บทบาทของยีน 14-3-3 จากกุ้งกุลาดำในการควบคุมออสโมลาริตี

นางสาวมณฑิรา แก้วดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

THE ROLE OF 14-3-3 GENES FROM BLACK TIGER SHRIMP *Penaeus monodon* ON OSMOTIC REGULATION

Miss Montira Kaeodee

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Academic Year 2008

Thesis Title	THE ROLE OF 14-3-3 GENES FROM BLACK TIGER SHRIMP *Penaeus monodon* ON OSMOTIC REGULATION
By	Miss Motira Kaeodee
Field of Study	Biotechnology
Thesis Principal Advisor	Professor Anchalee Tassanakajon, Ph.D.
Thesis Co-advisor	Siriporn Pongsomboon, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

S. Hannongbua

..... Dean of the Faculty of Science

(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Professor Aran Incharoensakdi, Ph.D.)

A. Tassanakajon

..... Thesis Principal Advisor

(Professor Anchalee Tassanakajon, Ph.D.)

Siriporn Pongsomboon

..... Thesis Co-advisor

(Siriporn Pongsomboon, Ph.D.)

..... Member

(Associate Professor Vichien Rimphanitchayakit, Ph.D.)

..... Member

(Associate Professor Sirirat Rengpipat, Ph.D.)

มณฑิรา แก้วดี : บทบาทของยีน 14-3-3 จากกุ้งกุลาดำในการควบคุมออสโมลาริตี (THE ROLE OF 14-3-3 GENES FROM BLACK TIGER SHRIMP *Penaeus monodon* ON OSMOTIC REGULATION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.ดร.อัญชลี ทัศนาขจร, อ. ที่ปรึกษาร่วม ดร.สิริพร พงษ์สมบูรณ์, 132 หน้า.

จากฐานข้อมูล EST ของกุ้งกุลาดำพบยีน 14-3-3 จำนวน 2 ไอโซฟอร์ม คือ 14-3-3A และ 14-3-3B จากการวิเคราะห์ลำดับกรดอะมิโนพบว่า 14-3-3A ประกอบด้วยรหัสสร้างโปรตีนที่ครบสมบูรณ์ ขณะที่ 14-3-3B ยังไม่ได้รหัสที่สมบูรณ์ ในงานวิจัยนี้ได้พยายามหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของยีน 14-3-3B จากกุ้งกุลาดำ โดยวิธี 5' RACE และ RT-PCR อย่างไรก็ตามไม่ประสบผลสำเร็จในการหาลำดับนิวคลีโอไทด์ที่สมบูรณ์แม้ว่าจะได้พยายามปรับสภาวะต่างๆในการทดลอง สาเหตุอาจเกิดจากโครงสร้างทุติยภูมิที่แข็งแรงที่ปลาย 5' ของเอ็มอาร์เอ็นเอของ 14-3-3B จากการวิเคราะห์ phylogenetic tree ของยีน 14-3-3 พบว่ายีน 14-3-3A จากกุ้งกุลาดำมีความคล้ายกับ 14-3-3 Zeta isotype ส่วนยีน 14-3-3B นั้นมีความคล้ายกับ 14-3-3 Epsilon isotype มากกว่า จากการศึกษาผลของความเค็มน้ำต่อการแสดงออกของยีน 14-3-3 โดยเทคนิค semi-quantitative RT-PCR พบว่า ยีน 14-3-3B มีระดับการแสดงออกที่สูงที่สุดในเหงือกของกุ้งที่เลี้ยงที่ระดับความเค็มน้ำต่ำที่ 3 ส่วนในพัน เมื่อทำการเปรียบเทียบกับกุ้งเลี้ยงที่ระดับความเค็มน้ำ 25 และ 40 ส่วนในพัน พบการแสดงออกที่ลดลงของยีน 14-3-3B เมื่อย้ายกุ้งจากการเลี้ยงที่ความเค็มน้ำต่ำไปเลี้ยงที่ความเค็มสูง สำหรับระดับการแสดงออกของยีน 14-3-3A เมื่อเปรียบเทียบระหว่างกุ้งที่เลี้ยงในระดับความเค็มน้ำทั้ง 3 ระดับดังกล่าว ไม่พบความแตกต่างหรือพบความแตกต่างอย่างมีนัยสำคัญเพียงเล็กน้อย และน่าสนใจที่พบว่ามีกุ้งบางกลุ่มเท่านั้นที่มีการแสดงออกของยีน 14-3-3B โดยกุ้งกลุ่มนี้สามารถปรับตัวในสภาวะเครียดจากความเค็มน้ำต่ำได้ดีกว่ากุ้งที่ไม่พบการแสดงออกของยีน 14-3-3B เมื่อทำการย้ายกุ้งจากระดับความเค็มน้ำ 40 ส่วนในพัน ไปยังน้ำระดับความเค็ม 3 ส่วนในพันเป็นระยะเวลา 4 วัน พบว่ากุ้งกลุ่มที่ไม่มีการแสดงออกของยีน 14-3-3B มีอัตราการตายในสองกลุ่มจากฟาร์มอำเภอแหลมสิงห์ที่ตรวจสอบเป็น 100 % ในขณะที่กุ้งกลุ่มที่มีการแสดงออกของยีนมีอัตราการตายเพียง 23 % ในกลุ่มแรก และ 57 % ในกลุ่มที่สองจากฟาร์มอำเภอท่าใหม่ นอกจากนี้พบว่าเมื่อทำการยับยั้งการแสดงออกของยีน 14-3-3B ด้วยเทคนิค RNA interference (RNAi) พบว่ากิจกรรมของเอนไซม์ ATPase ลดลง แสดงว่ายีน 14-3-3B น่าจะมีหน้าที่เกี่ยวข้องกับการควบคุมการทำงานของเอนไซม์ ATPase

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อ นิสิต.....

ปีการศึกษา.....2551..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4872412623: MAJOR BIOTECHNOLOGY
KEY WORD : *Penaeus monodon*/OSMOTIC REGULATION/RNAi INTERFERENCE/ 14-3-3 PROTEIN

MONTIRA KAEODEE: THE ROLE OF 14-3-3 GENES FROM BLACK TIGER SHRIMP *Penaeus monodon* ON OSMOTIC REGULATION. THESIS PRINCIPAL ADVISOR: PROF. ANCHALEE TASSANAKAJON, Ph.D., THESIS CO-ADVISOR: SIRIPORN PONGSOMBOON, Ph.D., 132 p.

The cDNAs encoding two different isoforms of 14-3-4 protein, namely 14-3-3A and 14-3-3B, were identified from the *Penaeus monodon* EST database. Sequence analysis revealed a complete coding sequence (CDS) of 14-3-3A while a partial sequence of 14-3-3B was obtained. In this study, we attempt to isolate a complete CDS of 14-3-3B cDNA using 5' Rapid Amplification of cDNA Ends (5' RACE) and reverse transcription (RT)- PCR. However, the complete CDS of 14-3-3B was not successfully isolated although several conditions were applied. This trouble was probably due to a strong secondary structure at the 5' end of 14-3-3B mRNA. A phylogenetic analysis of 14-3-3 sequences suggested that *P. monodon* 14-3-3A was closely related to 14-3-3 Zeta isotype whereas the 14-3-3B was more closely related to 14-3-3 Epsilon isotype. The effect of salinity on mRNA expression of the two isoforms of *P. monodon* 14-3-3 was determined in shrimp gill using a semi-quantitative RT-PCR. It was found that shrimps reared under low salinity (3ppt) showed higher expression of 14-3-3B mRNA than those acclimatized at high salinity (25 and 40 ppt). A significant decrease in the mRNA level of 14-3-3B was observed in shrimp transferred from 3 ppt to 40 and 25 ppt while the mRNA level of 14-3-3A remained unchanged or slightly changed over the salinity range tested. The results suggest that 14-3-3B probably plays a role in hypo-osmotic regulation in *P. monodon*. Interestingly, it was found that not all shrimp samples collected from different farms, expressed 14-3-3B isoform but shrimps that expressed 14-3-3B were more tolerant to sudden salinity transfer than those that did not. The cumulative mortalities of shrimps expressing 14-3-3B from two shrimp groups from farm I (Tamai district) was about 23 % and 57 % in day4 after low salinity stress while those of shrimps not expressing the gene in the two groups from farm II (Lamsing district) were 100 %. In addition, knockdown of 14-3-3B gene by RNA interference (RNAi) resulted in a significant decrease in total ATPase activity in shrimp gill suggested that 14-3-3B might be involved in the regulation of ATPase.

Field of study.....Biotechnology..... Student's signature.....Montira Kaeodee

Academic year.....2008..... Principal Advisor's signature.....A. Tassanakajon

Co-advisor's signature.....Siriporn Pongsomboon

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor Professor Dr. Anchalee Tassanakajon, and my co-advisor Dr. Siriporn Pongsomboon for their guidance, supervision, encouragement and supports throughout my study.

My gratitude is also extended to Professor Dr. Aran Incharoensakdi, Associate Professor Dr. Vichien Rimphanitchayakit and Associate Professor Dr. Sirirat Rengpipat for serving as thesis committees, for their valuable comments and also useful suggestions.

Thanks are also expressed to Dr. Premruethai Supungul and Dr. Piti Amparyap for very useful comments. Many thanks to all my friends of the Biochemistry Department especially in R728 for their helps in the laboratory and friendships that help me enjoy and happy through out my study.

Thank you for Kung Krabaen Bay Royal Development Study Center for treat sea water and data of shrimp farmer. Every shrimp farmer, especially, Mr. Aketanat (Tamai), Mr. Pramoth (Lamsing), Mrs. Ple (Lamsing) and Mr. Damrong (Kung Krabaen) for shrimp samples and suggestion of culture shrimp. Special thanks to all shrimps for sacrifice their lives for my research.

Finally, I would like to express my deepest gratitude to my parents and members of my family including Mr. Wichai Kaeodee, my father for their love, care, understanding and encouragement extended throughout my study.

CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EtBr	ethidium bromide
h	hour
kb	kilobase
M	molar
mg	milligram
ml	millilitre
mM	millimolar
MT	metric ton
ng	nanogram
nm	nanometre
O.D.	optical density
^{o}C	degree Celcius
ORF	open reading frame
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
sec	second
μγ	microgram
μλ	microlitre
Mμ	micromolar
UTR	untranslated region

CHAPTER I

INTRODUCTION

1.1 General introduction

The world cultured shrimp production in 2000 accounted for 56 % of *Penaeus monodon*, 17 % of *P. merguiensis*, 16 % of *P. vannamei* and 11 % of the others (Rosenberry, 2001). In Thailand, *P. monodon* have been intensively cultured for more than two decades. Approximately 60 % of the total harvest shrimp comes from cultivation. Shrimp farms and hatcheries are scattered along the coastal areas of Thailand. Southern provinces (Nakorn Sri Thammarat and Surat Thani) account for the majority while those in the East (Chanthaburi) and Central regions (Samut Sakhon and Samut Songkhran) comprise the minority in terms of number. The intensive farming system (85 %) has been used for *P. monodon* farming activity resulting in the consistent increase in the outcome production (Source: Department of Fisheries). However, the outbreak of infectious disease has a great impact on the decreasing of shrimp production in several countries including Thailand, Ecuador, Vietnam, Taiwan, etc. Since 2005, the great loss of shrimp production in Thailand has caused a change of the major farmed species from *P. monodon* to the white shrimp *Litopenaeus vannamei*. The black tiger shrimp production of Thailand fell down from 180,000 tons in 2004 to 19,000 tons in 2005 (Fig. 1.1). In 2008, the black tiger shrimp production is 60,000 tons while that of white shrimp is 550,000 tons.

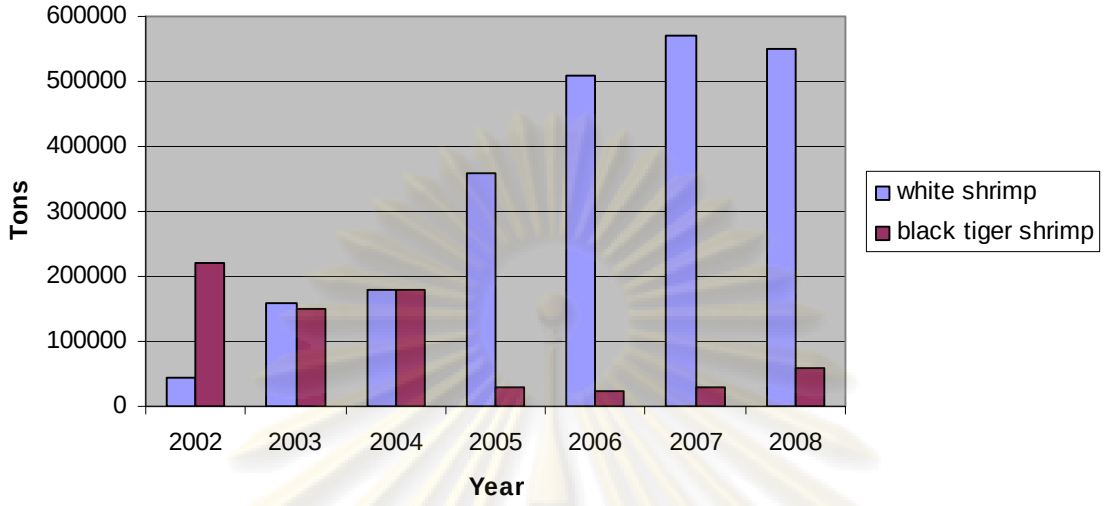


Figure 1.1 The shrimp production in Thailand between 2002 to 2008

(Source: http://www.shrimpcenter.com)

The United States of America is the major shrimp importers (Fig. 1.2). Approximately 33 % of shrimp was exported from Thailand in 2006 and 2007 which was worth about US$ 854 million.

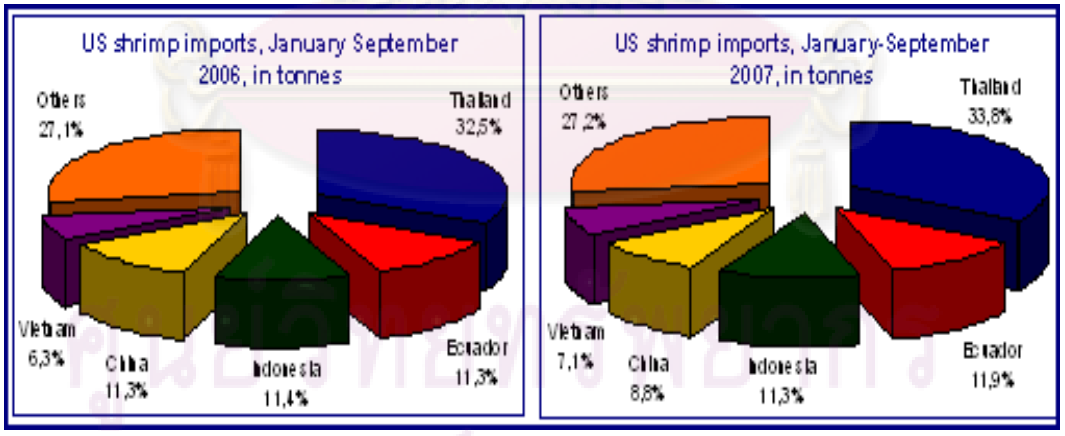


Figure 1.2 US shrimp imports by country, January-September 2006-2007

(Source: National Marine Fisheries Service)

P. monodon is markedly euryhaline and tolerates wide variations in salinity. They are considered as candidate species for culture in low saline areas preferably under extensive systems. Since culture of *P. monodon* in extremely high salinities to over 30 ppt may cause disease problems, particularly white spot syndrome virus or yellow head virus or luminescent bacteria. More shrimp farmers keep moving towards brackish water or freshwater areas (Chanratchakool, 2003; Laxminarayana, 2001). The culture of shrimp in freshwater will decreases disease problems and pollutant environment. The culture of *P. monodon* in freshwater has become a reality in India (Laxminarayana, 2001; Saha et al. 1999). Despite a high production of the white shrimp, the world market still requests for production of the black tiger shrimp for consumption. In the future, if Thailand can develop a method to successfully culture shrimp in low salinity or freshwater, the production might probably change towards the black tiger shrimp production.

1.2 Taxonomy of *Penaeus monodon*

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

Phylum Arthropoda

Subphylum Crustacea

Class Malacostraca

Subclass Eumalacostraca

Order Decapoda

Suborder Natantia

Infraorder Penaeidea

Superfamily Penaeoidea

Family Penaeidae Rafinesque, 1985

Genus Penaeus Fabricius, 1798

Subgenus Penaeus

Species monodon

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

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

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

1.3 Morphology

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

The abdomen has the obvious segmentation of invertebrates. A swimming legs or pleopods are the abdominal appendages. A pair of pleopods arises from each of the G abdominal segments. A tail fan comprises of a telson which bears the anus, and two uropods attach to the last (6^{th}) abdominal segment. The telson has a deep medication groove without dorso-lateral spines. A rapid ventral flexion of the abdomen with the tail fan produces the quick backward dart characteristic of shrimp (Anderson, 1993).

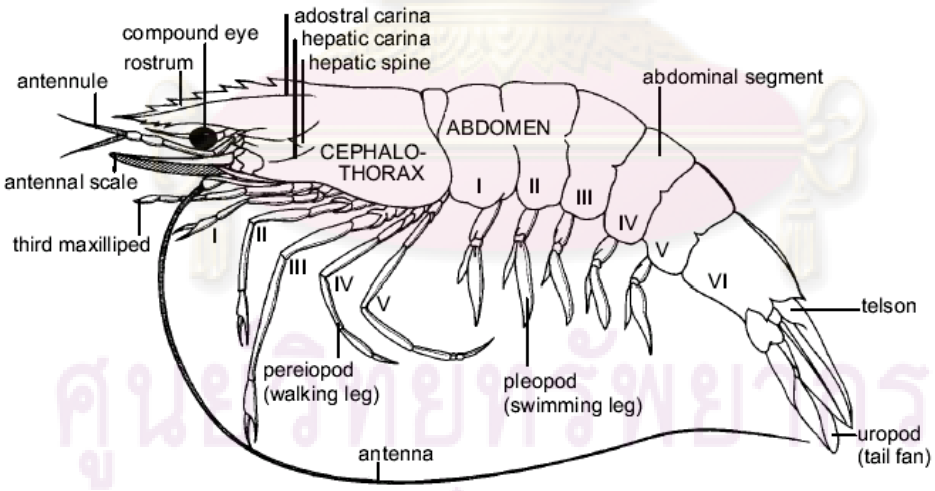


Figure 1.3 Lateral view of the external morphology of *P. monodon* (Primavera, 1990).

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

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

1.4 Life cycle

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

1.4.1 Larvae

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

Occurring offshore, they are planktonic in behavior. The body of the post-larvae is transparent with a dark brown streak from the tip of the antennular flagellum to the tip of the

telson. The 6th abdominal segment is relatively longer than the carapace length. The carapace length of the post-larvae varies between 1.2 and 2.2 mm. *P. monodon* enters nursery grounds during the last sub-stage of the post-larvae.

1.4.2 Juvenile

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

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

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

1.4.3 Subadult

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

A sex-size disparity occurs at almost 30 mm CL, and here after the size of females becomes greater than males. They migrate from nursery to spawning ground. During this

stage, first copulation takes place between males with minimum CL of 37 mm and females of 47 mm in the estuarine or inner littoral areas before migrating to the deeper water.

1.4.4 Adult

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

The maximum size of males recorded is 71mm CL, whereas the maximum recorded length of females is 81 mm CL, reaching 270 mm in body length or 260 g in weight. Carapace length varies between 37 and 71 mm in males and 47 and 81 mm in females. The diagram of the life history of *P. monodon* is shown in (Fig. 1.4).

