the body (King and Agre, 1996) while some (AQP0, AQP2, and AQP6) are tissue specific (Deen, Verdujk, and Knoers, 1994; Mulders, Preston, and Deen, 1995; Ma, Yang, and Verkman, 1996).

Distribution of PmAQP1 was determined by RT-PCR. It was found to express in hepatopancreas, gill, ovaries, testes, intestine, stomach and heart but not in haemocyte, epidermis, lymphoid organs and muscle. Gill appears to be the main location of PmAQP1 activity. This result was in agreement with the studies in European eel, *Anguilla anguilla* (Cutler and Cramb, 2002), Mozambique tilapia, *Oreochromis mossambicus* (Watanabe, Kaneko, and Aida, 2005) and adult silver sea bream, *Sparus sarba* (Deane and Woo, 2006). In silver sea bream, gill and spleen displayed modulated expression during salinity acclimation. However, in hypoosmotic and isoosmotic condition, AQP was approximately 5-8-fold greater than normal or hypersaline condition (Deane and Woo, 2006). Silver sea bream is highly euryhaline. They are able to tolerate salinities ranging from hypoosmotic to hypersaline with no appreciable changes in serum ions or tissue hydration (Woo and Kelly, 1995).

In hypoosmotic conditions, shrimp, which is similar to other aquatic animals, have to counteract the passive gain of water and loss of ions by producing dilute urine and actively taking up ions across the gills whereas in seawater and hypersaline conditions the passive gain of ions and loss of water is counteracted by increased drinking of seawater, absorbing water and ions across the gut, and secreting excess ions at the gills and kidney.

Table 4.2 Peptide sequence alignment of AQP genes around NPA boxes (underline)

Groups	Species	NPA1	NPA2
AQP-Z	*Coptotermes formosanus*	SGCHINPAVTCGL	TGASMNPA**RS**FGP
	Aedes aegypti	SGCHINPAVTIGL	TGASMNPA**RS**FGP
	Danio rerio	SGAHINPAVTVAM	TGASMNPA**RS**FGP
	Mus musculus	SGGHINPAVTVAM	TGASMNPA**RS**FGP
GlpF	*Caenorhabditis elegans*	SGGHFNPAVSIAF	LGYPINPA**RD**LGP
	Xenopus laevis	SGGHLNPAVTFAL	SGYAVNPA**RD**FGP
	Danio rerio	SGGHLNPAVTFAL	SGYAVNPA**RD**FGP

Table 4.3 Distribution of aquaporin genes

Organisms	GlpF group	Middle group	AQP-Z group	AQP8 group	Super AQP
Bacteria					
E. coli	1		1		
S. flexneri	1	1			
H. infuenzaae	1		1		
P. aeruginosa	1				
S. typhimurium			1		
B. abortus			1		
L. lactin		1			
B. subtili		1			
Yeast					
S. cervisiae	2		2		
Protozoa					
L. major	1		4		
T. cruzi			4		
T. brucei	3				
P. falcuparum			1		
C. parvum			1		
Nematode					
C. elegans	5		3		3
Plant					
A. thaliana	9		13	10	3
Insect					
D. melenogaster			7		1
Vertebrates					
Fugu	6		6	1	3
Chicken	3		5	1	2
Mouse	3		6	1	2
Human	4		6	1	2
Invertebrate					
P. monodon			1		

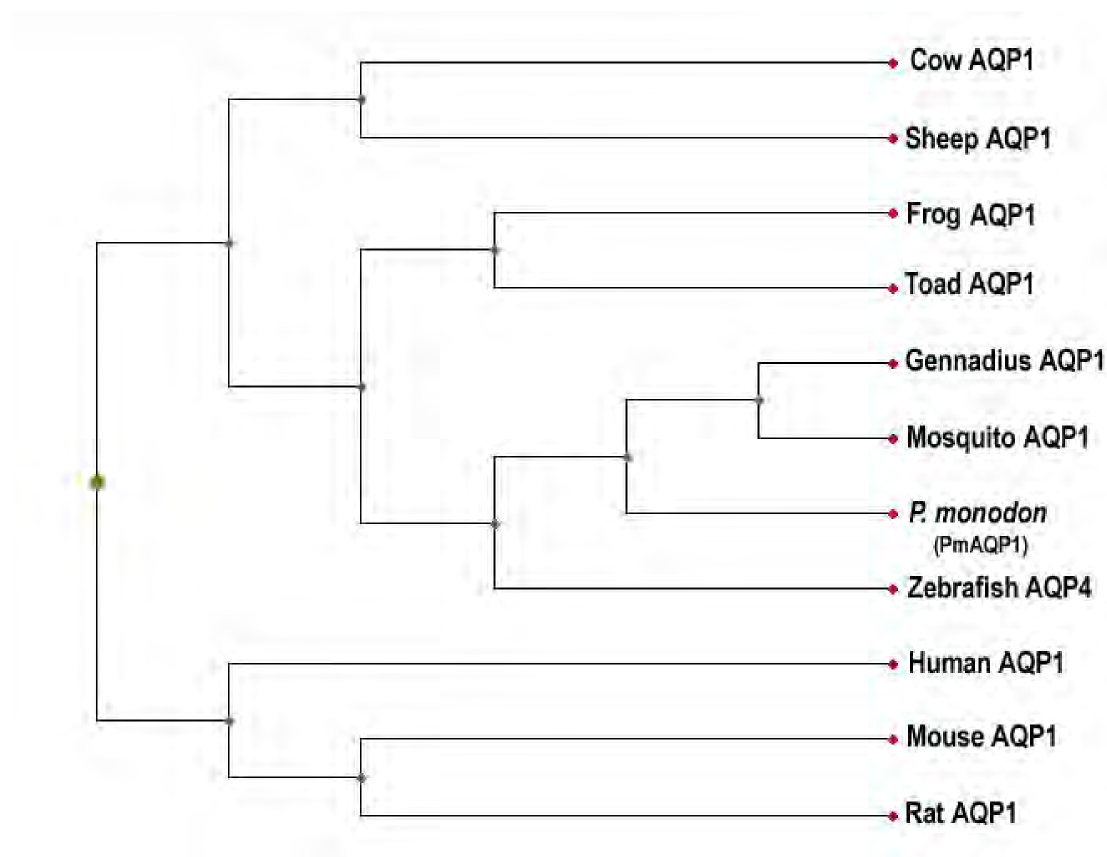

Figure 4.1 Phylogenetic trees showing the relation of shrimp (*P. monodon*) AQP to other knows AQPs. The relationships among cowAQP1: P47865, SheepAQP1: P56401, Frog: P50501, ToadAQP1: AAC69693, GennadiusAQP1:ABW96354, Mosquite: XP1656931, zebrafishAQP4: NP1003749, HumanAQP1: P29972, MouseAQP1: Q02013 and RatAQP1: P29975 were analyzed by the neighbour-joining method for their mature amino acid sequences, based on the alignment performed by Clustal W software.

4.3.1.2 Polymorphism of PmAQP1

Polymorphic analysis of PmAQP1 in this study was a preliminary study to roughly identify genetic variation of this gene in *P. monodon*. SSCP is a rapid and sensitive technique to discriminate DNA fragments of differing sequences. SSCP analysis discriminates single-stranded DNA fragments according to differences in their electrophoretic mobility due to sequence-dependent conformational changes of the fragments (Orita, et al., 1989).

Four synonymous SNPs (No amino acid change) were located at 430, 451, 562, 721 and one non-synonymous SNP that lead to amino acid changes in the protein product

was found at (579 T>G). High number of SNPs found within coding region of the gene indicates multiple loci of PmAQP1 gene and it can also be speculated that the variants of PmAQP1 might be associated with crucial disorder or diseases in *P. monodon*.

In human, reports on SNPs are far more informative than any investigated vertebrates. Numerous studies indicate that variants in AQP family members have been associated with many disease phenotypes. For examples, variants in AQP2 show partial loss of function (Canfield, et al., 1997, Mattia, et al., 2005), variants in AQP-4 reduce cellular water permeability (Sorani, et al., 2008), and a single amino acid substitution of AQP11 can cause lethal renal phenotype of sudden juvenile death syndrome (Tchekneva, et al., 2008).

An investigation into the levels and patterns of polymorphism in PmAQP1 is an important first step toward a better understanding of the evolutionary forces that have been operated on this gene in shrimp. Further investigation should be focused on identifying SNPs on AQP genes which are related to crucial phenotypes of the shrimp. This will be valuable for a better understanding of genetic diseases and help predict the diseases or chemical sensitivity in shrimp.

4.3.1.3 Expression pattern of PmAQP1 during stress condition

Heat acclimation involves numerous molecular and cellular changes. In present study, the expression pattern of PmAQP1 was investigated in shrimp induced by heat and during ammonia exposure. Transcriptional level of PmAQP1 gene was determined in shrimp exposed to ammonia using semi-quantitative RT-PCR. The result indicated that PmAQP1 gene responded to heat induction within 6 h. The ratio of expression level of PmAQP1 in heat induced shrimp was 1.7 fold higher than that of normal shrimp and maintained the level for more than 12 h after induction. The expression level of PmAQP1 began to decrease after 48 h and came down to normal level within 72 h.

After ammonia exposure, within 24 h, expression level of PmAQP1 was significantly up-regulated in heat induced shrimp while that of un-induced shrimp remained the same level as normal shrimp and increased significantly within 48 h. The levels of PmAQP1 from both heat induced and un-induced shrimp decreased to normal level within 72 h of exposure. These results show that PmAQP1 can be induced by both heat and ammonia treatments but PmAQP1 responses to heat quicker than ammonia, resulting in the higher and earlier expression of PmAQP1 in heat induced shrimp.

Most studies of AQPs in aquatic animals emphasize the role and importance of AQPs in water regulation during osmotic stress. For examples, Umenishi, Narikiyo, and Schrier (2004) reported that expression of AQP1 was raised by osmotic stress after 12 h and still increased until 24 h in a time-dependent manner. Deane and Woo, (2006) reported that AQP3 increased 5-8 folds higher in gills of hypoosmotic and isoosmotic acclimated sea bream in comparison to those maintained at seawater and hypersalinity. It was suggested that increased water channels were needed as a protective response against osmotic swelling and subsequent gill cell damage. Similar result was reported in European eel (Cutler and Cramb, 2002). In mammals, AQP3 is predominantly located in the basolateral membrane of renal collecting duct cells and it has been speculated that it could serve as an exit pathway for both water and urea during antidiuresis (Ishibashi, Nakornsri, and Nagai, 1994) whereas teleosts are mainly ammoniotelic (Anderson, 1995).

AQP are also involved in a variety of physiological and cellular functions under stress (Umenishi, et al., 2005; Dibas, et al., 2007; Sidhaye, et al, 2008; Nase, et al., 2008). Many roles of AQP are now considered to be exploited for clinical benefit (Agre, et al., 2002; Morishita, et al., 2004; Beitz, 2005). The expressions of some of these aquaporins increase near the time of birth and appear to be regulated by grown factor, inflammation, and osmotic stress (Verkman and Song, 2006). In invertebrates, increasing number of AQP was reported in various species and most of the studies supported the important role of AQP in cellular homeostasis (Amado, Freire, and Souza, 2006; Boyle, et al., 2007).

In present study, it has been shown that expression of PmAQP1 can be induced by heat and it is possibly one of key molecules that play important roles in cross tolerance process of *P. monodon* during ammonia exposure.

4.3.1.4 In vitro expression of recombinant PmAQP1.

Expression of recombinant clone of aquaporin (27.77 kDa) after induced by IPTG at 37 °C was examined. The protein found at the expected size after induced by IPTG for 3 h. The recombinant protein was stably expressed at 6, 12 h post IPTG.

4.3.2 Glucosamine-6-phosphate deaminase

4.3.2.1 Molecular characterization of Glucosamine-6-phosphate deaminase

Glucosamine-6-phosphate deaminase (GluN6P-deaminase, EC 3.5.99.6) is an enzyme formerly known as glucosamine-6-phosphate isomerase (EC 5.3.1.10). It

catalyzes the reversible conversion of glucosamine-6-phosphate (GluN6P) into D-fructose-6-phosphate (Fru6P) and ammonium. It is a hexameric enzyme with the identical subunits with the molecular weight of 29-33 kDa (Nakamura, et al., 2000). The *E. coli* GluN6P-deaminase is a *K*-type allosteric enzyme, which is activated by substrate cooperatively and by the allosteric activator, *N*-acetyl-glucosamine-6-phosphate (GluNAc6P). This enzyme has been currently characterized in a variety of organisms such as *Escherichia coli*, *Candida albicans*, *Musca domestica*, *Plasmodium falciparum*, *Canis familiaris*, *Bos taurus,* and *Homo sapiens*.

GluN6P-daminase involves regulation of amino sugars which are important building blocks for structural polysaccharides or sugar chains. Its role regarding stress responses in organisms is limit. Some evidences indicate that it plays an important role in hyperthermophilic organisms (Borys, Linzer, and Papoutsakis, 1993; Butler and Spier, 1984; Schachter, 1986; Andersen and Goochee, 1995; Yang and Butler, 2000).

PmGluN6P-deminase is a small enzyme. Its full length sequence can be obtained from EST transcript of haemocyte and gill heat shock libraries. PmGluN6P-deaminase is also a conserved protein. Sequence similarities between PmGluN6P-deaminase gene and that of other species are high. The close similarities include GluN6P-deminases of *Ciona intestinalis* (LOC100184260, 77% identity), *Culex quinquefasciatus* (CPIJ008074, 77% identity), *Aedes aegypti* (AAEL013877, 77% identity), and *Nasonia vitripennis* (LOC100115436, 76% identity).