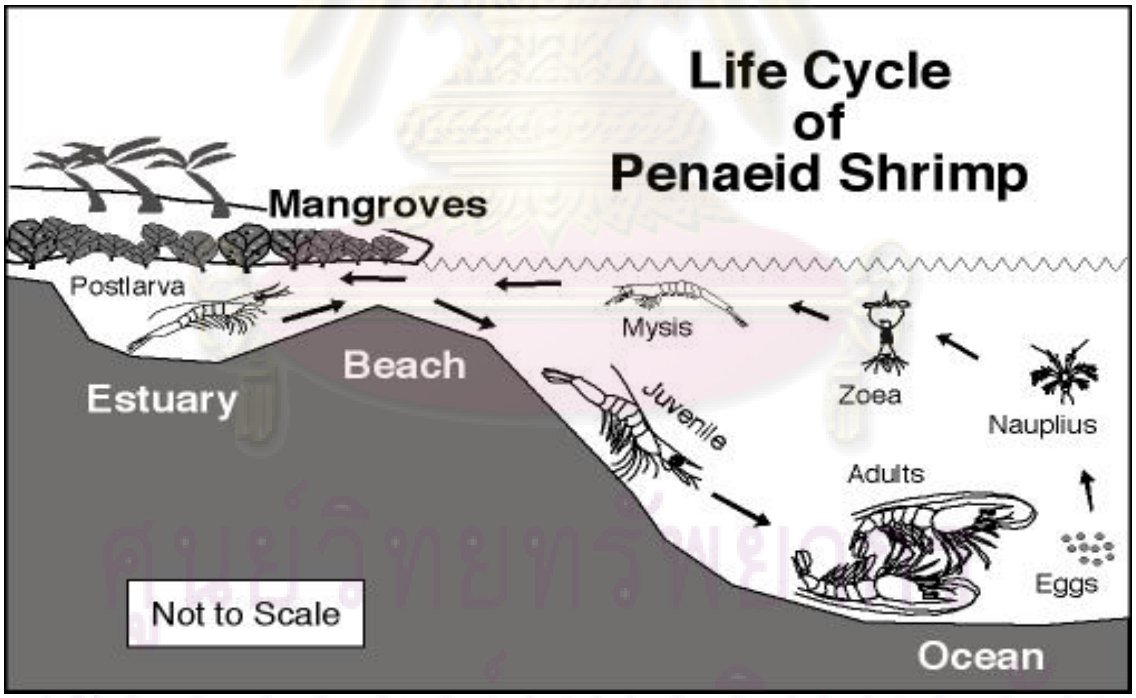


Figure 1.4 Diagrammatic representation of the life history of *P. monodon*

(Source: http://oceanworld.tamu.edu/resources/oceanography-book/invertebrates.htm)

1.5 Osmoregulation of crustaceans

Organisms living in estuaries and migrating to marine habitat as penaeid shrimp must be able to osmoregulate efficiently (Gilles, 1970; Gilles, 1979). Osmoregulation is one of the most important adaptive physiological processes permitting the successful establishment of a species in a given habitat (Charmantier, 1998).

Osmoregulation means the various strategies developed by the animal species to control their body water in the wide variety of possible environments which they have been aggressively inhabited. It implies detailed study of all the processes at work in the control of the thermodynamic activity of water in biological fluids, either intracellular or extracellular, of the animal species considered. This includes analyses of any kind of transport processes and metabolic correlates involved in the adjustment and the control of the level of both inorganic and organic constituents (Charmantier and Charmantier-Daures, 2001; Martinez et al., 2005).

For many years the composition of the haemolymph has been studied in various groups of crustacean as a function of the salinity environment. Several generalizations for the most representative patterns discovered in animals living in a range of media from concentrated sea water to fresh water have been explored.

Classification of organisms according to their osmoregulation capabilities divides the organisms into three groups: osmoconformers, hyper-osmoregulators and hypo-osmoregulators. The osmoconformers regulate their ionic concentration in haemolymph such that it is always equal to the surrounding seawater. These animals keep their body fluids isotonic to the external environments. The hyper-osmoregulators have higher ionic concentration in haemolymph than in surrounding medium while hypo-osmoregulators show lower ionic concentration in haemolymph than in external medium (Campbell and Jones, 1989; Castille and Lawrence, 1981; Charmantier and Charmantier-Daures, 2001; Gilles, 1970; Mantel and Farmer, 1983; Pequeux, 1995; Potts and Parry, 1964). Generally, *P. monodon* is a hypo-hyper osmoregulator. It exhibits hyperosmotic regulation to sea water at salinities below isosmotic concentrations and hypoosmotic regulation to those above

(Wuthisuthimethavee et al., 2005). There are some other crustaceans that show ability as hypo-hyper osmoregulation such as *Palemonetes varians*, and *Artemia salina* (Castille and Lawrence, 1981; Cheng and Liao, 1986; Ferraris et al., 1987; Knut, 1983). These organisms are extremely powerful osmoregulators and can cope with any fluctuation of salinity of the external medium that normally occurs in nature (Dall, 1985; Ferraris et al., 1986; Ferraris et al., 1987; Gilles, 1979; Motoh, 1981; Potts and Parry, 1964). The synthetic way of the osmotic regulation in freshwater and marine teleost fish is shown in Fig. 1.5.

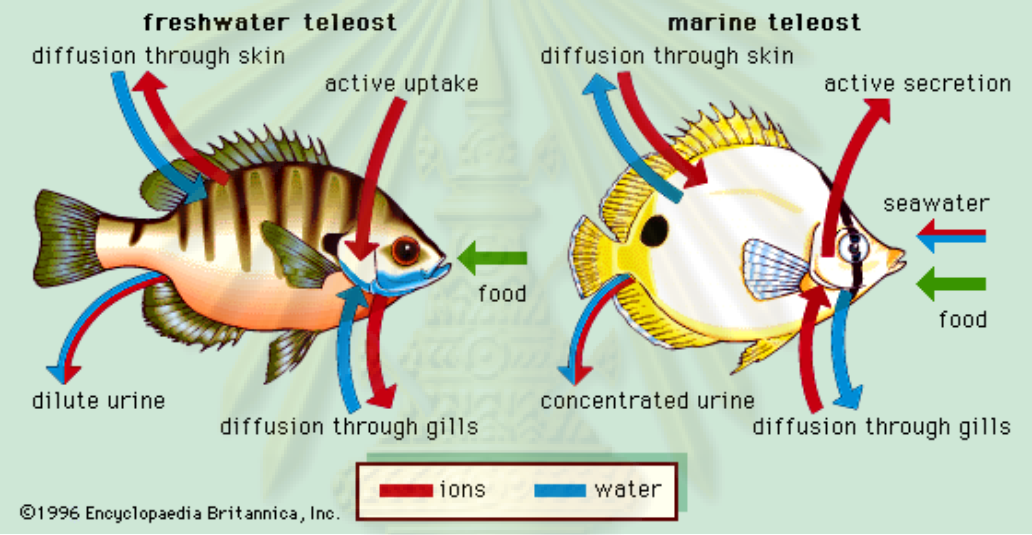


Figure 1.5 Osmotic regulations in freshwater and marine teleost fish.

(Source: Encyclopædia Britannica, Inc.)

Salinity is one of the main environmental factors affecting growth, survival and development of the black tiger shrimp. The black tiger shrimp is naturally distributed over a wide range of salinities during its life cycle. The life cycle includes a marine phase during the first stages of development, then a brackish or estuarine phase for postlarvae and juvenile stages, after which they migrate towards marine water at preadult or adult stages. The matured shrimp naturally migrates from brackish water to the deep sea, a place with high salinity level and environmentally consistency, for mating. Spawning and the developing shrimp larva returns to the nutrition-rich in-shore sea or mangrove swamp for growing. Since the salinity of brackish water fluctuates more than that of marine sea water, it is likely that the degree of euryhalinity, possibility of supporting rapid salinity changes, varies with the

age of the shrimps. *P. monodon* is an euryhaline crustacean which is able to regulate their body fluid composition with regard to the ambient medium by modifying surface permeability, urine production, and ionic transport (Ferraris et al., 1987; Spaning-Pierrot et al., 2000).

1.6 Osmoregulatory organs

Among the few comprehensive histological studies of osmoregulatory structures in decapod crustaceans, most have concerned the gills of crabs (Drach, 1930; Chen, 1933; Smyth, 1942) and shrimps such as *Palaemonetes varians* (Allen, 1892) *Crangon vulgaris* (Debaisieux, 1970) and *Penaeus aztecus* (Foster and Howse, 1978). Numerous studies hypothesize that the branchiostegite such as epipodite has an osmoregulatory function in *P. japonicus*. Moreover, previous studies found that the function of the antennal gland in penaeid shrimp has been associated with ion transport and osmoregulation. Therefore, these three organs are studied to elucidate genes involved in osmoregulation mechanism.

The antennal glands consist of the coelomosac, labyrinth, tubule and bladder, and their main function is urine production. By filtering the haemolymph, urine is produced and flows to the branchial chamber via the nephropore. In marine decapods, the antennal glands are involved in the control of haemolymph volume, hyporegulation of magnesium and sulfate in haemolymph, excretion of organic compounds and reabsorption of fluid, sugars and amino acids from the primary urine filtration (Riegel and Cook, 1975; Mantel and Farmer, 1983). The function of the antennal glands in penaeid shrimp have been associated with the differences of sodium and chloride concentrations between haemolymph and urine. Previously reports showed that antennal glands of *P. monodon* functioned to stabilize potassium concentrations by reabsorbing potassium from the primary urine and excreting sodium in exchange (Mantel and Farmer, 1983; Lin et al., 2000).

The epipodites, or mastigobranchs, are elongated, thin, biramous structures, attached to the coxopodites of some thoracic appendages. Previous study showed that the epipodites are probably involved in osmoregulatory mechanisms. The epithelial cells of epipodite contain numerous elongated mitochondrias which reveal the presence of the Na^+/K^+-ATPase.

This is further supported by the high level of the Na^+/K^+-ATPase activity measured in epipodites of adult *P. japonicus* (Bouaricha et al., 1991) that the epipodite of *P. japonicus* could be involved in osmoregulatory mechanism. Moreover, previous study found that the excretion of salts in *Cladocera* species was associated with epipodite cell.

The gills are specialized for several functions, including gas exchange, osmoregulation, acid-base balance and nitrogen excretion. Previous study reported that the gills are among the most permeable external surfaces of crustaceans, and they are considered the primary site for ionic and osmotic regulation (Robertson, 1960; Lockwood, 1962; Lockwood, 1968; Gilles, 1975; Croghan, 1976; Kirschner, 1979; Pequeux and Gilles, 1981; Pequeux and Gilles, 1988; Towle, 1984a). Numerous studies reported that the gill of crab species consists of two kinds, anterior and posterior gills that are histologically different. In *Eriocheir sinensis***,** the anterior gills have a thin, little-differentiated epithelium, probably involved in respiration, while the posterior ones play a key role in osmotic and ionic regulation (Luquet et al., 2002; Pequeux and Gilles, 1981; Pequeux and Gilles, 1988; Schoffeniels and Gilles, 1970). In juveniles and adults of crustacean species, the epithelium of the gill contains mitochondria that can be involved in osmoregulation. This is supported by the increase in the size of epithelium in *Penaeus aztecus* that are transferred to low or high salinities. Similar epithelia have also been reported in the posterior gills of *E. sinensis* (Barra et al., 1983).