Analysis shows that PmGluN6P-deaminase exhibits high sequence homology to *C. quinquefasciatus* Oscillin, a protein containing a Glucosamine_iso domain (Parrington, et al., 1996). Oscillin was a sperm derived GluN6P-deaminase homolog from hamster which was previously thought to be able to induce calcium oscillation in eggs at fertilization. However, latter evidences did not support the notion that oscillin was the sperm-specific factor responsible for calcium oscillations (Wolosker, et al., 1998; Nakamura, et al., 2000, Amireault and Dubé, 2000).

Phylogenic analysis of PmGluN6P-deaminase with similar GluN6P-deaminases from different species results in 2 main clusters; frog, fish, insects, and *P. monodon* are in one cluster and vertebrates such as rat and human are in another cluster (Fig 4.2).

PmGluN6P-deaminase was found to express in 9 tissues including haemocyte, hepatopancreas, epidermis, gill, ovary, testis, intestine, lymphoid organ, and stomach. The levels of PmGluN6P-deaminase present in gill, ovary, and testis appeared to be

greater than that of the others and undetectable level was observed in heart and muscle (Fig. 3.14).

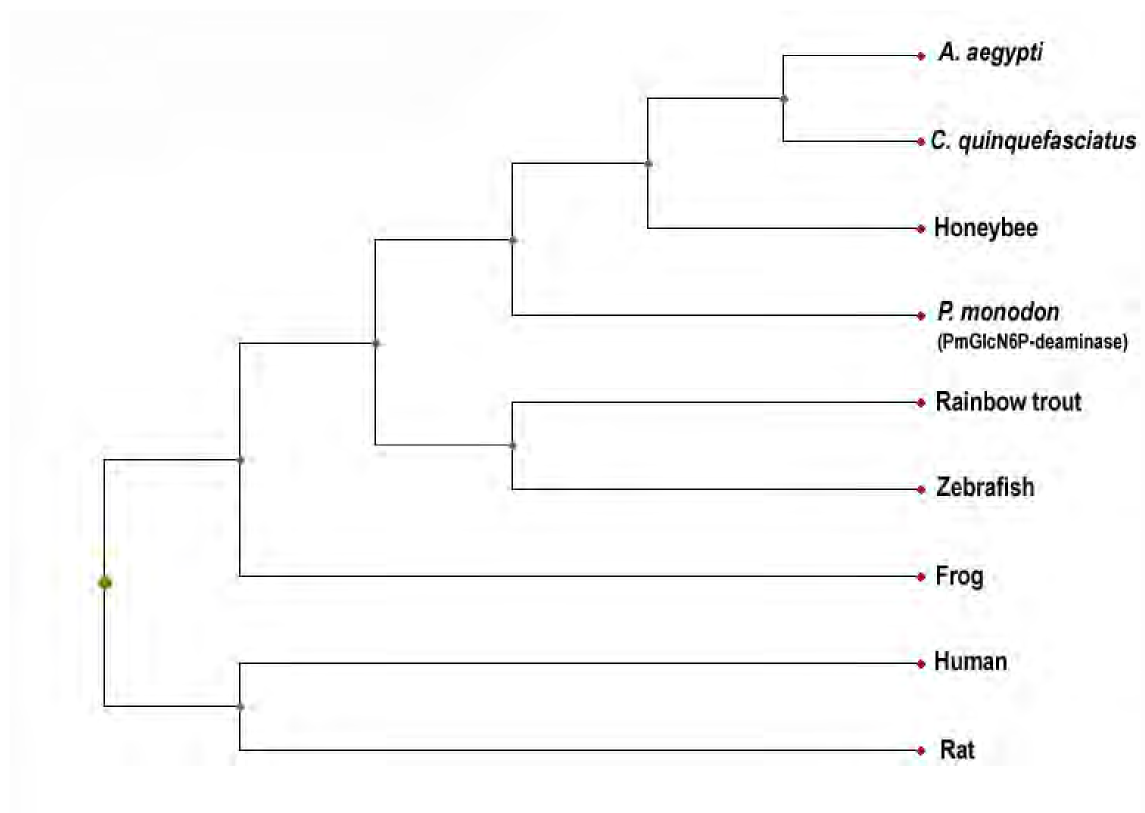

Figure 4.2 Phylogenetic trees of GluN6P-deaminase from *P. monodon* and others. Known GlcN6P-deaminases from *A.aegypti* (XP1657194), *C. quinuefasciatu*s (XP1849348), Honeybee (XP393026), Rainbow trout (ACO08728), Zebrafish (NP1017867), Frog (NP1083339), Human (NP612208), Rat (NP1099475), and Salmon (NP1134003) were analyzed by the neighbour-joining method using their mature amino acid sequences, based on the alignment performed by Clustal W software.

4.3.2.2 Expression pattern of PmGluN6P-deaminase

The expression patterns of GluN6P-deaminase in human was most abundant in spleen, ovary, kidney, uterus, and testis, while liver and heart displayed lower expression levels. The tissue distribution of mouse expressed oscillin exhibited the highest level of expression in testis and ovary (Nakamura, et al., 2000). In cell line, GlcN6Pdeaminase was induced by its substrate and from media ammonia (Cayli, et al., 1999).

Expression level of PmGluN6P-deaminase was high in various tissues except in heart and muscle. The transcription level of PmGluN6P-deaminase gene in juvenile *P. monodon* increased significantly after 12 h of post induction confirming the heat inducible capability of PmGluN6P-deaminase gene in *P. monodon*. However, no

significant change in its expression in shrimp during stress was detected, indicating that the activity of PmGluN6P-deaminase does not relate to the enhancement of ammonia stress tolerance in *P. monodon*.

Most documents regarding GluN6P-deaminase emphasize the involvement of this enzyme to critical conditions of growth and susceptibility in microorganisms (Yamada-Okabe and Yamada-Okabe, 2002; Komatsuzawa, et al., 2004; Tanaka, et al., 2005; Solar, et al., 2008; Jiang, et al., 2009). GlcN6P-deaminase plays crucial role in bacteria by catalyzing the first committed step in a biosynthetic pathway leading to amino sugar-nucleotide precursors of bacterial peptidoglycan. Inhibition of GluN6P deaminase precluded peptidoglycan biosynthesis and resulted in a strong bacteriolytic effect.

In hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1, a novel glucosamine-6-phosphate deaminase, GlmD*Tk*, was identified. It contained a highly conserved domain similar to the one in GlcN6P syntheses which played a central role in the migration of ammonia. Its expression was induced by the chitin degradation intermediate, diacetylchitobiose. GluN6P-deaminase and related enzymes in KOD1 possess high thermostability. It is expected that these enzymes can be useful catalysts for conversion of the unused biomass. In *P. monodon*, PmGluN6P-deaminase shows no significant evidence on its role in enhancing ammonia tolerance. However, its expression responds to heat induction, speculating that it might relate to different route of stress response.

4.3.3 C-type lectin domain containing protein (PmCLP)

4.3.3.1 Molecular characterization of PmCLP

C-type lectins are commonly found in almost all living organisms. They are capable of binding to specific carbohydrates, therefore, involving important role in non-self recognition and clearance of invading microorganisms (Dodd and Drickamer, 2001; Vasta, et al., 2004). There are numerous reports on their participation in various immune responses of invertebrates, including phagocytosis (Kaneko, et al., 2002; Luo, et al., 2006; Sierra, et al., 2005), antibacterial activity (Schroder, et al., 2003), proPO activation (Yu and Kanost, 2000), encapsulation and melanization (Ling and Yu, 2006), and nodule formation (Koizumi, et al., 1999).

C-type lectins were originally described as a group of Ca^{2+}-dependent (C-type) carbohydrate-binding proteins that differ from other lectins (Drickamer, 1988). C-type lectins with diverse overall architecture generally contain homologous carbohydrate-

recognition domains (CRDs) that mediate sugar binding. Now, proteins that contain domains with sequence similarity to C-type lectin domain are included into this group (Zelensky and Gready, 2005). Many proteins containing Ca-dependent (C-type) lectin-like domains (CTLDs) are also related to immunity. These include collectins, coagulation factor binding protein, IgE Fc receptor and NK cell receptor (Iwanaga and Lee, 2005; Zelensky and Gready, 2005).

Ca^{2+} is a direct ligand in mammalian C-type lectin carbohydrate binding as demonstrated by mannose-binding protein (MBP) (Weis, Drickamer, and Hendrickson, 1992). Most C-type lectins are known to be calcium dependent and they are sensitive to divalent chelators. However, some animal C-type lectins do not require calcium to function. For example, OLABL, a C-type lectin from the eggs of shishamo smelt (*Osmerus* (*Spirinchus*) *lanceolatus*), does not require Ca^{2+} for lectin activity (Hosono, et al., 2005) and several insect C-type lectins also do not require calcium for their binding activity, but do require it for agglutinating activity (Yu, et al., 2006; Shin, et al., 2000).

Lectins found in invertebrates are known to be less conserved when compared to vertebrate lectins (Robinson, et al., 2006). In shrimp, several C-type lectins have also been purified and characterized (e.g. Ratanapo and Chulavatnatol, 1990; Fragkiadakis and Stratakis, 1995; Kondo, Itami, and Takahashi, 1998; Sritunyalucksana, Cerenius, and Söderhäll, 1999; Cominetti, et al., 2002; Maheswari, Mullainadhan, and Arumugam, 2002; Alpuche, et al., 2005; Yang, et al., 2007; Rittidach, Paijit, and Utarabhand, 2007; Sun, et al., 2008). Serum lectins of *P. indicus* (Maheswari, Mullainadhan, and Arumugam, 1997) and *P. paulensis* (Marques and Barracco, 2000) do not require cations (Ca^{2+} and Mg^{2+}) for their activities. Sun, et al. (2008) demonstrated that agglutination activity of the mature Fc-hsL, C-type lectin found in *F. chinensis*, was calcium-dependent, but binding and antimicrobial activities were calcium-independent.

Function and carbohydrate specificity of C-type lectins can be predicted using the presence of their motifs. Four Ca^{2+}-binding sites have been identified in different groups of C-type lectins. Sites 1, 2, and 3 are found in the rat MBP-A (mannose-binding protein A) and sites 1, 2 and 4 are seen in human ASGPR-I (Zelensky and Gready, 2005). Ca^{2+}-binding site 2 is directly involved in carbohydrate binding. Most known CRDs contain a conserved carbohydrate-binding site. The EPN motif in the CRD is specific for binding to mannose and site 2 Ca^{2+}, while some other C-type lectins containing a QPD motif which are specific to galactose. Mutation analyses reveal that carbohydrate-binding specificity could be changed from mannose to galactose by conversion of the EPN sequence to QPD

(Drickamer, 1992). However, not all C-type lectins containing an EPN or QPD motif bind to Ca^{2+}. For example, OLABL possesses a conserved Ca^{2+}-binding site 2 (EPN-WND), but it does not require Ca^{2+} for binding to β-galactoside (Hosono, et al., 2005). AJL-2 from Japanese eel also contains an EPN motif but binds to lactose in a calcium-independent manner (Tasumi, et al., 2002).

In *P. monodon,* a number of C-type lectins have been reported. These include PmAV (Luo, et al., 2003, 2007), PmL (Luo, et al., 2006), and PmLT (Ma, et al., 2008). PmAV contains a single C-type lectin-like domain (CTLD) with no signal peptide coding sequence (Luo, et al., 2003). PmL contains a C-type lectin domain (CTLD) with motif QPD and shows calcium-dependent activities. No potential glycosylation site exists in the PmL amino acid sequence (Luo, et al., 2006). PmLT consists of 2 conserved carbohydrate recognition domains (CRDs). The first CRD contains a QPD motif with specificity for binding galactose, while the second CRD contains a EPN motif for binding to mannose (Ma, et al., 2008). The overall homology of PmLT with other animal lectins was low. The dual-CRD structural organization of PmLT is similar to that of immunolectins from the insect, *M. sexta*, which functions as a pattern recognition protein in innate immunity (Yu, et al., 2002, 2005; Yu and Kanost, 2004).

The results in this study reveal that C-type lectin domain containing protein identified in *P. monodon* (designated as PmCLP) shares very little similarity to other shrimp lectins (*F. chinensis* 17% identity, *P. monodon* 16% identity and *L. vannamei* 11% identity). Instead, PmCLP is more closely to CTLD genes found in several fish (BLAST result). Phylogenetic analysis also indicates that PmCLP belongs to different phylogenetic tree clusters (Fig 4.3). PmCLP contains a single CTLD with 4 highly conserved cysteine residues (Cys65, Cys133, Cys148, and Cys156) (Fig 4.4), a classical characteristic of C-type lectin. However, PmCLP does not contain EPN/QPD or WND motif, which has been predicted to be important for $Ca^{2+/}$carbohydrate binding (Zelensky and Gready, 2005). Also, 4 conserved residues for Ca^{2+} binding sites are not present in PmCLP, suggesting that it is a Ca^{2+} independent lectin and its binding specificity is probably different from other *P. monodon* lectins reported earlier.

Although, prediction of Ca^{2+}/carbohydrate binding properties of an uncharacterized sequence with the presence of these motifs is a useful simplification, it should be noted that the absence of the motifs associated with Ca^{2+}-binding site does not indicate clearly that the CTLD is incapable of binding Ca^{2+}. Similarly, the presence of these motifs does not guarantee lectin activity for the CTLD, as there are numerous

examples of CTLDs that contain the conserved motifs but are not known to bind monosaccharides (Zelensky and Gready, 2005).

Growing evidences indicates that many protein modules containing part or all of the C-type CRD motif serve functions other than saccharide recognition. This motif is recognized as C-type lectin-like domains (CTLDs), reflecting their similarity to CRDs of C-type lectins without necessarily implying common function (Weis, et al., 1992). The structures of a few CTLDs have now been established (Drickamer, 1988; Drickamer and Dodd, 1999). Overall similarity in fold is the most obvious feature of the structures between CRD and CTLD proteins. However, comparisons also reveal important differences in fold, in interactions with Ca^{2+} and in oligomerisation (Drickamer, 1999).

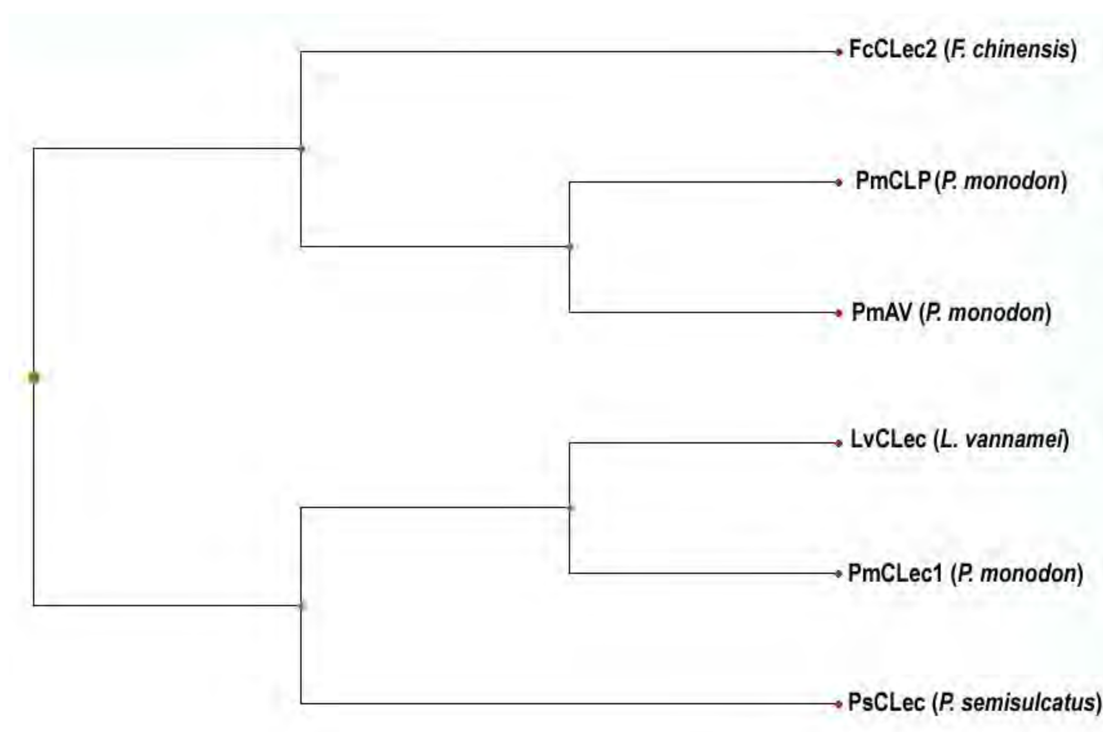

Figure 4.3 Phylogenetic trees showing the relation of PmCLP and other shrimp C-type lectins. The relationships among lectins of *F. chinensis* (FcCLec2: ABA54612.1), *P. monodon* (PmVA: AAQ75589.1), *L. vannamei* (LvCLec: ABI97374.1), *P. monodon* (PmCLec1: ABI97373.1), and *P. semisulcatus* (PsCLec: ABI97372.1) were analyzed by the neighbour-joining method using mature amino acid sequences, based on the alignment performed by Clustal W software.

```
FcCLec          ---------MKFLAPVILTTLISVAASASVR--ATECPSPYEPLDETRCIFLDAFVSYTW 49
PmAV            ----MRHTILVFLSLGVVGSAVATSYEKSANDSKAVCYSPYTAIAD-RCLFVDHQTDGSW 55
PmCLP           MTSLTTSLLFTATSLLFTAAYLVLVPSLAVSQLQRNCPDNYVLLADKCYGFRRTVTD--W 58
                         :   :  .  : :    . :.      * . *  : :    *    ..  *

FcCLec          QETVDLCKSHGGEILTIEDCETFALVYDYIRSQDVTKGKHYWLGATDEVEEGTWKFVNNR 109
PmAV            YDMREYCNLINGDFLKLDDANLLTDIVEYITYQ-VGVNRDYWIGGSDENHEGLWLWTDGT 114
PmCLP           NNARATCLNENADLTSVLTTQEYTEILAHLAAN---YPGVYWVGGATS-NQGAWRWVASG 114
                 :    *   ..::  .:   :  :  :  ::  :        **:*.: . .:* * :. .

FcCLec          LTPMGIPYWG-----VNEPNNGNTYNCAMMHASYNHYWYDAACGSKYNPICLKNY----- 159
PmAV            LMRTGVPLWYHCTSISQQPDGGSSENCAVMRWDSFYHIHDVSCYTSRSVICESRTH---- 170
PmCLP           -APMNEQWWG-------GDHTPTSQRCAYFCSHTRKYWS-STCGVSKNFICEKSAETEAE 165
                    .   *          .  .: .** :      :    :*  . . ** .

FcCLec          ------------------------------------------------------------
PmAV            ------------------------------------------------------------
PmCLP           SGEEFEPEGDTDPENESDEADAERIPSLPSVRAELRSGVSSTTQNMACLVYLLSLCAVMV 225
```

Figure 4.4 Multiple protein sequences alignment of similar C-type lectins from shrimp. Highlight shows 4 highly conserved cysteine residues (Cys 65, Cys 133, Cys 148, and Cys 156) in CTLD.

4.3.3.2 Expression pattern of PmCLP

Hepatopancreas is one of the most important organs in humoral immune responses of shrimp. It contains highly specialized cells and phagocytes that also function in cellular immune responses (Gross, et al., 2001). In this study, PmCLP was expressed constitutively in all tested tissues of *P. monodon* but it appeared that the expression in hepatopancreas was slightly lesser than that found in other tissues which quite contrasted to the results of other shrimp lectins. For examples, PmAV, *P. monodon* C-type lectin involved in antibacteria and antiviral defenses, was highly expressed in the hepatopancreas (Luo, et al., 2003, 2006, 2007; Sun, et al., 2008). cDNA sequences of PmLT and PmLec were also obtained from hepatopancreas (Ma, et al., 2008; Luo, et al., 2006).

The expression level of PmCLP from heat induced shrimp reached its peak and was significantly different from un-induced shrimp after 72 h of exposure and did not respond significantly to WSSV challenge. These results are different from most reports on shrimp lectins which tend to respond much sooner to almost all invaders. Liu, et al., (2007) reported that the expression of Fclectin in hemocytes increased gradually in the first 3 h post-challenge with WSSV and reached the maximums at 3 h post-challenge compare to that of the control animals and then decreases from 6 h to 48 h post infection. Sun, et al., (2008) demonstrated that *Fc-hsL* was expressed constitutively at a relatively low level in the hepatopancreas and increased to a higher level at 24 h after shrimp were

challenged with bacteria. When shrimp were challenged with WSSV, *Fc-hsL* expression in the hepatopancreas decreased at 2 h and gradually recovered to the original level from 6 h to 12 h post-infection, and then decreased again at 24 h post-injection.

However, the late response of PmCLP expression is similar to pathogenic response of *PmAV*. Its expression started to increase from day 2 and reached its peak after 4 days of infection (Luo, et al., 2003, 2007). The late response of PmCLP suggests that function of PmCLP might be different from other lectins.

C-type lectins have diverse functions. In addition to agglutinating activity and opsonic effects, some C-type lectins have antibacterial activities. Numerous studies reported on microbial activities of lectins. These included a SV lectin from a snake venom that exhibited antimicrobial activity against Gram-positive and Gram negative bacteria (Radis-Baptista, et al., 2006), surfactant proteins A (SP-A) and D (SP-D) that inhibited proliferation of Gram-negative bacteria (Wu, et al., 2003), a galactose-binding lectin from the tunicate *Polyandrocarpa misakiensis* (Suzuki, et al., 1990). Several shrimp lectins have also been proved to possess microbial activity; Fc-hsL showed strong antimicrobial activity against Gram-positive bacteria and some fungi, and moderate activity against Gram-negative bacteria (Sun, et al., 2008), *P. monodon* PmAV has anti-viral activity (Luo, et al., 2003), *L. vannamei* LvLT with two CRDs may play a role in white spot syndrome virus (WSSV) infection (Ma, et al., 2007), *F. chinensis* Fclectin, which also has two CRDs, is expressed in haemocyte and is up-regulated upon challenge with WSSV (Liu, et al., 2007).

Lectins with more than one CRD have been identified in decapods and other invertebrates. It has been demonstrated that lectins with multimeric CRD enhance their ability to agglutinate cells or to form precipitates with glycolconjugates in a manner similar to antigen-antibody interactions, suggesting that the number of CRDs provide broader specificity for divers pathogens (Ma, et al., 2008; Sun, Fournier, and Zhang, 2009).

PmCLP is a unique lectin in *P. monodon*. The results show that PmCLP can be detected in all tissues and it is different from all known shrimp lectins. Its expression responses to virus challenge. Further investigate on carbohydrate specificity and function of PmCLP will extend knowledge for understanding the crucial role of novel lectins in *P. monodon*.

CHAPTER V

CONCLUSION

1. Acute toxicities of WSSV to *P. monodon* have been determined. Number of WSSV particles used in this study is determined using semi-quantitative PCR. The calculated value of 96 h LC_{50} of WSSV is 1.24 x 10^{9} viral particles/ml.

2. Toxic effect of ammonia to *P. monodon* has been measured. The values of 96 h LC_{50} of total ammonia-N and NH_3-N are 22.57 and 0.69 mg-N/l, respectively. The result indicates that *P. monodon* is more sensitive to ammonia when compared to other penaeid shrimp.

3. Pre-heat treatment at 35°C (5-7 °C higher than ambient temperature) for 2 h can increase 15 and 20% more tolerance in heat induced shrimp under WSSV and ammonia stresses, respectively. This indicates that heat induction enhances cross tolerance in *P. monodon*.

4. Full length cDNA sequences of aquaporin (PmAQP1), gulcosamine-6-phosphate deaminase (PmGluN6P-deaminase), and C type lectin domain containing protein (PmCLP) genes have been firstly identified and characterized in *P. monodon*. PmAQP1 composes of 786 bp ORF encoding a putative polypeptide of 261 amino acid residues. PmGluN6P-deaminase composes of 948 bp ORF encoding a putative polypeptide of 315 amino acid residues. PmCLP composes of 678 bp ORF encoding a putative polypeptide of 225 amino acid residues.

5. Polymorphic analysis shows variations between the sequences of PmAQP1 genes detected among individual while no difference between sequences of PmGluN6P-deaminase and PmCLP genes is observed.

6. Expression of PmAQP1 gene distributes in many tissues including hepatopancreas, gill, ovaries, testes, intestine, stomach and heart where gill is the main location for

PmAQP1 activity. PmAQP1 is not detectable in haemocyte, epidermis, lymphoid organs and muscle.

7. Expression level of PmAQP1 gene in the gill of *P. monodon* can be significantly up-regulated by heat induction (35 °C for 2 h) within 6 h after induction and reaches its peak after 24 h of post induction. Expression of PmAQP1 gene in normal shrimp also responds to ammonia treatment but it significantly responds almost 24 h slower and reaches its peak after 48 h of post exposure. It is suggested that the quick response and higher up-regulation of PmAQP1 gene in heat induced shrimp might play a functional role in the enhancement of ammonia stress tolerance in *P. monodon*.

8. Recombinant PmAQP1 at the molecular weight of 27.77 kDa is successfully produced using pET17b System. The recombinant protein will be useful for antibody construction and further used as biological tool for determining health status of the shrimp.

9. PmGluN6P-deaminase constitutively expresses in haemocyte, hepatopancreas, epidermis, gill, ovaries, testes, intestine, lymphoid organs and stomach but it is undetectable in heart and muscle. The levels of PmGluN6P-deaminase present in gill, ovaries and testes appear to be greater than the other tissues.

10. Expression of PmGluN6P-deaminase gene is induced by heat treatment (35 °C for 2 h) within 12 h of post induction. However, no significant difference of its expression between heat-induced and normal shrimp is observed after ammonia stress. This indicates that PmGluN6P-deaminase might not be directly involved in ammonia stress response in *P. monodon*.

11. Expression of PmCLP is highly detected in all tested tissues. These include haemocyte, hepatopancreas, epidermis, gill, ovaries, testes, intestine, lymphoid organs, stomach, heart, and muscle.

12. PmCLP gene does not significantly respond to WSSV challenge. However, up-regulation of PmCLP gene responding to heat induction can be detected after 72 h of post induction. The late response of PmCLP gene to pathogen is quite different from

other shrimp lectins but similar to that of PmAV, a *P. monodon* antimicrobial lectin. Evidence from gene expression pattern in stressed shrimp combined with the unique molecular characteristic of PmCLP indicates that PmCLP might be involved in pathogenic response of *P. monodon* but the role of PmCLP might be different from other known shrimp lectins.

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APPENDICES

APPENDIX A

1. LB Broth (per Liter)

10 g of NaCl

10 g of tryptone

5 g of yeast extract

Add deionized H_2O to a final volume of 1 liter. Adjust to pH 7.0 with 5 N NaOH and autoclave.