1.7 Significance of osmoregulation mechanism in the black tiger shrimp

As describe above, the black tiger shrimp is able to survive and grow in a wide range of salinity from 2 to 45 ppt. However, extremely high or low salinity always causes more problems than the suitable salinities, which range from 15 to 25 ppt. Culture in extremely high salinities over 30 ppt may cause disease problems, particularly white spot or yellow head virus and luminescent bacteria. In low salinity at 3 to 8 ppt, in fact that the shrimp can grow for a period of 4 months or a little bit more but no more growth appears to be significant transfer (Chanratchakool, 2003). In addition, the current domesticated broodstock after growing out of the broodstock-sized shrimp for gonad and sperm development still depends upon the stimulation of high salinity level before mating. Structural and functional characterization of salinity stress responsive genes has contributed to a better understanding of how shrimp response and adapt to salinity stress. An understanding of the osmoregulatory mechanism would be useful for the production of broodstock and shrimp farming. Moreover, genes controlling osmoregulatory system in *P. monodon* can be applied for the selection of low-salinity tolerant shrimp strain.

1.8 Characterizations of 14-3-3 proteins

Members of the 14-3-3 proteins family form a group of highly conserved 30 kDa acidic proteins expressed in a wide range of organisms and tissues. The proteins exist primarily as homo- and heterodimers in all eukaryotic cells. The name 14-3-3 was given to an abundant mammalian brain protein family due to its particular elution and migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis. The 14-3-3 proteins elute in the 14^{th} fraction of bovine brain homogenate from the authors "homemade" DEAE cellulose column and fractions 3.3 in the latter step (Aitken, 2006). In humans, there are seven distinct 14-3-3 genes denoted β, γ, ε, η, σ, τ (θ), and ζ (as well as a number of potential pseudogenes), while yeast and plants contain between 2 and 15 genes. It was subsequently shown that while many 14-3-3 interacting proteins contain RSXpSXP (mode I) and RXY/FXpSXP (modeII) binding motifs (Table 1), many others do not,

indicating that additional sequences and modes of interaction/contacts also allow 14-3-3 binding. Well over half of the 14-3-3 binding proteins identified to date use phosphorylated sequences which are reasonably close matches to the optimal 14-3-3 consensus motifs. The motifs are clearly not absolute, however, since the presence of a non-phosphorylated S immediately following R within the first motif, and a P two positions C-terminal to the phosphorylated S or T in both motifs, though strongly favored, is not required for peptide and protein binding to 14-3-3 (Yaffe, 2002 and Sehnke, DeLille et al.,2002). The interior of the L-structure is composed of four helices: H3 and H5, which contain many charged and polar amino acids, and H7 and H9, which contain hydrophobic amino acids. These four helices form the concave amphipathic groove that interacts with target peptides. An alignment of all currently known full-length isoforms provides evidence that this groove is over 70 % conserve (Figs. 1.6 and 1.7).

Table1. Observed and Putative Interactions of 14-3-3 Client Proteins (Aitken, 2006)

Protein	Binding Site(s)	Accession No.
R/KXXpSXP (mode-1)		
Nitrate reductase	KKxSTP	AAA32830
Vacuolar ATP synthetase catalytic subunit A	RKVSGP RGVSVP	O23654
ATP synthase β chain	RFLSQP	P19366
Calcium-dependent protein kinase, AK1	RTESKP	Q06850
Starch synthase III	RYGSIP	CAA16796
β-Amylase[a]	RYPSYP	P25853
Protein kinase AFC2[a]	RNGSPP	P51567
Protein kinase 54K[a]	RQGSPP REQSIP	S71169
Magnesium-chelatase subunit CHLI[a]	RFDSNP	P16127
COP1 regulatory protein[a]	RNISQP	P43254
2-cys peroxiredoxin BAS1[a]	RTLSSP	Q96291
Homeobox-Leu zipper protein ATHB-5[a]	RSPSPP	P46667
R/KXXXpSXP (mode-2)		
13-lipoxygenase Lox-2	KPSDSKP	L37358
Other		
H^+ ATPase	YpTV	P19456

[a] Putative interaction.

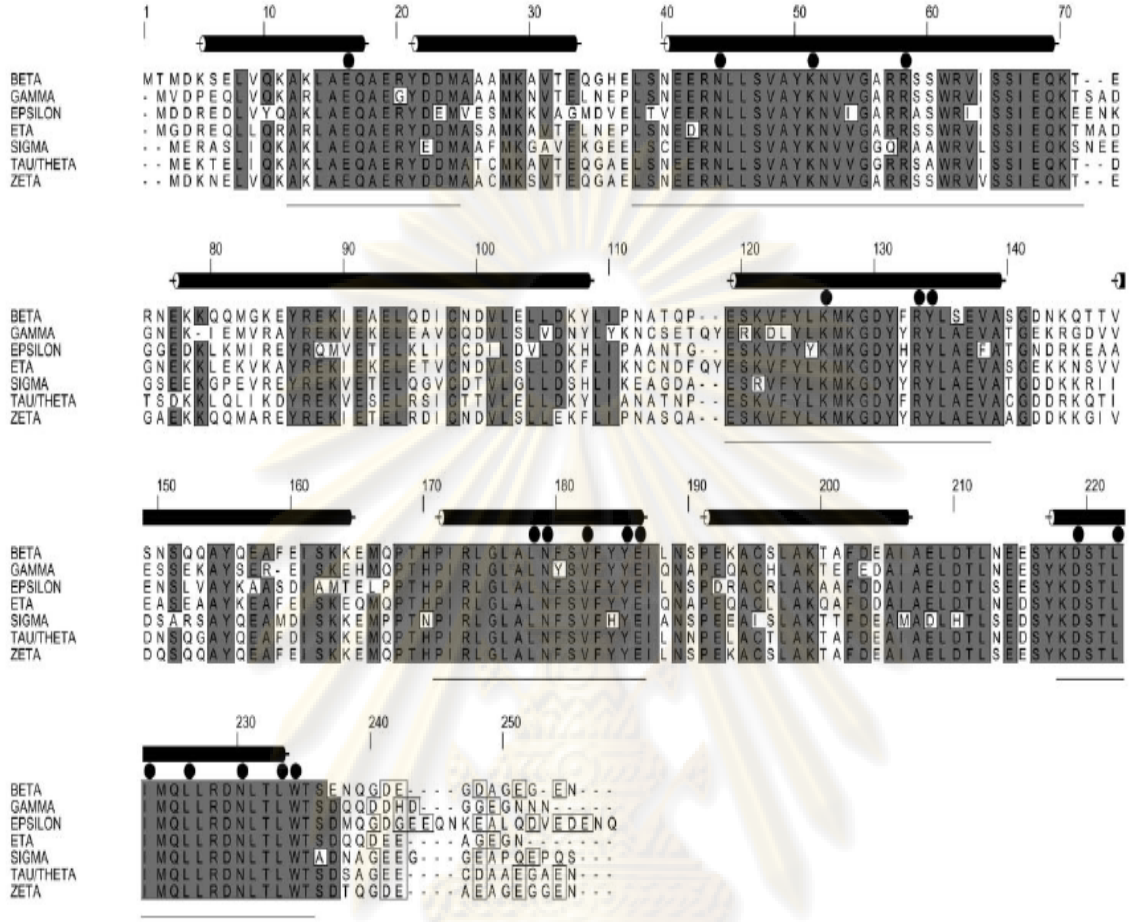


Figure 1.6 Sequence alignments of human 14-3-3 isotypes. Residues conserved in at least six of the seven isotypes are shaded gray. The structure of 14-3-3 is indicated by helices above the alignment. Five conserved sequence blocks within the 14-3-3 family, as defined by Wang and Shakes, are indicated by a thin line below the alignment. Residues within the binding cleft that interact with peptide ligands or with the serotonin N-acetyl transferase molecule are indicated by filled circles. Acidic residues within the divergent C-termini are boxed (Yaffe, 2002).

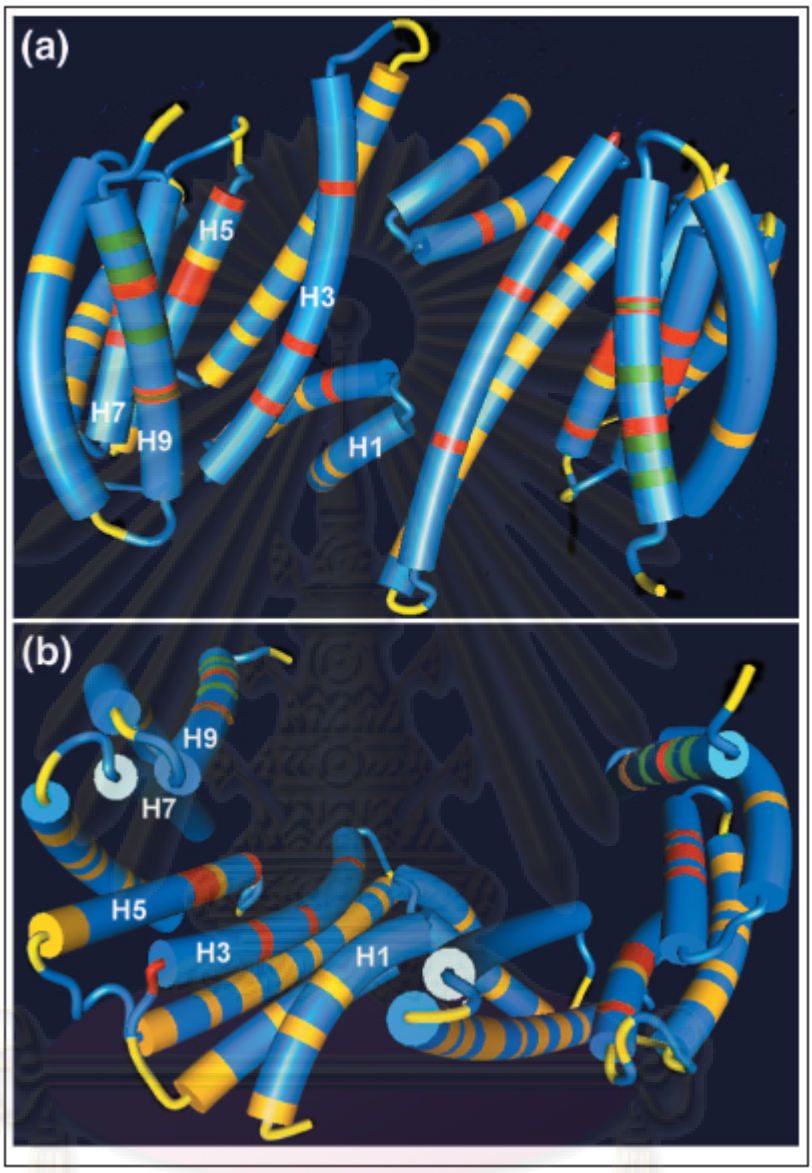


Figure 1.7 The crystal structure of 14-3-3s. The model shown is derived from the human 14-3-3 ζ isoform (PDB1QJB) and is shown from (a) the top and (b) one side as visualized by the 3Dmol software found in the Vector NTI 7.0 Suite. Helix numbers are denoted from H1 near the amino terminus to H9 near the carboxyl terminus. Red and blue areas correspond to residues of 100 % identity and high conservation, respectively, and correspond to colors on the alignment (Fig. 1.6). Yellow areas correspond to regions of reduced similarity and green areas indicate the nuclear export signal (Ferl, et al.,2002).