2. LB Agar (per Liter)

- 10 g of NaCl
- 10 g of tryptone
- 5 g of yeast extract
- 20 g of agar

Add deionized H_2O to a final volume of 1 liter. Adjust to pH 7.0 with 5 N NaOH and autoclave. After, pour into petri dishes (~25 ml/100 mm plate)

3. LB-Ampicillin Agar (per Liter)

- Prepare 1 liter of LB agar. Autoclave and cool to 55 ºC
- Add 50 ml of filter-sterilized ampicillin
- Pour into petri dishes (~25 ml/100-mm plate)

4. 1x TAE Buffer

- 40 mM Tris-acetate
- 1 mM EDTA

5. SOB Medium (Per liter) :

- Bacto-tryptone 20 g
- Yeast extract 5 g
- NaCl 0.5 g

6. Ampicillin

Stock solution. 25 mg/ml of the sodium salt of ampicillin in water. Sterilize by filtration and store in aliquots at –20 ºC

7. 5 M NaCl

Dissolve 292.2 g of NaCl in 800 ml of H_2O. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

8. 1 M $MgCl_2$

Dissolve 203.3 g of $MgCl_2 \cdot 6H_2O$ in 800 ml of H_2O. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

9. 3 M Sodium acetate (pH 5.2)

Dissolve 408.1 g of sodium acetate $\cdot$ $3H_2O$ in 800 ml of H_2O. Adjust pH to 5.2 with glacial acetic acid. Adjust volume to 1 liter. Dispense into aliquots and sterilize by autoclaving.

10. 10% Sodium dodecyl sulfate (SDS) (also called sodium lauryl sulfate)

Dissolve 100 g of electrophoresis-grade SDS in 900 ml of H_2O. Heat to 68 ºC to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust volume to 1 liter. Dispense into aliquots.

11. Ethidium bromide 10 mg/ml

Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4 ºC.

12. TE pH 8.0

- 10 mM Tris · Cl (pH 8.0)
- 1 mM EDTA (pH 8.0)

13. Tris-Borate (TBE)

-Working solution

- 0.089 M Tris-borate
- 0.089 M boric acid
- 0.002 M EDTA

- Concentrated stock solution (5x)

Per liter:

- Tris base	54 g
- Boric acid	27.5 g
- 0.5 M EDTA (pH 8.0)	20 ml

14. Gel-Loading Buffer Type II

- 10x buffers
- 0.25% bromophenol blue
- 0.25% xylene cyanol
- 25% Ficoll (type 400) in H_2O
- Store at room temperature.

15. 10x TEN buffers

- 0.1 M Tris-Cl (pH 8.0)

- 0.01 M EDTA (pH 8.0)

- 0.1 M NaCl

16. Glycerol (10% v/v)

Dilute 1 volume of molecular-biology-grade glycerol in 9 volume of sterile pure H_2O. Sterilize the solution by passing it through a prerinsed 0.22 µM filter. Store in 200-ml aliquots at 4 °C

17. IPTG (20% w/v, 0.8 M)

IPTG is isopropylthio-B-D-galactoside. Make a 20% solution of IPTG by dissolving 2 g of IPTG in 8 ml of distilled H_2O. Adjust the volume of the solution to 10 ml with H_2O and sterilize by passing it through a 0.22 µM disposable filter. Dispense the solution into 1-ml aliquots and store them at -20 °C

18. X-gal solution (2% w/v)

X-gal is 5-bromo-4-chloro-3-indolyl-B-d-galactoside. Make a stock solution by dissolving X-gal in dimethylformamide at a concentration of 20 mg/ml solution. Use a glass or polypropylene tube. Wrap the tube containing the solution in aluminum foil to prevent damage by light and store at -20 ºC. It is not necessary to sterilize X-gal solution by filtration.

19. 10% (w/v) Ammonium persulfate

Ammonium persulfate (sigma) 1.0 g is dissolved in 10 ml of dH_2O.

20. Resolving gel buffers : 3 M Tris-HCl pH 8.8

Tris 36.3 g is dissolved in 40 ml of dH_2O, adjusted with 1 M HCl to pH 8.8 and adjusted to 100 ml final volume with dH_2O.

21. Stacking gel buffers : 0.5 M Tris-HCl pH 6.8

Tris 6 g is dissolved in 40 ml of dH_2O, adjusted with 1 M HCl to pH 6.8 and adjusted to 100 ml final volume with dH_2O.

22. 30.8% (w/v) Acrylamide-bisacrylamide (per 100 ml)

Acrylamide	30.0 g
Bis-acrylamide	0.8 g

23. TEMED (N,N,N',N'-tetramethyl ethlenediamine)

This reagent is commercial available.

24. 15% Resolving gel

Acrylamide-bisacrylamide (30:0.8)	5 ml
dH_2O	2.40 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml
10% SDS	100 µl
10% Ammonium persulfate	75 µl
TEMED	5 µl

25. 3.9 % Stacking gel

Acrylamide-bisacrylamide	0.65 ml
dH_2O	3.05 ml
0.5 M Tris-HCl (pH 6.8)	1.25 ml
10% SDS	50 µl
10% Ammonium persulfate	50 µl
TEMED	10 µl

26. 10x Running buffers: 0.25 M Tris-HCl, 1.92 M glycine, 1% (w/v) SDS pH 8.3

Tris	30.3 g
Glycine	144.0 g
SDS	10 g

Dissolve and adjust to 1000 ml with dH_2O.

27. 4x Sample buffer: 0.0625 M Tris-HCl pH 6.8, 8%(w/v SDS, 40%) (v/v) glycerol and 0.005% Bromophenol blue)

SDS	0.8 g
Glycerol	4.0 ml
Stacking gel buffer	5.0 ml
Bromophenol blue	0.5 mg

Dissolve and adjust the volume to 10 ml with dH_2O. One ml of 2-mercaptoethanol (2-ME) is added to 9 ml of 4x sample buffer for reducing condition.

28. Staining solution of SDS-PAGE

Coomassie brilliant blue	0.25 g
Methanol	45 ml
dH_2O	45 ml
Glacial acetic acid	10 ml

29. Destaining solution

Glacial acetic acid	100 ml
Methanol	300 ml

dH_2O	600 ml

30. Transfer buffer: 20 mM Tris-HCl pH 8.3, 150 mM Glycine, 20% (v/v) methanol

Tris	1.211 g
Glycine	5.63 g

Dissolve and adjust to 400 ml with dH_2O and add 100 ml of methanol

31. Blocking buffer (1% BSA)

BSA	1 g

Dissolve and adjust to 100 ml with PBS.

32. Phosphate Buffer Saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Dissolve in 800 ml of dH_2O, adjust pH to 6.8 and adjust to 1000 ml final volume with dH_2O

33. 0.1 % DEPC- dH_2O

Diethyl pyrocarbonate 97 %	1 g

Add dH_2O to 1000 ml and incubate overnight at 37 °Cthen autoclave.

34. 40 % polyacrylmide gel solution (75:1)

Acrylamide	197.35 g

Bis-acrylamide	2.63 g

Dissolve in 300 ml of dH_2O and adjust to 500ml final volume with dH_2O.

35. Fix/Stop solution

Glacial acetic acid	200 ml
dH_2O	1800 ml

36. Staining solution of SSCP

Silver nitrate	1.5 g
37 % formaldehyde	2.25 ml
dH_2O	1497.75 ml

Dissolve silver nitrate in 200 ml of dH_2O, add 37 % formaldehyde and adjust to 1500 ml final volume with dH_2O.

37. Developing solution

Sodium carbonates	90 g
37 % formaldehyde	2.25 ml
10 mg/ml Sodium thiosulfate	600 ml

38. 6x loading dye

40% Sucrose	40 g
0.25% xylene cyanol	0.25 g
0.25% bromophenol blue	0.25 g

Dissolve in 50 ml of dH_2O and adjuste to 100 ml final volume with dH_2O.

39. 98% Formamide dye

0.5 M EDTA	1 ml

0.025% bromophenaol blue	0.05 g
0.025% xylene cyanol	0.05 g
10 mM NaOH	750 µl
98 % formamide	49 ml

Dissolve in 30 ml of 98 % formamide and adjust to 50 ml final volume with 98 % formamide.

APPENDIX B

Nucleotide sequences of the heat induced genes from cDNA libraries. The highlights show the position of 5' and 3' RACE-PCR primer.

1. Aquaporin (GL-H-S01-0227-LF)

ATCACCGGATCCTCGATGAACCCGGCAAGGAGCTTCGGTCCAGCGGTCATTTCCGGTCTCTGGCAGGAC
CACTGGGTGTACTGGGCTGGTCCTATTCTTGGTGGTCTCGCAGCCGCTCTAATCTACTCCTACGTTTTC
CGAGCTCCCAAGGACGCCGCTGCTTATGACGTGGAGATGGACAACTATAACAAGAGAACCAACAATGCT
TAAATGGAAAAGATCTACTGGCTCGGCCCTATCATGGGCGGTCTCCTCGCGGGCCGTATTTACAAAATA
ACCTGGCCAGGAGATGACGAAGCCGAAGAGGAAAACAACCACGGGAAGAACAAGAAGGAGAGCGTAAAG
AACGGCGAGAAAGAGAGGAACGGCGAGAGAGAGAAGAGTGACTGTAGGTACGAGCTGACCATCCCCATC
GACGACCTCACTGGGCATCAGTTCCAAAAAGATCAGTATAGTTGCCGCTTGAGTGAGAAGGACGACCCA
CACCATCTCATGGAAGAGGAAGAGGAATACGATATGAATGAAGAGGAAGAGGAGAAGAAGAAGTTGGTC
GATGTGGAGAAGGTGGTTATTGTTAAGTCTCAGAAAAAGAATGAAGAGGAGGAGATACAAGAGAAGGAA
AACGACTGTGTGATATCGGAGAGGACGACCGTTATATAAGTGTCATTTCGGGTCTTTAGTCCTTCATTA
GCTACAATAAGACTAAACCATTTTTTTCCACCAATTTT

2. Gulcosamine-6-phosphate deaminase (GL-H-S01-0118-LF)

GGAGAAGGACATTCGTTGGCAATTGCAAGAAGAAGATGCGTCTGGTTATCTTAGACGATGCGGGCAGAG
TGGCCGAGTGGGCAGCGAGATATATCATCAAACGGATCAACGACTTTAAACCAAACCCAGAGAAGTTCT
TTGTCCTTGGCTTGCCCACAGGAAGCACTCCATTGGGCACTTACCAAAGACTAGTGCAATATCATAAAG
CAGGAAAGATTTCATTCAAATATGTCAAGACGTTTAACATGGACGAATATGTTGGTATCCCAAGGGACC
ACTCCCAGAGCTATCACACCTTCATGTGGGAGAATCTCTTCAAGCACATAGACATTGACCCTGCTAATG
CACACATTCTGGACGGCAATGCACAGGACCTGGAGAAAGAGTGCCAGTTGTATGAGCAGAAGATTAAGG
AAGCCGGTGGGATCGAGTTATTTATGGGAGGTATAGGCCCAGATGGCCACATTGCTTTCAATGAGCCAG
GCTCTAGCCTTGTTTCGCGAACCCGTGTCAAGACACTCAACCAAGAGACCATCACAGCCAATGCCCGTT
TCTTTAACAATGACATGTCTAAAGTTCCAAAGCAGTCACTTACTGTGGGCGTTGGGACTGTTATGGATG
CCAGAGAGGTAATGATCCTTATCACGGGATCACACAAGGCCTATGCCCTTCACATGGCGATTGAGGAAG
GGGTCAACCACATGTGGACTGTATCAGCATTCCAGCAACACCCCCGCACCCTTATGCTCTGTGATGAGG
ATGCGACTCTTGAGCTGAAGGTCAAGACTGTCAAGTACTTTAAGGATTTGTGGTCGGTGCACAGCAAAC
TCATTGACGACCCAATGATGGCATCGTTTCAAGAGTCAAGGATGAAGCCGTTTCGTGCTTTTCACAGAA
ATTGTCGCAGTGGTGGTGGTCATTCATTTCTTTACGGTGGAGGCTTAATGATAAAAGATTTTGAAATCA
TATTCCGAATACGGTAATGGTGCTATTGATCATTGAGTAAGATTGAGTTTTTATTTTTGGTGAATTATT
TTATAAAAAAAAAAAAAAAAAAA

3. C type lectin-like protein (HC-H-S01-0706-LF)

AACAAGTCTTTTATTCACCGCTGCTTATCTGGTCTTGGTGCCCAGCCTTGCGGTCTCACAACTACAGAG
GAATTGCCCAGACAACTATGTGCTCTTGGCTGACAAATGCTATGGCTTTCGGCGCACAGTGACAGACTG
GAATAACGCCCGTGCAACTTGTCTGAACGAGAATGCTGACCTGACCTCCGTTCTCACGACTCAAGAGTA
CACGGAGATTCTGGCGCATCTGGCTGCTAACTACCCCGGCGTGTACTGGGTTGGAGGCGCCACGAGTAA
CCAGGGCGCGTGGAGGTGGGTGGCTTCGGGCGCGCCCATGAATGAGCAGTGGTGGGGCGGCGACCACAC
CCCCACTTCACAACGCTGCGCCTACTTCTGCTCCCATACTCGCAAATACTGGAGCAGCACATGCGGGGT
GTCCAAGAACTTCATCTGCGAAAAAAGTGCGGAGACCGAAGCCGAATCAGGAGAAGAATTTGAACCAGA
GGGGGACACCGATCCGGAAAATGAATCGGACGAAGCAGACGCTGAGAGGATACCTTCCCTTCCCTCTGT
AAGGGCGGAGCTGCGGTCTGGAGTTTCGTCCACCACACAAAACATGGCCTGTCTCGTTTATCTGTTGAG
TCTTTGTGCAGTCATGGTCTAATACAGATATATGGGACTTGTGAGCCAGTGACTAAAATGCCTTTTCAA
ACCCCATGTGAAGCTCTACGTAACCAT

APPENDIX C

Nucleotide comparison of *P. monodon* aquaporin using BLASTX

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                                                               Score     E
Sequences producing significant alignments:                   (Bits)  Value

dbj|BAG72254.1|  aquaporin [Coptotermes formosanus]            266    8e-69
dbj|BAG72255.1|  aquaporin [Coptotermes formosanus]            265    2e-68
ref|XP_002425393.1|  Aquaporin AQPAe.a, putative [Pediculus hu...  254    3e-65
sp|Q9NHW7.2|AQP_AEDAE  RecName: Full=Aquaporin AQPAe.a         254    5e-65
ref|XP_001656931.1|  aquaporin-1 [Aedes aegypti] >gb|EAT45185....  254    5e-65 UG
gb|AAF64037.1|AF218314_1  aquaporin [Aedes aegypti]            253    1e-64
gb|ABV60346.1|  putative aquaporin [Lutzomyia longipalpis]     246    8e-63
emb|CAX48991.1|  water and ammonia transporting aquaporin [Lum...  244    3e-62
ref|XP_319584.4|  AGAP008842-PA [Anopheles gambiae str. PEST] ...  242    2e-61 UG
dbj|BAH47554.1|  aquaporin AQP-Gra1 [Grapholita molesta]       239    1e-60
sp|Q25074.1|AQP_HAEIX  RecName: Full=Aquaporin; AltName: Full=...  238    2e-60
ref|XP_002063396.1|  GK21884 [Drosophila willistoni] >gb|EDW74...  231    3e-58 G
gb|ABW96354.1|  aquaporin 1 [Bemisia tabaci]                   229    2e-57
gb|ACO10737.1|  Aquaporin AQPAe.a [Caligus rogercresseyi]      228    2e-57
ref|XP_001607940.1|  PREDICTED: similar to ENSANGP00000016718 ...  228    2e-57 UG
emb|CAI11692.1|  novel protein similar to vertebrate aquaporin...  228    2e-57 G
ref|NP_001003749.1|  aquaporin 4 [Danio rerio] >gb|AAH78213.1|...  228    2e-57 UG
ref|XP_002429480.1|  Aquaporin AQPcic, putative [Pediculus hum...  228    3e-57
ref|XP_002006444.1|  GI21049 [Drosophila mojavensis] >gb|EDW10...  227    7e-57 G
sp|Q23808.1|AQP_CICVR  RecName: Full=Aquaporin AQPcic >emb|CAA...  226    9e-57
ref|NP_001036919.1|  aquaporin [Bombyx mori] >dbj|BAD69569.1| ...  226    1e-56 UG
ref|XP_002091206.1|  GE13520 [Drosophila yakuba] >gb|EDW90918....  224    3e-56 G
ref|XP_002138347.1|  GA24443 [Drosophila pseudoobscura pseudoo...  224    4e-56 G
ref|XP_001958771.1|  GF12394 [Drosophila ananassae] >gb|EDV355...  224    6e-56 G
ref|XP_001986479.1|  GH20503 [Drosophila grimshawi] >gb|EDW013...  223    1e-55 G
ref|XP_972862.1|  PREDICTED: similar to aquaporin [Tribolium c...  223    1e-55 UG
ref|NP_523697.1|  drip, isoform B [Drosophila melanogaster] >r...  221    3e-55 UG
ref|XP_001976060.1|  GG22646 [Drosophila erecta] >gb|EDV56460....  221    5e-55 G
ref|XP_002033403.1|  GM20426 [Drosophila sechellia] >gb|EDW474...  219    1e-54 G
ref|XP_002050138.1|  GJ21971 [Drosophila virilis] >gb|EDW61331...  219    2e-54 G
ref|XP_624531.1|  PREDICTED: similar to Drip CG9023-PB, isofor...  218    3e-54 UG
ref|NP_001124421.1|  hypothetical protein LOC100174805 [Xenopu...  215    3e-53 UG
gb|EDL01602.1|  aquaporin 4, isoform CRA_c [Mus musculus]      212    2e-52 G
ref|NP_033830.2|  aquaporin 4 [Mus musculus] >gb|AAL73545.1|AF...  212    2e-52 UG
gb|AAZ03394.1|  Aqp4-Luc fusion protein [Reporter vector pmuAq...  212    2e-52
gb|AAL73546.1|AF469169_1  aquaporin-4 M23X isoform [Mus muscul...  212    2e-52 G
sp|P55088.2|AQP4_MOUSE  RecName: Full=Aquaporin-4; Short=AQP-4...  212    2e-52 G
gb|AAO38843.1|  aquaporin 4 M23 isoform [Ovis aries]           212    2e-52 G
ref|NP_001009279.1|  aquaporin 4 [Ovis aries] >gb|AAO21366.1| ...  212    2e-52 UG
ref|XP_001505227.1|  PREDICTED: similar to aquaporin [Ornithor...  211    5e-52 UG
ref|NP_851346.1|  aquaporin 4 [Bos taurus] >sp|O77750.3|AQP4_B...  210    8e-52 UG
dbj|BAA33583.1|  aquaporin-4 [Bos taurus] >dbj|BAA89291.1| aqu...  210    8e-52 G
pdb|2ZZ9|A  Chain A, Structure Of Aquaporin-4 S180d Mutant At ...  209    1e-51 S
pdb|3GD8|A  Chain A, Crystal Structure Of Human Aquaporin 4 At...  209    2e-51 S
ref|NP_001103893.1|  aquaporin 4 [Sus scrofa] >gb|ABW34385.1| ...  209    2e-51 UG
ref|XP_512074.2|  PREDICTED: aquaporin 4 isoform 3 [Pan troglo...  209    2e-51 UG
```

Sequence	Description	Score	E value
ref|XP_001097311.1|	PREDICTED: aquaporin 4 isoform 4 [Macaca ...	209	2e-51
ref|XP_001097418.1|	PREDICTED: aquaporin 4 isoform 5 [Macaca ...	209	2e-51
dbj|BAD96404.1|	aquaporin 4 isoform a variant [Homo sapiens]	209	2e-51
gb|AAB41570.1|	mercurial-insensitive water channel 3 [Mus mus...	209	2e-51
gb|AAC50284.1|	mercurial-insensitive water channel	209	2e-51
gb|AAB41569.1|	mercurial-insensitive water channel 2	209	2e-51
ref|NP_004019.1|	aquaporin 4 isoform b [Homo sapiens] >gb|AAB...	209	2e-51
gb|AAC52112.1|	mercurial-insensitive water channel	209	2e-51
gb|AAA84923.1|	mercurial-insensitive water channel >gb|AAB415...	209	2e-51
ref|NP_001641.1|	aquaporin 4 isoform a [Homo sapiens] >sp|P55...	209	2e-51
gb|ABO09756.1|	AQP4e [Expression vector pcDNA3-AQP4e] >gb|ABO...	208	2e-51
ref|XP_001494330.1|	PREDICTED: similar to aquaporin-4-A [Equu...	208	2e-51
dbj|BAF02790.1|	aquaporin-x5 [Xenopus laevis]	208	2e-51
sp|Q5I4F9.1|AQP4_NOTAL	RecName: Full=Aquaporin-4; Short=AQP-4...	208	2e-51
ref|NP_001079331.1|	aquaporin [Xenopus laevis] >gb|AAN75455.1...	208	2e-51
gb|AAZ03395.1|	Aqp4-Luc fusion protein [Reporter vector praAq...	208	2e-51
ref|NP_001135838.1|	aquaporin 4 isoform 2 [Rattus norvegicus]...	208	2e-51
ref|NP_036957.1|	aquaporin 4 isoform 1 [Rattus norvegicus] >s...	208	2e-51
emb|CAX48992.1|	water-specific aquaporin [Lumbricus rubellus]	208	3e-51
ref|XP_855456.1|	PREDICTED: similar to Aquaporin 4 (WCH4) (Me...	207	4e-51
ref|XP_001633187.1|	predicted protein [Nematostella vectensis...	207	5e-51
gb|AAK66823.1|	aquaporin 4 isoform 1 [Dipodomys merriami]	207	5e-51
sp|Q923J4.1|AQP4_DIPME	RecName: Full=Aquaporin-4; Short=AQP-4...	207	5e-51
ref|XP_001363585.1|	PREDICTED: similar to aquaporin [Monodelp...	207	7e-51
ref|XP_968342.1|	PREDICTED: similar to aquaporin [Tribolium c...	207	7e-51
gb|AAR06953.1|	aquaporin-2 [Coturnix coturnix]	207	7e-51
ref|XP_002196027.1|	PREDICTED: aquaporin 4 [Taeniopygia guttata]	206	9e-51
emb|CAX48970.1|	aquaporin [Eisenia andrei]	205	2e-50
gb|AAL73511.1|AF465730_1	aquaporin-4 [Coturnix coturnix]	205	3e-50
ref|XP_001656932.1|	aquaporin [Aedes aegypti] >gb|ABF18340.1|...	204	4e-50
gb|AAA17730.1|	mercurial-insensitive water channel	204	4e-50
ref|XP_394391.1|	PREDICTED: similar to CG7777-PA, isoform A [...	204	6e-50
ref|NP_001004765.1|	aquaporin 4 [Gallus gallus] >dbj|BAD46731...	204	6e-50
ref|NP_001085391.1|	MGC79006 protein [Xenopus laevis] >gb|AAH...	202	1e-49
ref|NP_001088210.1|	hypothetical protein LOC495037 [Xenopus l...	202	2e-49
ref|NP_001015749.1|	aquaporin 2 (collecting duct) [Xenopus (S...	202	2e-49
dbj|BAE43321.1|	unnamed protein product [Mus musculus]	200	9e-49
ref|NP_001108003.1|	aquaporin 2 (collecting duct) [Equus caba...	199	1e-48
gb|AAV65290.1|	aquaporin-1 [Passer domesticus]	199	1e-48
ref|XP_002196264.1|	PREDICTED: aquaporin 1 (Colton blood grou...	197	4e-48
ref|XP_002213012.1|	hypothetical protein BRAFLDRAFT_214280 [B...	197	4e-48
dbj|BAF62090.1|	aquaporin [Polypedilum vanderplanki]	197	4e-48
ref|NP_001005829.1|	aquaporin 1 (Colton blood group) [Xenopus...	197	4e-48
ref|NP_001137369.1|	aquaporin-X12 [Xenopus laevis] >dbj|BAH03...	197	6e-48
ref|NP_999619.1|	aquaporin 1 [Sus scrofa] >sp|Q6PQZ1.3|AQP1_P...	197	7e-48
ref|NP_000477.1|	aquaporin 2 [Homo sapiens] >sp|P41181.1|AQP2...	196	1e-47
ref|XP_002215983.1|	hypothetical protein BRAFLDRAFT_78258 [Br...	196	2e-47
ref|XP_001193877.1|	PREDICTED: similar to Si:ch211-192k9.1 [S...	195	2e-47
gb|AAH19966.1|	Aquaporin 2 [Mus musculus]	195	2e-47
gb|AAB71414.1|	aquaporin [Mus musculus]	195	2e-47
sp|P50501.1|AQPA_RANES	RecName: Full=Aquaporin FA-CHIP >gb|AA...	195	2e-47
ref|NP_033829.3|	aquaporin 2 [Mus musculus] >sp|P56402.2|AQP2...	195	2e-47
gb|AAI28706.1|	Aquaporin 2 (collecting duct) [Rattus norvegic...	195	3e-47

Nucleotide comparison of gulcosamine-6-phosphat deaminase *P. monodon* using BLASTX

Sequences producing significant alignments:		Score (Bits)	E Value	
ref|XP_002130881.1|	PREDICTED: similar to glucosamine-6-phosp...	448	5e-124	UG
ref|XP_001600716.1|	PREDICTED: similar to glucosamine-6-phosp...	443	1e-122	UG
ref|XP_001849348.1|	glucosamine-6-phosphate isomerase [Culex ...	442	3e-122	UG
ref|XP_001657194.1|	glucosamine-6-phosphate isomerase [Aedes ...	442	3e-122	UG
ref|XP_001657190.1|	glucosamine-6-phosphate isomerase [Aedes ...	439	3e-121	UG
ref|XP_393026.2|	PREDICTED: similar to Oscillin CG6957-PA, is...	437	1e-120	UG
ref|NP_001096324.1|	glucosamine-6-phosphate isomerase 2 [Xeno...	434	6e-120	UG
ref|XP_002003579.1|	GI17992 [Drosophila mojavensis] >gb|EDW13...	432	2e-119	G
ref|XP_001988955.1|	GH10296 [Drosophila grimshawi] >gb|EDW038...	431	8e-119	G
ref|XP_001657191.1|	glucosamine-6-phosphate isomerase [Aedes ...	431	8e-119	G
ref|NP_001083339.1|	glucosamine-6-phosphate isomerase 2 [Xeno...	429	2e-118	UG
ref|XP_684147.2|	PREDICTED: similar to glucosamine-6-phosphat...	428	4e-118	UG
ref|NP_001017867.1|	glucosamine-6-phosphate deaminase 1 [Dani...	428	4e-118	UG
ref|XP_002220801.1|	hypothetical protein BRAFLDRAFT_123320 [B...	427	9e-118	UG
ref|XP_002429415.1|	glucosamine-6-phosphate isomerase, putati...	427	1e-117	
ref|XP_001143245.1|	PREDICTED: similar to glucosamine-6-phosp...	427	1e-117	UG
ref|NP_001099475.1|	glucosamine-6-phosphate deaminase 2 [Ratt...	426	2e-117	UG
ref|XP_001951069.1|	PREDICTED: similar to glucosamine-6-phosp...	426	2e-117	UG
ref|XP_553256.3|	AGAP009305-PA [Anopheles gambiae str. PEST] ...	426	2e-117	UG
ref|XP_001917218.1|	PREDICTED: similar to glucosamine-6-phosp...	426	3e-117	UG
ref|XP_849417.1|	PREDICTED: similar to glucosamine-6-phosphat...	426	3e-117	UG
ref|XP_002018624.1|	GL25853 [Drosophila persimilis] >gb|EDW36...	425	3e-117	G
ref|XP_001504008.1|	PREDICTED: similar to glucosamine-6-phosp...	425	3e-117	UG
ref|NP_001073756.2|	glucosamine-6-phosphate deaminase 1 [Bos ...	425	3e-117	UG
ref|NP_612208.1|	glucosamine-6-phosphate deaminase 2 [Homo sa...	425	3e-117	UG
ref|NP_001033104.1|	glucosamine-6-phosphate deaminase 2 [Mus ...	425	3e-117	UG
ref|XP_001357485.1|	GA19983 [Drosophila pseudoobscura pseudoo...	425	3e-117	G
ref|XP_001139968.1|	PREDICTED: similar to KIAA0060 [Pan trogl...	425	4e-117	UG
dbj|BAA06544.2|	KIAA0060 [Homo sapiens]	425	4e-117	G
ref|NP_001068824.1|	glucosamine-6-phosphate deaminase 2 [Bos ...	424	6e-117	UG
ref|XP_001364721.1|	PREDICTED: hypothetical protein [Monodelp...	423	1e-116	UG
gb|ABM06146.1|	glucosamine-6-phosphate deaminase 1 [Bos taurus]	423	1e-116	G
ref|XP_002052909.1|	GJ19587 [Drosophila virilis] >gb|EDW65064...	423	2e-116	G
ref|XP_001961801.1|	GF15148 [Drosophila ananassae] >gb|EDV310...	423	2e-116	G
ref|NP_005462.1|	glucosamine-6-phosphate deaminase 1 [Homo sa...	423	2e-116	UG
dbj|BAB70977.1|	unnamed protein product [Homo sapiens]	422	2e-116	G
dbj|BAF83375.1|	unnamed protein product [Homo sapiens]	422	3e-116	G
dbj|BAE40396.1|	unnamed protein product [Mus musculus]	422	3e-116	G
sp|O88958.2|GNPI1_MOUSE	RecName: Full=Glucosamine-6-phosphate...	422	4e-116	G
sp|Q64422.1|GNPI1_MESAU	RecName: Full=Glucosamine-6-phosphate...	421	5e-116	
ref|XP_002197439.1|	PREDICTED: similar to glucosamine-6-phosp...	421	6e-116	UG
ref|XP_790227.2|	PREDICTED: similar to Glucosamine-6-phosphat...	421	6e-116	UG
ref|NP_036067.2|	glucosamine-6-phosphate deaminase 1 [Mus mus...	421	6e-116	UG

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ref|NP_001127467.1|  glucosamine-6-phosphate deaminase 1 [Pong...  421   6e-116
ref|NP_001134003.1|  Glucosamine-6-phosphate isomerase [Salmo ...  421   8e-116
ref|NP_001128467.1|  glucosamine-6-phosphate deaminase 1 [Ratt...  421   8e-116
ref|XP_002037963.1|  GM18555 [Drosophila sechellia] >gb|EDW543...  420   1e-115
ref|XP_420726.2|  PREDICTED: hypothetical protein [Gallus gallus]  420   1e-115
ref|NP_608938.1|  oscillin, isoform A [Drosophila melanogaster...  420   1e-115