1.9 Cellular contexts of 14-3-3 activities

A wide array of biological functions involving kinase-mediated signal transduction, growth and developmental regulation, and response to environmental stress has been attributed to members of the 14-3-3 family. The notable recurrent themes in these diverse systems are the involvement of protein–protein interactions, divalent cations, kinases, and phosphatases, and the role of 14-3-3s continues to center on direct participation in signal transduction events (Morrison, 1994; Morrison, 1995). Although not exhaustively complete, this survey highlights these themes and roles.

1.9.1 Kinase-mediated signal transduction

Signal transduction via protein phosphorylation is a common pathway for many organisms. Perhaps the best studied of these is the GTP-dependent Ras pathway. The stimulation of cell division by extracellular growth factors involves the receptor-based production of active Ras, which then turns on a series of protein kinases, including Raf-1, which activate enzymes in the nucleus that are critical for message transduction, including the transcription factors. In 1994, several investigators independently identified 14-3-3s within the Ras pathway as activators of Raf-1 (Fantl et al., 1994), and 14-3-3 proteins were found to associate with Raf-1 in vivo while completing its activation and recruitment to the membrane (Freed et al., 1994; Irie et al., 1994). Since these initial reports, other kinases have been shown to either bind to or be activated by 14-3-3s (Maru and Witte, 1991; Reuther et al., 1994; Liu et al., 1997), including the notable calcium dependent protein kinase (CDPK) (Camoni et al., 1998).

1.9.2 Growth and developmental signaling

In several organisms, it has become apparent that 14-3-3s play a pivotal role in the growth and development of the cell. A clear example of this comes from the study of the yeast *Saccharomyces cerevisiae* 14-3-3s, known as BMH1 and BMH2 (van Heusden et al., 1995). Strains with disrupted BMH genes grow more slowly on minimal medium, and double mutants are lethal. Normal growth rate, however, can be rescued by heterologous expression

of one of several 14-3-3s, including four different Arabidopsis isoforms (van Heusden et al., 1996). Examples of the involvement of 14-3-3s in higher order growth and development are available, especially for *Drosophila melanogaster*, in which the *leonardo* mutation demonstrates that 14-3-3s play a role in synaptic growth and learning (Skoulakis and Davis, 1996, 1998; Broadie et al., 1997; Li et al., 1997). The presence of an inherited variant of a human 14-3-3 has been associated with early-onset schizophrenia (Toyooka et al., 1999).

1.9.3 Structure and movement

There are examples of 14-3-3 proteins interacting with proteins that would not normally be considered enzymes or be subjected to signal-induced transitions in activity. Keratin intermediate filaments are expressed in simple-type epithelia and are responsible for cell structural integrity, and 14-3-3s associate with keratins where they act as solubility factors (Liao and Omary, 1996). Other structural roles for 14-3-3s are indicated by the localization of 14-3-3s to the mitotic spindle apparatus, by associations with centrosomes (Pietromonaco et al., 1996), and by interactions with other cell scaffold–type proteins (Dellambra et al., 1995; Du et al., 1996). In certain contexts, 14-3-3s serve to alter the subcellular localization of their clients. The 14-3-3s contain a nuclear export signal, such that interaction between 14-3-3s and clients within the nucleus serves to assist in the nuclear export of the client (Rittinger et al., 1999; McKinsey et al., 2000).

1.9.4 Response to stress

The study of stress induction in different organisms has led to the identification of 14-3-3s as integral components of response pathways. Environmental conditions affect 14-3-3s directly, because external stimuli such as cold and increased salt have led to altered regulation of plant 14-3-3s (Kidou et al., 1993; Chen et al., 1994; Jarillo et al., 1994). Biological interactions between organisms also engage 14-3-3s, as in the *Pseudomonas aeruginosa* exotoxin S, which requires a cellular 14-3-3 for activity (Fu et al., 1993). A direct relationship exists between 14-3-3s and the receptor for the wilt-inducing phytotoxin fusicoccin (FC) (Oecking et al., 1994; Aducci et al., 1995; de Boer and Korthout, 1996).

Interactions with 14-3-3s regulate the H^+-ATPase under normal physiological conditions via blue light excitation in guard cells (Emi et al., 2001), and the complex between 14-3-3s and the H^+-ATPase forms a binding site for FC. And marine observations from fish euryhaline teleost (*Fundulus heteroclitus*), the results showing an increase of 14-3-3a in gill epithelium of fish transferred from SW to FW, suggest that 14-3-3a could be a major factor for the activation of H^+-ATPase, downregulation of $Na^{+/}K^+$-ATPase, and inhibition of the Cl-channel in gill epithelial cells of euryhaline fish transferred from SW to FW and, therefore, represent a molecular master regulator for osmotic adaptation and the configuration of flexible ion transport mechanisms in the same tissue. Hypothetical schematic of one aspect of 14-3-3a function in gill epithelial cells of euryhaline teleosts acclimated to freshwater (FW)(Fig. 1.8) or seawater (SW)(Fig. 1.8). Active NaCl absorption across gill epithelial cells of FW-acclimated fish is energized by an apical H^+-ATPase. A homologous H^+-ATPase is activated by 14-3-3 in plants and it is possible that the induction of 14-3-3 in gills of FW-acclimated fish serves to stimulate NaCl absorption via activation of the H^+-ATPase. In SW-acclimated fish 14-3-3 is down regulated. Because 14-3-3 inhibits Cl–channels such down regulation could be necessary for active NaCl secretion, which depends on the activity of an apical Cl-channel. Notably, 14-3-3 proteins regulate many more phospho-proteins than just transporters and channels and are likely to be key regulators of other aspects of adaptive cell differentiation in chloride and pavement cells (Kultz, Chakravarty et al., 2001) 14-3-3 in *Xenopus laevis* oocytes protects the oocytes from osmotic stress, which was attributed to its inhibition of an endogenous oocyte chloride current (Kohn, Chakravarty et al., 2003).

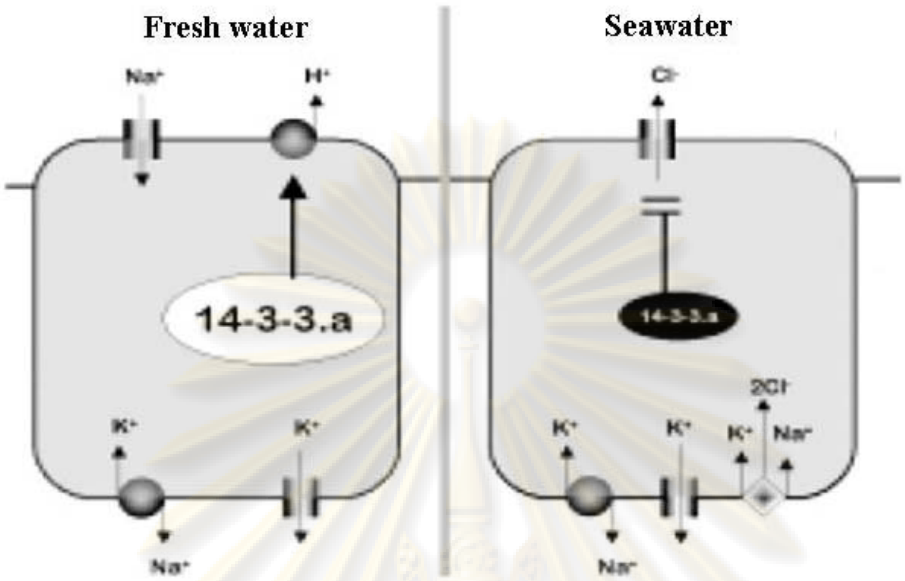


Figure 1.8 Hypothetical schematic of one aspect of 14-3-3a function in gill epithelial cells of euryhaline teleosts acclimated to freshwater (FW, left panel) or seawater (SW, right panel) (Kultz, Chakravarty et al., 2001).

1.10 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a rapid and quantitative method in analyzing the level of expression of gene. It is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts (Carding et al., 1992; Marone et al., 2001). The RNA cannot serve as a template for PCR, reverse transcription was combined with PCR to make RNA into a complementary DNA (cDNA) suitable for PCR. The combination of both techniques is colloquially referred to as RT-PCR (Fig. 1.9). The necessity to reverse transcribes mRNA into a cDNA prior to subjecting the RNA template to PCR is given by the fact that the polymerase used in PCR is a DNA-dependent polymerase. Reverse transcription of mRNA requires choosing a reverse transcriptase, a means of priming the mRNA to initiate polymerization and supplying optimal condition for the enzymatic reaction. Reverse transcriptase are RNA-dependent DNA polymerases which have been used predominantly to catalyze first strand synthesis (synthesis of a complementary DNA-cDNA), but are also capable of synthesizing a DNA strand complementary to a primed single

stranded DNA. The RT-PCR can be used for semi-quantitative also known as relative or quantitative analysis.

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

1.11 ATPase assay

H^+-ATPases are enzymes that couple the hydrolysis or synthesis of ATP to a transmembrane movement of protons. H^+-ATPases are found in nearly all cells and are divided into three distinct classes: the plasma membrane type, the vacuolar or lysosomal type, and the FoFI type, which is present in mitochondria and chloroplasts. ATPase activity can be monitored spectrophotometrically by use of enzyme systems that couple ATP hydrolysis to the oxidation of NADH. The coupled enzyme ATPase assay is based on the conversion of phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase (PK) coupled to the conversion of pyruvate to lactate by lactate dehydrogenase (LDH). The latter step requires NADH which is oxidized to NAD^+. NADH absorbs strongly at 340 nm but NAD^+ does not, enabling the utilization of NADH to be followed by monitoring absorbance at 340 nm. The decrease in OD_{340} can be converted into ATPase activity where 1 molecule of NADH oxidized to NAD^+

corresponds to the production of 1 molecule of ADP by the motor ATPase. The reaction of ATPase activity is shown in Fig. 1.10.