ref|XP_002089206.1|  GE25482 [Drosophila yakuba] >gb|EDW88918....  420   1e-115
ref|XP_002069268.1|  GK21107 [Drosophila willistoni] >gb|EDW80...  419   2e-115
ref|XP_001968817.1|  GG25080 [Drosophila erecta] >gb|EDV57876....  419   2e-115
gb|AAC36739.1|  glucosamine-6-phosphate isomerase [Mus musculus]   419   2e-115
ref|NP_001083469.1|  hypothetical protein LOC398943 [Xenopus l...  417   7e-115
dbj|BAB30428.1|  unnamed protein product [Mus musculus] >gb|ED...  416   2e-114
ref|XP_535222.2|  PREDICTED: similar to Glucosamine-6-phosphat...  416   2e-114
gb|ABU41056.1|  glucosamine-6-phosphate isomerase [Lepeophthei...  416   3e-114
gb|AAH15532.1|  Glucosamine-6-phosphate deaminase 2 [Homo sapi...  416   3e-114
dbj|BAD93141.1|  glucosamine-6-phosphate deaminase 2 variant [...  414   6e-114
gb|ACO12114.1|  Glucosamine-6-phosphate isomerase [Lepeophthei...  413   1e-113
gb|EDL37803.1|  mCG10526, isoform CRA_b [Mus musculus]             413   1e-113
ref|NP_001006156.1|  glucosamine-6-phosphate deaminase 1 [Gall...  413   1e-113
ref|XP_858777.1|  PREDICTED: similar to glucosamine-6-phosphat...  413   1e-113
ref|XP_001364124.1|  PREDICTED: similar to glucosamine-6-phosp...  411   5e-113
gb|ACO08728.1|  Glucosamine-6-phosphate isomerase [Oncorhynchu...  410   9e-113
emb|CAF99071.1|  unnamed protein product [Tetraodon nigroviridis]  408   6e-112
ref|XP_001091343.1|  PREDICTED: glucosamine-6-phosphate deamin...  407   1e-111
ref|XP_975109.1|  PREDICTED: similar to GA19983-PA [Tribolium ...  407   1e-111
dbj|BAD32157.1|  mKIAA0060 protein [Mus musculus]                  406   2e-111
gb|ACO14963.1|  Glucosamine-6-phosphate isomerase [Caligus cle...  404   1e-110
gb|ACO14633.1|  Glucosamine-6-phosphate isomerase [Caligus cle...  401   5e-110
ref|XP_002154646.1|  PREDICTED: similar to Glucosamine-6-phosp...  401   7e-110
ref|XP_001639105.1|  predicted protein [Nematostella vectensis...  399   2e-109
gb|AAW26554.1|  SJCHGC01037 protein [Schistosoma japonicum]        389   3e-106
gb|ACO11189.1|  Glucosamine-6-phosphate isomerase [Caligus rog...  387   8e-106
gb|ACO10743.1|  Glucosamine-6-phosphate isomerase [Caligus rog...  387   1e-105
ref|XP_002118174.1|  hypothetical protein TRIADDRAFT_33724 [Tr...  382   4e-104
ref|XP_002078245.1|  GD23349 [Drosophila simulans] >gb|EDX0383...  377   1e-102
ref|XP_001666325.1|  hypothetical protein CBG21221 [Caenorhabd...  375   3e-102
ref|XP_001378101.1|  PREDICTED: similar to glucosamine-6-phosp...  375   4e-102
ref|NP_499758.1|  hypothetical protein T03F6.3 [Caenorhabditis...  374   7e-102
ref|XP_995500.1|  PREDICTED: similar to mKIAA0060 protein [Mus...  373   2e-101
emb|CAF94912.1|  unnamed protein product [Tetraodon nigroviridis]  372   4e-101
ref|XP_001749463.1|  hypothetical protein [Monosiga brevicolli...  362   3e-98
ref|XP_002482467.1|  glucosamine-6-phosphate deaminase, putati...  354   7e-96
ref|XP_368619.1|  hypothetical protein MGG_00625 [Magnaporthe ...  353   1e-95
ref|XP_001938488.1|  glucosamine-6-phosphate deaminase [Pyreno...  353   2e-95
ref|XP_001586257.1|  hypothetical protein SS1G_12835 [Scleroti...  352   4e-95
ref|XP_002148212.1|  glucosamine-6-phosphate deaminase, putati...  351   8e-95
gb|AAO49718.1|  putative glucosamine-6-phosphate isomerase [Ho...  350   1e-94
ref|YP_002669069.1|  glucosamine-6-phosphate isomerase [Vibrio...  347   1e-93
ref|XP_001556554.1|  hypothetical protein BC1G_05323 [Botryoti...  347   2e-93
ref|YP_001142592.1|  glucosamine-6-phosphate deaminase [Aeromo...  347   2e-93
ref|YP_856064.1|  glucosamine-6-phosphate deaminase [Aeromonas...  346   3e-93
ref|YP_002395994.1|  glucosamine-6-phosphate deaminase [Vibrio...  345   4e-93
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Nucleotide comparison of C type lectin like protein *P. monodon* using BLASTX

Sequences producing significant alignments:	Score (Bits)	E Value	
gb|ACF77004.1| C-lectin-A [Dicentrarchus labrax]	61.6	6e-08	
gb|AAI53394.1| Si:ch211-154o6.6 protein [Danio rerio]	61.6	6e-08	G
emb|CAK04178.1| novel protein with a Lectin C-type domain [Da...	61.6	6e-08	G
ref|XP_002239162.1| hypothetical protein BRAFLDRAFT_129759 [B...	61.2	8e-08	G
ref|XP_001235106.1| PREDICTED: similar to Macrophage mannose ...	60.5	1e-07	G
ref|NP_001123743.1| hypothetical protein LOC100170489 [Xenopu...	58.9	4e-07	UG
gb|ACS16044.1| C-type lectin [Ctenopharyngodon idella]	58.5	5e-07	
gb|ACI67923.1| C-type lectin domain family 4 member E [Salmo ...	58.5	5e-07	
ref|XP_002206239.1| hypothetical protein BRAFLDRAFT_68724 [Br...	58.2	6e-07	G
gb|ACI33556.1| CD209 antigen-like protein E [Salmo salar] >gb...	57.8	8e-07	
ref|XP_002119389.1| PREDICTED: similar to mannose receptor, p...	57.8	8e-07	G
ref|NP_001117051.1| C type lectin receptor A [Salmo salar] >g...	57.8	8e-07	UG
ref|XP_002208905.1| hypothetical protein BRAFLDRAFT_208012 [B...	57.4	1e-06	G
ref|XP_002218125.1| hypothetical protein BRAFLDRAFT_222515 [B...	57.4	1e-06	G
ref|XP_784754.2| PREDICTED: hypothetical protein [Strongyloce...	57.4	1e-06	UG
gb|AAZ29608.1| C-type lectin [Penaeus monodon]	56.6	2e-06	
dbj|BAD93253.1| CLEP [Oryzias latipes]	56.2	2e-06	
emb|CAG03717.1| unnamed protein product [Tetraodon nigroviridis]	55.8	3e-06	
ref|XP_002207811.1| hypothetical protein BRAFLDRAFT_205740 [B...	55.5	4e-06	G
ref|NP_001118105.1| CD209-like protein [Oncorhynchus mykiss] ...	55.5	4e-06	UG
gb|AAA49617.1| antifreeze polypeptide (AFP) precursor	55.1	6e-06	
gb|AAA49618.1| antifreeze protein	55.1	6e-06	
sp|Q66S03.1|NATTE_THANI RecName: Full=Nattectin; Flags: Precu...	55.1	6e-06	
pdb|2AFP|A Chain A, The Solution Structure Of Type Ii Antifre...	55.1	6e-06	S
sp|P05140.2|ISP2_HEMAM RecName: Full=Type-2 ice-structuring p...	55.1	6e-06	
ref|XP_873119.1| PREDICTED: similar to C-type lectin domain f...	54.7	7e-06	UG
emb|CAF89942.1| unnamed protein product [Tetraodon nigroviridis]	54.7	7e-06	
ref|NP_001123444.1| CD209 molecule [Sus scrofa] >gb|ACC63374....	54.3	9e-06	UG
ref|XP_001519978.1| PREDICTED: similar to hCG16425, partial [...	54.3	9e-06	G
ref|XP_002208901.1| hypothetical protein BRAFLDRAFT_208006 [B...	53.9	1e-05	G
ref|XP_001105418.1| PREDICTED: similar to C-type lectin, supe...	53.9	1e-05	UG
dbj|BAB83835.1| CLEP [Oryzias latipes]	53.9	1e-05	
gb|ACJ64661.1| hypothetical protein A043-D8 [Acropora millepora]	53.5	2e-05	
ref|XP_002120905.1| PREDICTED: similar to mannose receptor [C...	53.5	2e-05	UG
ref|XP_001641575.1| predicted protein [Nematostella vectensis...	53.5	2e-05	G
gb|ACQ59051.1| Nattectin precursor [Anoplopoma fimbria]	53.1	2e-05	
gb|ACQ58373.1| C-type lectin domain family 4 member E [Anoplo...	53.1	2e-05	
gb|ACQ58341.1| Nattectin precursor [Anoplopoma fimbria] >gb|A...	53.1	2e-05	
ref|XP_002219477.1| hypothetical protein BRAFLDRAFT_224792 [B...	53.1	2e-05	G
gb|EDM01743.1| similar to RIKEN cDNA 9830005G06 (predicted), ...	53.1	2e-05	G
ref|XP_607489.3| PREDICTED: similar to mannose receptor, C ty...	53.1	2e-05	UG
ref|XP_511600.2| PREDICTED: mannose receptor, C type 2 [Pan t...	53.1	2e-05	UG
ref|NP_006030.2| mannose receptor, C type 2 [Homo sapiens]	53.1	2e-05	UG
ref|XP_001116053.1| PREDICTED: similar to mannose receptor, C...	53.1	2e-05	UG
dbj|BAA31684.2| KIAA0709 protein [Homo sapiens]	53.1	2e-05	G
ref|NP_001102824.1| C-type lectin domain family 9, member a [...	53.1	2e-05	UG
gb|AAD30280.1|AF134838_1 endocytic receptor Endo180 [Homo sap...	53.1	2e-05	G
dbj|BAE44113.1| serum lectin isoform 1 [Verasper variegatus]	53.1	2e-05	
sp|Q9UBG0.1|MRC2_HUMAN RecName: Full=C-type mannose receptor ...	53.1	2e-05	G

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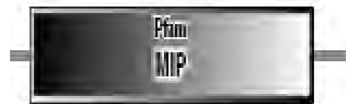
prf||2022211A  asialoglycoprotein receptor                              53.1    2e-05
ref|XP_002230900.1|  hypothetical protein BRAFLDRAFT_226848 [B...      52.8    3e-05  G
pdb|2ZIB|A  Chain A, Crystal Structure Analysis Of Calcium-Ind...      52.8    3e-05  S
ref|XP_873855.2|  PREDICTED: similar to C-type lectin domain f...      52.8    3e-05  UG
ref|XP_687939.3|  PREDICTED: similar to mannose receptor, C ty...      52.8    3e-05  UG
emb|CAN88727.1|  novel protein similar to human and mouse mann...      52.8    3e-05  G
dbj|BAF37106.1|  type II antifreeze protein [Brachyopsis rostr...      52.8    3e-05
dbj|BAE45334.1|  C-type lectin [Spirinchus lanceolatus]                52.8    3e-05
gb|ACO13705.1|  C-type lectin domain family 4 member E [Esox l...      52.4    4e-05
gb|ABO26597.1|  putative perlucin 8 [Haliotis discus discus]           52.4    4e-05
dbj|BAF34211.1|  serum lectin isoform 4 [Verasper variegatus]          52.4    4e-05
dbj|BAA19862.1|  Incilarin B [Incilaria fruhstorferi]                  52.4    4e-05
gb|ACJ24350.1|  dectin-1 [Sus scrofa]                                  52.0    5e-05
ref|NP_001139338.1|  C-type lectin domain family 7, member A [...      52.0    5e-05  UG
gb|ACN44183.1|  dendritic cell-associated C-type lectin 1 isof...      52.0    5e-05  G
gb|ACN44182.1|  dendritic cell-associated C-type lectin 1 isof...      52.0    5e-05  G
ref|XP_001339584.2|  PREDICTED: C-type lectin, superfamily mem...      52.0    5e-05  UG
gb|EDM06333.1|  collagen-binding factor Endo180 [Rattus norveg...      52.0    5e-05  G
gb|EDL34236.1|  mannose receptor, C type 2, isoform CRA_a [Mus...      52.0    5e-05  G
emb|CAM24831.1|  mannose receptor, C type 2 [Mus musculus] >em...      52.0    5e-05  G
emb|CAM14206.1|  novel protein [Danio rerio]                           52.0    5e-05
ref|XP_001168677.1|  PREDICTED: C-type lectin, superfamily mem...      52.0    5e-05  UG
gb|AAX63905.1|  C-type lectin protein [Fenneropenaeus chinensis]       52.0    5e-05
ref|NP_032652.3|  mannose receptor, C type 2 [Mus musculus] >e...      52.0    5e-05  UG
gb|AAI16643.1|  Mannose receptor, C type 2 [Mus musculus] >gb|...      52.0    5e-05  G
gb|AAC52729.1|  lectin lambda                                          52.0    5e-05  G
sp|Q64449.2|MRC2_MOUSE  RecName: Full=C-type mannose receptor ...      52.0    5e-05  G
ref|NP_001019858.1|  mannose receptor, C type 2 [Rattus norveg...      52.0    5e-05  UG
ref|XP_002230895.1|  hypothetical protein BRAFLDRAFT_226860 [B...      51.6    6e-05  G
ref|XP_001068599.1|  PREDICTED: similar to CD209a antigen [Rat...      51.6    6e-05  G
gb|AAT73604.1|  AICL-like 1 [Anas platyrhynchos]                       51.6    6e-05
ref|XP_543823.2|  PREDICTED: similar to C-type lectin domain f...      51.6    6e-05  UG
ref|XP_002248354.1|  hypothetical protein BRAFLDRAFT_132727 [B...      51.2    8e-05  G
ref|XP_001521561.1|  PREDICTED: similar to osteoclast inhibito...      51.2    8e-05  UG
gb|ABQ40396.1|  codakine isoform 2 [Codakia orbicularis]               51.2    8e-05
ref|XP_001372164.1|  PREDICTED: similar to QDED721 [Monodelphi...      51.2    8e-05  UG
ref|XP_001376444.1|  PREDICTED: similar to mannose receptor, C...      51.2    8e-05  UG
ref|XP_854186.1|  PREDICTED: similar to C-type lectin superfam...      51.2    8e-05  UG
gb|ACN11189.1|  Nattectin precursor [Salmo salar]                      50.8    1e-04
ref|NP_001123945.1|  immunity adhesion receptor CD209L2 [Pan t...      50.8    1e-04  UG
ref|XP_001639026.1|  predicted protein [Nematostella vectensis...      50.8    1e-04  G
dbj|BAF34210.1|  serum lectin isoform 3 [Verasper variegatus]          50.8    1e-04
dbj|BAE44114.2|  serum lectin isoform 2 [Verasper variegatus]          50.8    1e-04
emb|CAE18170.1|  C-type lectin 2 like protein [Crassostrea gigas]      50.8    1e-04
ref|NP_997228.1|  C-type lectin domain family 9, member A [Hom...      50.8    1e-04  UG
gb|EDM04974.1|  asialoglycoprotein receptor 1, isoform CRA_d [...      50.4    1e-04  G
gb|EDM04972.1|  asialoglycoprotein receptor 1, isoform CRA_b [...      50.4    1e-04  G
gb|EDL12528.1|  asialoglycoprotein receptor 1, isoform CRA_c [...      50.4    1e-04  G
dbj|BAF62976.1|  hypothetical protein [Gallus gallus]                  50.4    1e-04
```

APPENDIX D

Result of *domain prediction* Using SMART Program

1. Aquporin



Confidently predicted domains and features:

Name	Begin	End	E-value
Pfam:MIP	16	234	3.10e-96

2. Gulcosamine-6-phosphate deaminase

Confidently predicted domains and features:

Name	Begin	End	E-value
intrinsic disorder	1	12	-
Pfam:Glucosamine_iso	15	250	2.30e-171
intrinsic disorder	292	307	-

3. C type lectin like protein

Confidently predicted domains and features:

Name	Begin	End	E-value
signal peptide	1	33	-
CLECT	37	157	1.18e-12
intrinsic disorder	162	196	-
intrinsic disorder	202	211	-

APPENDIX E

1. Partial nucleotide sequence of aquaporin gene of SSCP band 1

CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTGG
GCACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAG
GCTACGCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCGTCCA
TCGCGCAGGCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCG
CCATGCTTGTTGCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCC
AGTGCCTTGGTGCCATCGTAGGGGCTGCCATTCTGAAGGGCGTGACCCCCGCAGACA
TCCAGGGGTCCCTCGGCATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT

2. Partial nucleotide sequence of aquaporin gene of SSCP band 2

ATGCCTGGGCCGTGTCAATCTTCTCATTTCTCAGGGTCATGCCGAGGGACCCCTGGA
TGTCTGCGGGGGTCACTCCCTTCAGAATGGCAGCCCCTACGATGGCACCAAGGCACT
GGCACACGATGTAGATGAGTGCCCGGATGACAGAGACATGACGAGCAACAAGCATGG
CGCATGTCACAGCTGGGTTGATATGACAGCCAGAGACATGACCGACGGCCTGCGCGA
TGGACGCCACGGTGACCCCGAAGGCGAGCGAGATCTGGACGACGCTGGGGGCGTAGC
CTTCGCTCCAGGAGCCGATGCAGGAGCCGCAGCCGATGAACACGAGGAACATGGTGC
CCAGGAACTCCGCCAGGAAGGCTTTCCACACGCGGCGGTCGTTCAGGAGTTCGCCGA

3. Partial nucleotide sequence of aquaporin gene of SSCP band 3

CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTAG
GCACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAG
GCTACGCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCGTCCA
TCGCGCAGGCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCG
CCATGCTTGTTGCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCC
AGTGCCTTGGTGCCATCGTAGGGGCTGCCATTCTGAAGGGCGTGACCCCCGCAGACA
TCCAGGGGTCCCTCGGCATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT

4. Partial nucleotide sequence of aquaporin gene of SSCP band 4

CGGGCGAACTCCTGAACGACCGCCGCGTGTGGAAAGCCTTCCTGGCGGAGTTCCTGG
GCACCATGTTCCTCGTGTTCATCGGCTGCGGCTCCTGCATCGGCTCCTGGAGCGAAG
GCTACGCCCCCAGCGTCGTCCAGATCTCGCTCGCCTTCGGGGTCACCGTGGCATCCA
TCGCGCAGGCCGTCGGTCATGTCTCTGGCTGTCATATCAACCCAGCTGTGACATGCG
CCATGCTTGTTGCTCGTCATGTCTCTGTCATCCGGGCACTCATCTACATCGTGTGCC
AGTGCCTTGGTGCCATCGTAGGGGCTGCCATTCTGAAGGGAGTGACCCCCGCAGACA
TCCAGGGGTCCCTCGGCATGACCCTGAGAAATGAGAAGATTGACACGGCCCAGGCAT

5. Partial nucleotide sequence of aquaporin gene of SSCP band 5

TGCCTGGGCCGTGTCAATCTTCTCATTTCTCAGGGTCATGCCGAGGGACCCCTGGAT
GTCTGCGGGGGTCACTCCCTTCAGAATGGCAGCCCCTACGATGGCACCAAGGCACTG

GCACACGATGTAGATGAGTGCCCGGATGACAGAGACATGACGAGCAACAAGCATGGC
GCATGTCACAGCTGGGTTGATATGACAGCCAGAGACATGACCGACGGCCTGCGCGAT
GGATGCCACGGTGACCCCGAAGGCGAGCGAGATCTGGACGACGCTGGGGGCGTAGCC
TTCGCTCCAGGAGCCGATGCAGGAGCCGCAGCCGATGAACACGAGGAACATGGTGCC
CAGGAACTCCGCCAGGAAGGCTTTCCACACGCGGCGGTCGTTCAGGAGTTCGCCCGA

6. Partial nucleotide sequence of aquaporin gene of SSCP band 6

ATGCCTGGGCCGTGTCAATCTTCTCATTTCTCAGGGTCATGCCGAGGGACCCCTGGA
TGTCTGCGGGGGTCACTCCCTTCAGAATGGCAGCCCCTACGATGGCACCAAGGCACT
GGCACACGATGTAGATGAGTGCCCGGATGACAGAGACATGACGAGCAACAAGCATGG
CGCATGTCACAGCTGGGTTGATATGACAGCCAGAGACATGACCGGCGGCCTGCGCGA
TGGACGCCACGGTGACCCCGAAGGCGAGCGAGATCTGGACGACGCTGGGGGCGTAGC
CTTCGCTCCAGGAGCCGATGCAGGAGCCGCAGCCGATGAACACGAGGAACATGGTGC
CCAGGAACTCCGCCAGGAAGGCCTTCCACACGCGGCGGTCGTTCAGGAGTTCGCCGA

APPENDIX F

Standard curve of ammonium-nitrogen

Concentration ($mg\ NH_4^+$-N/L)	**Absorbance (A640)**
0.01	0.01
0.05	0.03
0.1	0.07
0.5	0.49

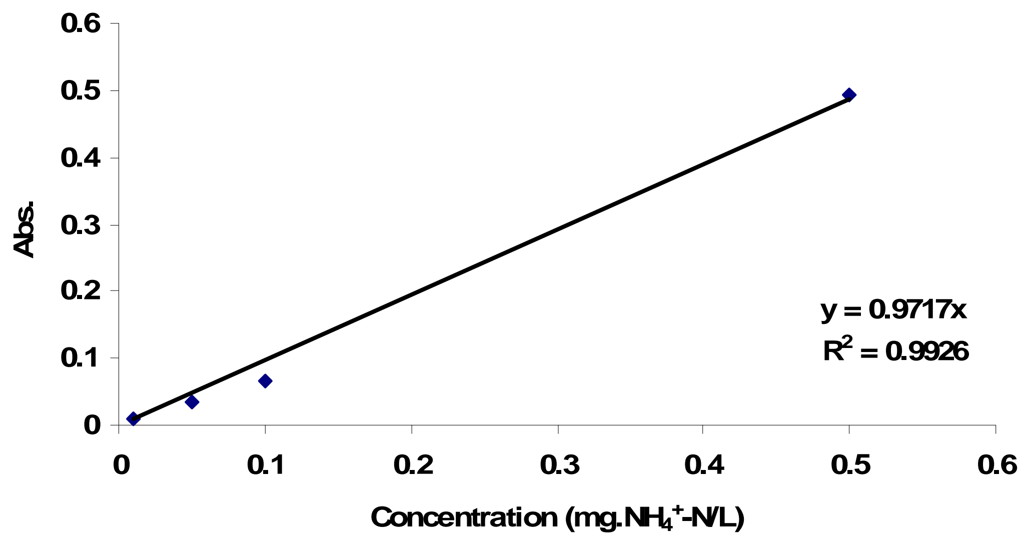


Figure E1 Standard curve of ammonium-nitrogen

Standard curve of nitrite-nitrogen

Concentration ($mg\ NO_2^+$-N/L)	Absorbance (A534)
0.01	0.02
0.05	0.13
0.1	0.26
0.5	1.32