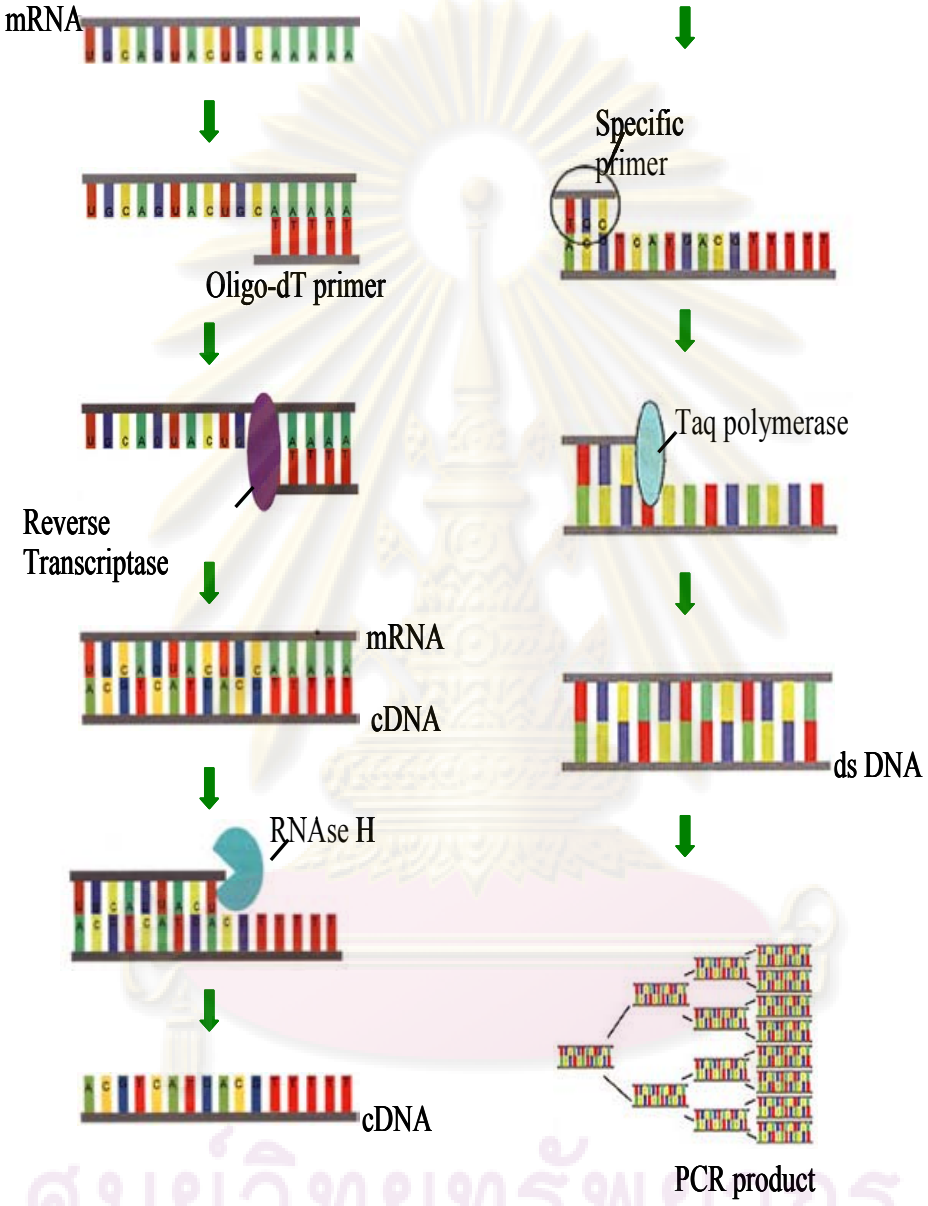


Figure 1.9 Overview of the RT-PCR technique

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

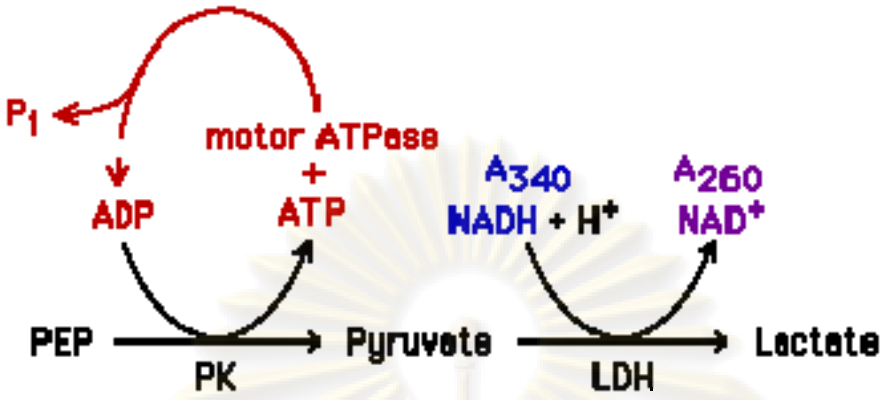


Figure 1.10 Reaction of ATPase activity

(Source: http://www.proweb.org/kinesin/Methods/ATPase_assay.html)

1.12 RNAi interference technique

The RNA interference (RNAi) pathway was originally recognized in *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA) leading to sequence-specific gene silencing. It soon turns out that RNAi is not restricted to nematode and can be induced in Drosophila, Trypanosoma, and vertebrates. Similar phenomena had been observed in plants and fungi. These phenomena were called co-suppression (also termed post-transcriptional gene silencing, PTGS) and quelling, respectively. Co-suppression was shown later to be mediated by dsRNA as a quide molecule. Establishing pathways including RNAi, co-suppression (PIGS), and quelling is now collectively called RNA silencing and appears to be present in most, if not all, eukaryotic organisms. The common key player in RNA silencing is small RNA of 21 to 28 nucleotides (nt) in length. Two classes of small RNAs are involved in RNA silencing: small interfering RNAs (siRNA) and microRNAs (miRNAs). Because of the exquisite specificity and efficiency, RNAi has drawn much attention as a powerful gene knockdown technique.

RNAi is mediated by small interfering RNAs (siRNAs) that are generated from long dsRNAs of exogenous or endogenous origin. Long dsRNAs are cleaved by a ribonuclease III (RNase III) type protein Dicer. Dicer homologues can be found in *C.elegans*, Drosophila,

plants, and mammals, suggesting that small RNA-mediated regulation is evolutionarily ancient and may have critical biological roles. The siRNA generated by Dicer is a short (~22-nt) RNA duplex with 2-nt overhang at each 3' end (Fig. 1.11). Each strand contains a 5' phosphate group and a 3' hydroxyl group. The siRNA is incorporated into a nuclease complex called RISC (RNA induced silencing complex) that targets and cleaves mRNA that is complementary to the siRNA. The intitial RISC containing a siRNA duplex is still inactive until it is transformed into an active form (RISC), which involes loss of one strand of the duplex by an RNA helicase activity. The identity of the RNA helicase is currently unknown. Dicer has a conserved helicase domain but it remains to be seen whether Dicer actually catalyzes this reaction.

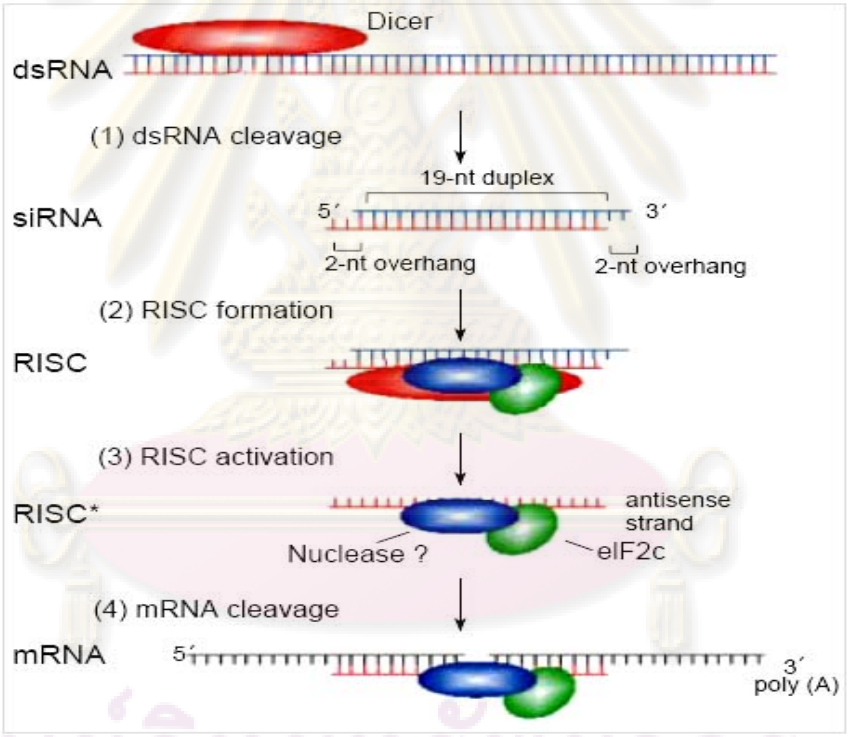


Figure 1.11 Current models for RNA interference. RNAi process can be divided into four stages: (1) dsRNA cleavage by Dicer and generation of siRNA duplex, (2) recruitment of RNAi factors and formation of RISC (RNA-induced silencing complex), (3) siRNA unwinding and RISC activation and (4) mRNA targeting and degradation (V. Narry Kim, 2003)

1.13 Rapid amplification of cDNA ends (RACE)

Rapid amplification of cDNA ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3' or the 5' -end of the mRNA. RACE procedures have been used for amplification and cloning of rare mRNAs that may escape, or prove challenging for, conventional cDNA cloning methodologies. Additionally, RACE may be applied to existing cDNA libraries. Random hexamerprimed cDNA has also been adapted to 5' RACE for amplification and cloning of multiple genes from a single first strand synthesis reaction. Products generated by the 3' and 5' RACE procedures may be combined to generate full-length cDNAs. Lastly, the RACE procedures may be utilized in conjunction with exon trapping methods to enable amplification and subsequent characterization of unknown coding sequences.