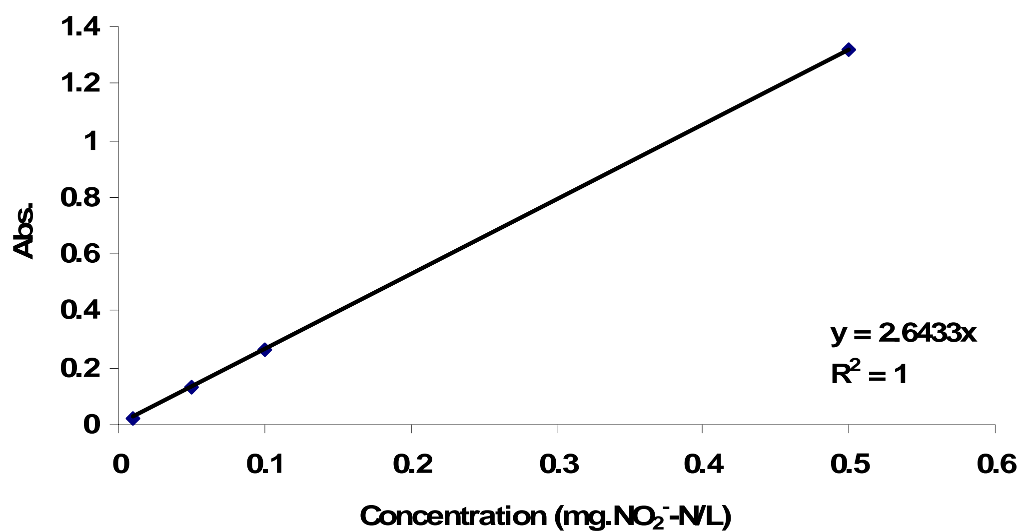


Figure E2 Standard curve of nitrite-nitrogen

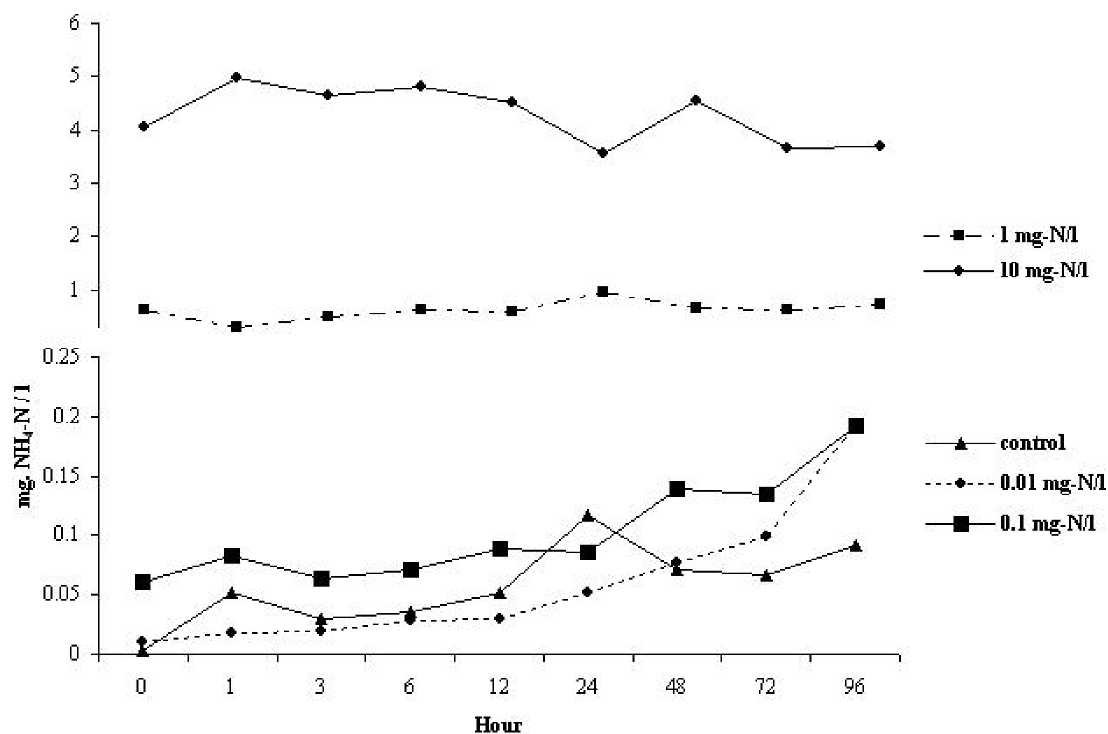


Figure E3 The concentration value of NH_3-N (unionized) changed in each hour.

Table E1 Ammonia concentrations of water during ammonia toxicity test.

Hour	Concentration (mg-N/L)					SD(mg-N/L)				
	control	0.01	0.1	1	10	control	0.01	0.1	1	10
0	0.00	0.01	0.06	0.63	4.05	0.00	0.00	0.00	0.01	0.029
1	0.05	0.02	0.08	0.30	4.98	0.02	0.00	0.00	0.01	0.00
3	0.02	0.02	0.06	0.48	4.64	0.01	0.00	0.00	0.01	0.01
6	0.03	0.03	0.07	0.63	4.83	0.01	0.00	0.00	0.01	0.01
12	0.05	0.03	0.089	0.60	4.52	0.00	0.00	0.00	0.001	0.01
24	0.12	0.05	0.09	0.94	3.58	0.02	0.00	0.00	0.021	0.02
48	0.07	0.08	0.14	0.66	4.54	0.00	0.00	0.00	0.001	0.01
72	0.07	0.10	0.13	0.63	3.66	0.01	0.00	0.00	0.001	0.00
96	0.09	0.19	0.19	0.73	3.69	0.01	0.01	0.00	0.001	0.02

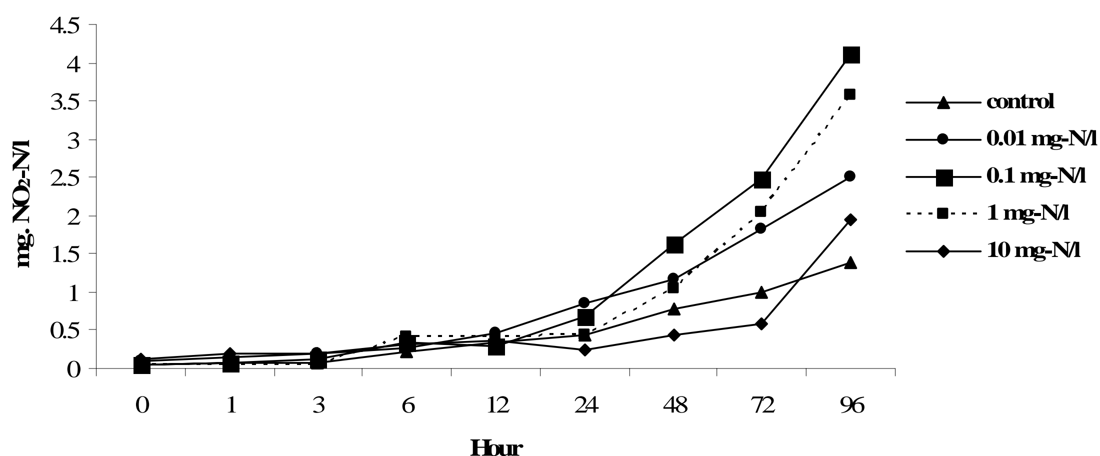


Figure 3.21 The concentration value of nitrite changed in each hour.

Table B4 Nitrite concentrations of water during ammonia toxicity test.

Hour	Concentration (mg-N/L)					SD(mg-N/L)				
	control	**0.01**	**0.1**	**1**	**10**	**control**	**0.01**	**0.1**	**1**	**10**
0	0.06	0.09	0.05	0.05	0.12	0.01	0.05	0.01	0.00	0.01
1	0.07	0.14	0.07	0.05	0.19	0.01	0.05	0.01	0.00	0.06
3	0.08	0.19	0.11	0.06	0.20	0.01	0.05	0.02	0.00	0.03
6	0.21	0.26	0.35	0.41	0.32	0.05	0.05	0.05	0.01	0.15
12	0.35	0.45	0.28	0.42	0.37	0.05	0.11	0.04	0.01	0.33
24	0.44	0.84	0.68	0.43	0.23	0.10	0.18	0.08	0.02	0.01
48	0.78	1.16	1.64	1.05	0.44	0.19	0.04	0.19	0.04	0.01
72	1.00	1.82	2.48	2.04	0.57	0.26	0.13	0.45	0.06	0.02
96	1.40	2.50	4.11	3.59	1.94	0.35	0.12	0.581	0.10	0.21

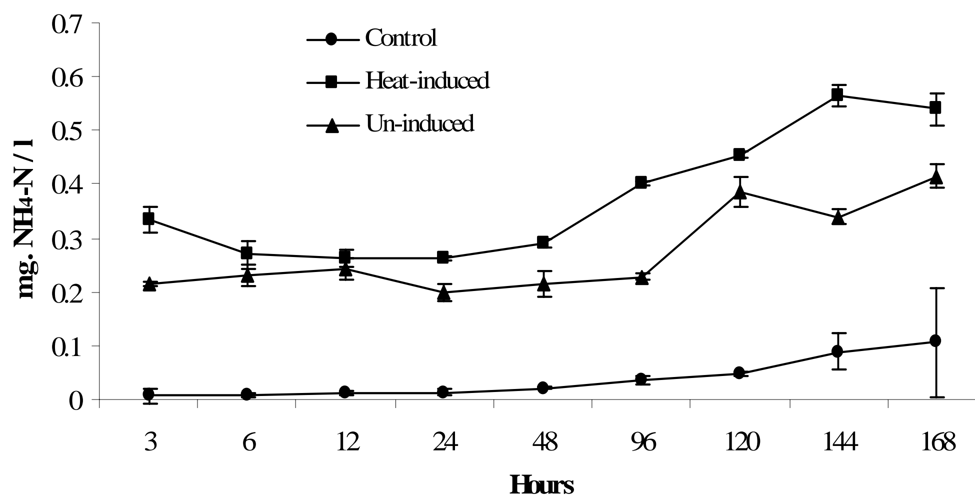


Figure 3.22 The concentration value of NH_3-N (unionized) changed in each hour.

Table B5 Ammonia concentrations of water during stress tolerance study.

Hour	Concentration (mg-N/L)			SD		
	Control	Un-induced	Heat-induced	Control	Un-induced	Heat-induced
0	0.01	0.21	0.33	0.014	0.00	0.02
1	0.01	0.23	0.27	0.01	0.02	0.03
3	0.01	0.24	0.26	0.01	0.02	0.01
6	0.01	0.20	0.26	0.01	0.01	0.01
12	0.02	0.21	0.29	0.00	0.02	0.01
24	0.03	0.23	0.40	0.01	0.01	0.00
48	0.04	0.39	0.45	0.01	0.03	0.01
72	0.09	0.34	0.56	0.03	0.01	0.02
96	0.11	0.41	0.54	0.10	0.02	0.03

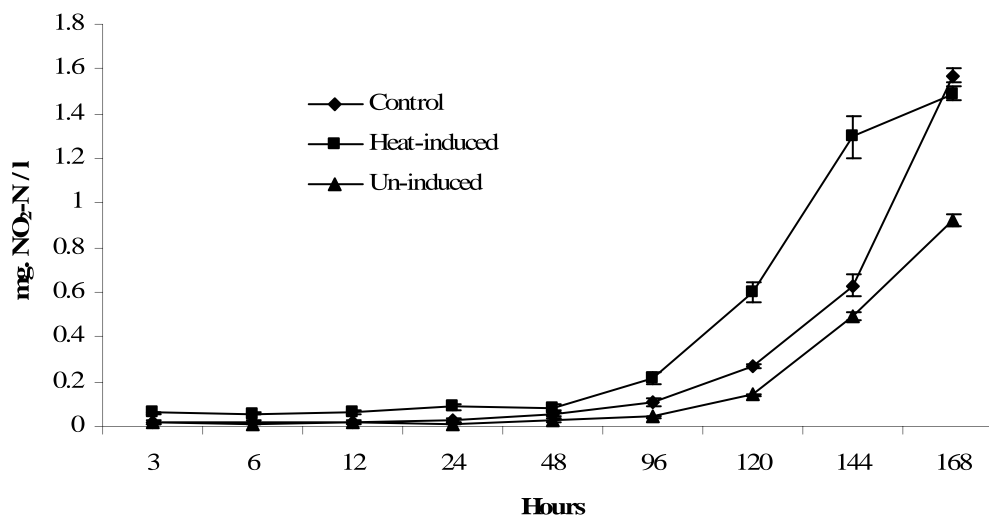


Figure 3.23 The concentration value of nitrite changed in each hour.

Table B6 Nitrite concentrations of water during stress tolerance study.

Hour	Concentration (mg-N/L)			SD		
	Control	Un-induced	Heat-induced	Control	Un-induced	Heat-induced
0	0.01	0.01	0.06	0.01	0.01	0.00
1	0.02	0.01	0.06	0.00	0.00	0.01
3	0.02	0.01	0.06	0.01	0.01	0.01
6	0.03	0.01	0.08	0.01	0.00	0.01
12	0.05	0.03	0.08	0.01	0.00	0.01
24	0.11	0.04	0.21	0.02	0.01	0.03
48	0.27	0.14	0.60	0.01	0.01	0.04
72	0.63	0.50	1.26	0.04	0.02	0.10
96	1.57	0.92	1.49	0.03	0.03	0.03

BIOGRAPHY

Miss Pranee Peaydee was born on April 30, 1984 in Lopburi Province, Thailand. She graduated with the degree of Bachelor of Science in Faculty of Marine Technology from Burapha University. She has studied for a degree of Master degree of science at faculty of Biotechnology, Chulalongkorn University.

Publication from this thesis

1. **Peaydee, P.,** Petkon S., Puanglar, N., and Menasveta, P. 2007. Cloning and characterization of gulcosamine-6-phosphate deaminase gene in black tiger shrimp, *Penaeus monodon*. The 33 th Congress on Science and Technology of Thailand. Nakhon Si Thammarat, Thailand. (Poster presentation).
2. **Peaydee, P.**, Puanglarp, N., Klinbunga, S., and Menasveta , P.2008. Cloning and characterization of aquaporin gene in black tiger shrimp, *Penueus monodon.* The 20 th Annual Meeting and International Conference of the Thai Society for biotechnology. Mahasarakham, Thailand. (Poster presentation).
3. **Peaydee, P**., Puanglarp, N., Klinbunga, S., and Menasveta, P. 2008. Tissue distribution and expression level of glucosamine-6-phosphate deaminase gene in black tiger shrimp, *Penaeus monodon* under heat induction. The 34 th Congress on Science and Technology of Thailand. Bangkok, Thailand. (Oral presentation).