1.14 Previous studies

From the *P. monodon* EST Database (http://pmonodon.biotec.or.th), we identified 103 clones of a putative 14-3-3 like protein from the cDNA libraries of *P. monodon*. The partial sequence of the cDNAs showed two different isoforms of 14-3-3 like protein, the first isoform, namly 14-3-3A represented by 99 clones identified from all tissues showing the highest similarity to 14-3-3 like protein of *P. monodon* (100 % identity) and *Apis mellifera* (90 % identity). The second isoform namely 14-3-3B, represented by 4 clones identified from gill-epipodite cDNA library showing the highest similarity to 14-3-3 like protein of *Tetrahymena thermophila* B210 (71 % identity). In addition, specific expression in osmoregulatory tissues such as gills suggests that 14-3-3B may be important for salinity adaptation of *P. monodon*.

1.15 Objectives of the thesis

The aims of the thesis were to characterize the two isoforms of *P. monodon* 14-3-3 protein, and examine the expression of *P. monodon* 14-3-3 in response to change in salinity. Furthermore, the cumulative mortalities of shrimps express and not express the 14-3-3B gene when transfers from high salinity to low salinity were determined. In addition, the function of 14-3-3B was further investigated by gene silencing and assayed for the ATPase activity of the 14-3-3B silenced shrimp compared with the control shrimps. This will lead to a better understanding in osmoregulation of *P. monodon.*

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

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

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

Balance: Satorius 1702 (Scientific Promotion Co.)

Bind Silane (Pharmacia, USA)

-20°C Freezer (Whirlpool)

-80°C Freezer (ThermoForma)

Gel Documention System (GeneCam FLEX1, SynGene)

GS Gene Linker™: UV chamber (Bio-RAD Laboratories)

Gene Pulser (Bio-RAD)

Incubator 37°C (Memmert)

LABO Autoclave (SANYO)

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

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

Minicentrifuge (Costar, USA)

Medical X-ray film (Kodax, USA)

Nipro disposable syringes (Nissho)

Orbital shaker SO3 (Stuart Scientific, Great Britain)

PCR Mastercycler (Eppendorf AG, Germany)

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

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

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

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

Refrigerated microcentrifuge MIKRO 22R (Hettich Zentrifugen, Germany)

Spectrophotometer: Spectronic 2000 (Bausch & Lomb)

Spectrophotometer DU 650 (Beckman, USA)

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

Sterring hot plate (Fisher Scientific)

Touch mixer Model # 232 (Fisher Scientific)

Vertical electrophoresis system (Hoefer™ miniVE)

Water bath (Memmert)

White/UV transilluminator: UVP ImageStore 7500 (Mitsubishi Electric Corporation, Japan)

96-well plate (Bio-RAD)

2.1.2 Chemicals and reagents

Absolute ethanol, C_2H_5OH (BDH)

Acrylamide, C_3H_5NO (Merck)

Adenosine-5'-triphosphate potassium salt (ATP) (Sigma)

Agarose (Sekem)

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

Bacto agar (Difco)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Boric acid, BH_3O_3 (Merck)

Bromophenol blue (Merck, Germany)

Chloroform, $CHCl_3$

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

Dihydronicotinamide adenine dinucleotide (NADH), $C_{21}H_{27}N_7O_{14}P_2Na_2$ (Sigma)

Dimethyl sulfoxide (DMSO), C_6H_6SO (Amresco)

1,3-diazacyclopenta-2,4-diene (Imidazole), $HC_3H_3N_2$ (Sigma)

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

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

Ethidium bromide (Sigma)

Ficoll^{TM} 400 (Amersham)

Formaldehyde (BDH)

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

GeneRuler™ 100bp DNA ladder (Fermentus)

Glucose (Merck)

Glycerol, $C_3H_8O_3$ (BDH)

4-2-hydroxyethy-1-piperazineethanesulfonic acid (HEPES), $C_8H_{18}N_2O_4S$ (USB)

Isopropanol (Merck)

Kodak Tri-Xpan400 film

Magnesium Chloride, $MgCl_2$ (Merck)

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

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

Phenol:chloroform:isoamyl alcohol (Sigma)

Phospho(enol)pyruvic acid monopotassium salt (PEP-K), $C_3H_4KO_6P$ (Fluka)

Poly CG (Sigma)

Potassium chloride, KCl (BDH)

RNA markers (Promega)

Sodium acetate, CH_3COONa (Merck)

Sodium chloride, NaCl (BDH)

Sodium citrate, $Na_3C_6H_5O_7$ (Carlo Erba)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide, NaOH (Eka Nobel)

Sucrose (Merck)

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

Trizol reagent (Gibco BRL)

Xylene cyanol FF, $C_{25}H_{27}N_2O_6S_2Na$ (Sigma)

2.1.3 Bacterial strains

Escherichia coli strain XL-I blue

2.1.4 Kits and enzymes

HiYield™ Gel/PCR Mini Kit (RBC).

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

NucleoSpin® Extract II Kits (MACHEREY-NAGEL)

QIAprep spin miniprep kit (QIAGEN)

SMART™ RACE cDNA Amplification Kit (Clontech)

T & A Cloning vector Kit (RBC)

T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega)

Advantage® 2 Polymerase Mix (Clontech)

*Bam*HI (Biolabs)

*Eco*RI (Biolabs)

*Hin*dIII (Biolabs)

*Not*I (Biolabs)

Pyruvate Kinase/Lactic Dehydrogenase enzymes from rabbit muscle (Promega)

RNase A (Sigma)

RQ1 RNase-free DNase (Promega)

Taq DNA polymerase (Fermentus)

2.1.5 Software

BlastX (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)

Clustal X (Thompson, 1997)

GENETYX (Software Development Inc.)

SECentral (Scientific & Educational Software)

SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi.GENOMIC=1)

2.2 Shrimp samples

P.monodon juveniles (approximately 3 months old, about 15 to 20 g of body weight) were used for most studies. For RNAi experiments and determination of survival of shrimp under low salinity stress, small juveniles (approximately 1 to 1.5 months old, about 3 to 5 g of body weight) were used. All shrimps were from commercial farms located in Chuntaburi Province, Thailand. Shrimps were reared at salinity of 25 ppt and water temperature between 26 to 32 °C for two weeks in the laboratory prior to initiation of transfer experiments. Ten individual shrimps from each group were acclimatized at 3 ppt for 1 day and then investigated for the presence or absence of 14-3-3B mRNA by RT-PCR prior to conducting of experiments.

2.3 Tissue collection and total RNA preparation

Gill, epipodite, antennal gland, heart, hemocytes, hepatopancreas, intestine, lymphoid gland and eyestalk were isolated separately from an individual shrimp and immediately frozen in liquid nitrogen (-176 °C) in order to preserve the intact tissues. The samples were briefly homogenized by a pestle in 1 ml of ice-cold Trizol reagent (Gibco BRL). The homogenate was stored at room temperature for 5 to 10 min to permit complete dissociation of nucleoprotein complexes. After that, 200 µl of chloroform were added and vigorously shaken for 15 sec and incubated at room temperature for 3 min. The samples were centrifuged at 12,000xg for 15 min at 4 °C. The colorless upper aqueous phase containing total RNA was transferred to a new 1.5 ml microcentrifuged tube. Total RNA was precipitated by the addition of 500 µl of isopropanol. Then, the mixture was incubated at room temperature for 10 min and centrifuged at 12,000xg for 10 min at 4 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75 % ethanol. The RNA pellet was kept under 75 % ethanol until used. When required, the sample was centrifuged at 12,000xg for 15 min at 4 °C. The supernatant was removed. The RNA pellet was air dried about 30 min. The total RNA was dissolved with an appropriate amount of diethyl pyrocarbonate (DEPC) – treated water.

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

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

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

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

2.4 Formaldehyde-agarose gel electrophoresis

The quality of the extracted RNA was analyzed by formaldehyde-agarose gel electrophoresis as following described. A 1.2 % (w/v) formaldehyde agarose gel was prepared in 1x MOPS buffer (final concentration of 0.2 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0). The gel slurry was heated until completely dissolving and placed to cool down at room temp before formaldehyde (0.66 M final concentration) was added. Then, the melted formaldehyde-agarose gel was poured into a chamber set and applied the comb. The RNA marker and RNA samples were prepared under the denaturing condition. The RNA sample in DECP-treated water, 7.4 M of formamide, 1.64 M of formaldehyde, 1x MOPS and DECP-treated water to a final volume of 12 µl were heated at 70 °C for 10 min and the mixtures were immediately chilled on ice. After that, three microliters of the 5x RNA loading dye buffer containing 50 % (v/v) glycerol, 1 mM EDTA, pH 8.0, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF and 0.025 % (w/v) ethidium bromide was added to each sample and loaded to formaldehyde-agarose gel. Electrophoresis was run in 1x MOPS buffer at 100 volts 45 to 50 min. Sizes of RNA were visualized under a UV transiluminator by comparing with a standard RNA marker (Promega).

2.5 DNase treatment of total RNA samples

The obtained total RNA was further treated with RQ1 RNase-free DNase(Promega, 1 units/5 µg of the total RNA) at 37 °C for 30 min to remove the contaminating chromosomal DNA. Then, the RNA pellets were purified by phenol/chloroform extraction following by ethanol precipitation. Briefly, the reaction volume was adjusted to 40 µl with DEPC-treated water, 250 µl of Trizol reagent were added and vortex for 10 sec. Two hundred microliters of chloroform was then added and vigorously shaken for 15 sec. The resulting mixture was stored at room temperature for 2 to 5 min and centrifuged at 12,000xg for 15 min at 4 °C. The RNA in upper phase was precipitated by isopropanol and washed by 70 % (v/v) ethanol. After that, RNA pellet was briefly air-dried and dissolved with an appropriate amount of DEPC- treated water. The concentration of DNA-free total RNA was determined as described in 2.4

2.6 First-stranded cDNA synthesis

The first stranded cDNA was synthesized from 1 μg of total RNA using an ImProm-II™ Reverse Transcription system kit (Promega). Total RNA was combined with 0.5 μg of oligo (dT_{15}) primer and appropriate DECP-treated water in final volume of 0.5 μl. The reaction was incubated at 70 °C for 5 min and immediately placed on ice for 5 min. After that, 4 μl of 5x reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of dNTP Mix (10 mM each), 20 units of Ribonuclease inhibitor and 1 μl of ImProm-II reverse transcriptase were added and gently mixed. The reaction mixture was incubated at 25 °C for 5 min and at 42 °C for 60 min. Then, the reaction was incubated at 70 °C for 15 min to terminate reverse transcriptase activity. All cDNA samples were stored at -20 °C until ready for use.

2.7 Primer designation

PCR primers were designed based on nucleotide sequences of the selected EST clones using the SECentral program (Scientific & Educational Software). Each pair of upstream and downstream primers had closely similar Tm values, and they were checked for minimal self-priming and upper/lower dimer formation. A housekeeping gene, β-actin was used as an internal control (Table 2.1).

Table 2.1 Primer sequences, annealing temperatures and cycle numbers for semi-quantitative RT-PCR

Clone name	Primer sequence*	Annealing Temperature (°C)	Cycle	Product size (bp)
14-3-3A	F =5'CCACAAGGGCGGGATTTA 3'	57	25	267
	R =5'CTGGGTGCTCTGCCTATT 3'			
14-3-3B	F= 5'CGAAGAACTGCCTGGAGAGCCCTCAGC 3'	62	27[a], 33[b], 35[c]	402
	R= 5'GCAACTCCGGGGTCCTCATTGACT 3'			
β-actin	F=5' GCTTGCTGATCCACATCTGCT 3'	55	25	317
	R=5' ACTACCATCGGCAACGAGA 3'			

*F = Forward primer, R = Reverse primer

Table 2.1 (continued)

A= Cycle of amplification for response to salinity stress study of shrimp group II
B= Cycle of amplification for response to salinity stress study of shrimp group I
C= Cycle of amplification for tissue specific expression and checking the presence of 14-3-3B mRNA

2.8 Semi-quantification of 14-3-3 mRNA expression by RT-PCR

Semi-quantitative RT-PCR was used to examine tissue specific expression and expression pattern in response to salinity stress of two isoforms of 14-3-3 mRNA. Total RNA from shrimp tissues was extracted and then subjected to cDNA synthesis as described as above. The cDNA was then ready to use in RT-PCR assay using β-actin as the internal control. The primer sequences, annealing temperatures and cycle numbers for RT-PCR are shown in Table 2.1. Amplification reactions were performed in a 25 µl total volume containing 1 µl cDNA sample, 1x PCR buffer, 0.2 µM each dNTP, 0.8 µM each primer and 0.025 units *Taq* DNA polymerase (Fermentus). Cycle profile was a denaturation step of 95 °C for 2 min, followed by 25 or 35 cycles (depending on experiment) of 95 °C for 30 sec, optimum annealing temperature for 30 sec, 72 °C for 45 sec with a final extension phase of 72 °C for 5 min. The PCR amplification was carried out in a Mastercycler® PCR thermal cycler (Eppendorf, Germany). The reactions were performed in triplicated.

PCR products were determined by electrophoresis on 1.2 % agarose gels. Two percent (w/v) of agarose gel was prepared using 1x TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0). The slurry of agarose in TBE buffer was melted in microwave oven until completely dissolved. The solution was allowed to cool at 55 to 60 °C before pouring into a casting tray with a well comb. After hardening, the gel was submerged in a chamber containing an enough amount of 1xTBE buffer covering the gel for approximately 0.5 centimeters.

The PCR products were mixed with 2 µl of the 10x loading dye (0.25 % bromophenol blue and 25 % Ficoll in water) before loading into the well. A DNA ladder (100 bp marker) was used as standard DNA markers. Electrophoresis was carried out in 1 x TBE buffer at 100

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

The intensity of bands was measured and normalized relative to that of β-actin using the commercial image analysis software package (GeneSnap and GeneTools, SynGene). The data were then subjected to statistical analysis for comparison between groups.

2.9 Identification and characterization of the full-length cDNA of 14-3-3B

Rapid Amplification of cDNA ENDs (RACE) is used widely for the preparation of full-length cDNA. However using RACE technique was not successful to identify the complete 14-3-3B cDNA sequences. Thus we modify to identify the complete cDNA of 14-3-3B by amplification of *P. monodon* 14-3-3 with specific primers as described below.

Rapid amplification of cDNA ends (RACE)

2.9.1 First-stranded RACE-cDNA synthesis

The first-stranded RACE-cDNA was synthesized according to the manufacturer's instructions (Fig. 2.1). Briefly, mRNA (2 μg) obtained from shrimp gill were mixed with SMART II™ A oilgonucleotide and 5' -RACE CDS primer (for 5' RACE cDNA library). The mixtures were pre-heated for 10 min at 80 ^{o}C and immediately cooled on iced water for 10 min. The first strand cDNA was synthesized using PowerScript reverse transcriptase at 42 ^{o}C for 2 h in a hot-lid thermal cycle. The reaction contains 2 μl of 5x First-Strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl and 30 mM $MgCl_2$), 1 μl of 20 mM DTT, 1 μl of 10 mM dNTP Mix and 1 μl of BD PowerScript™ Reverse Transcriptase. Finally, the RACE cDNA were diluted with 250 μl of Tricine-EDTA buffer (10 mM Tricine-KOH, pH 8.5 and 1 mM EDTA and heated at 72 ^{o}C for 10 min. The obtained 5' - RACE cDNA were kept at -20 ^{o}C until used.

2.9.2 Primer designation

For 14-3-3B, the 5' gene-specific primer (5' RACE) was designed from EST sequence of a 14-3-3B from the gill-epipodite cDNA library of *P. monodon* (http://pmonodon.biotec.or.th) using SECentral program (Scientific & Educational Software). Primer sequences for RACE-cDNA synthesis and RACE-PCR were listed in Table 2.2. The gene-specific primers designed here produce overlapping RACE products as shown in Fig. 2.2.

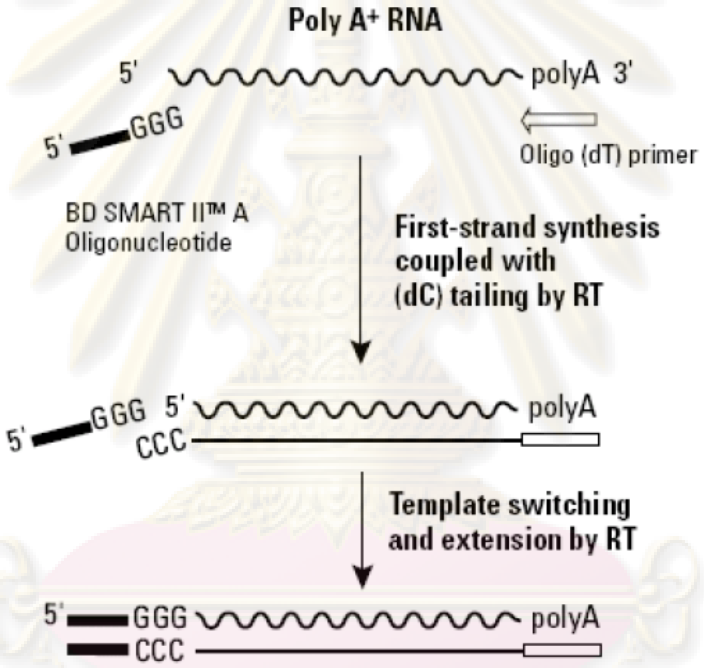


Figure 2.1 Principle of SMART™ RACE-cDNA syntheses (Clontech). First-stranded cDNA was synthesized using a modified oligo(dT) primer. The BD PowerScript™ reverse transcriptase exhibits terminal transferase activity after it reaches the end of the mRNA template, adding several dC residues to the 3' end of the first-strand cDNA. The BD SMART II A Oligonucleotide contains a terminal stretch of G residues that anneal to the dC-rich cDNA tail and serves as an extended template for BD PowerScript Reverse Transcriptase.

(Source: SMART™ RACE cDNA Amplification kit User Manual)

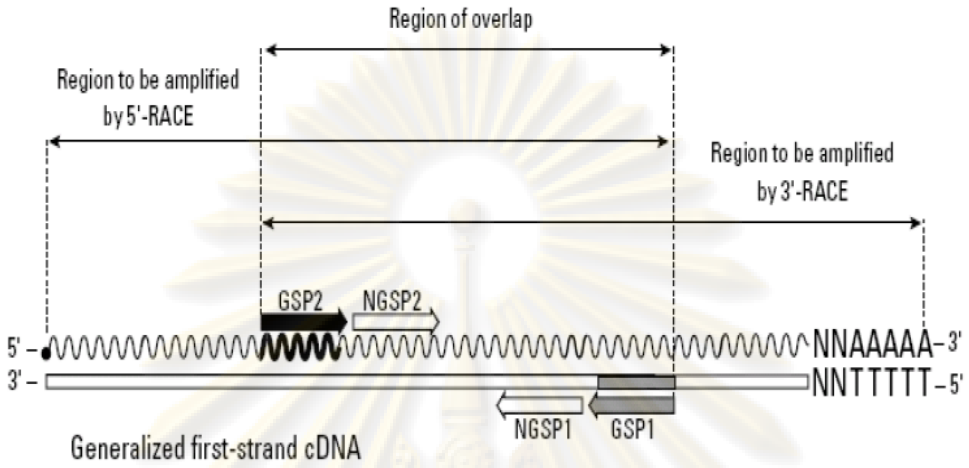


Figure 2.2 The location of gene specific primer within gene (Clontech)

(Source: SMART™ RACE cDNA Amplification kit User Manual)

Table 2.2 Primer sequences for RACE-cDNA synthesis and RACE-PCR

Primer name	Sequence	Tm (°C)
5' RACE		
5' gsp14-3-3BRACE-1	5' GCTGAGGGCTCTCCAGGCAGTTCTTCG 3'	74
Nested5' gsp14-3-3BRACE	5' GGTCAGCATTAGCGTTGACAACC 3'	66
5' gsp14-3-3BRACE-2	5' GCAACTCCGGGGTCCTCATTGACT 3'	70
Kits		
RACE-cDNA synthesis		
SMART II™ A Oligonucleotide	5' AAGCAGTGGTATCAACGCAGAGTACGCGGG 3'	
5'-RACE CDS	5' (T)25V N* 3'	
RACE-PCR		
10x Universal Primer A (UPM)	Long : 5' CTAATACGACTCACTATAGGGCAAGCAGTG GTATCAACGCAGAGT 3' Short : 5' CTAATACGACTCACTATAGGGC 3'	
Nested Universal Primer (NUP)	5' AAGCAGTGGTATCAACGCAGAGT 3'	

2.9.3 Rapid amplification of cDNA ends (RACE)-PCR

The 5' gsp14-3-3BRACE-1, 5' gsp14-3-3BRACE-2 and UPM primers were used for 5' RACE-PCR. The amplification reaction for GC-rich amplicon was performed using Advantage® 2 Polymerase Mix (Clontech) in the presence of 2.5 % DMSO. 5' RACE-PCR of 14-3-3B was performed in a 50 µl reaction volume containing 1x PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl, 0.001 % gelatin), 1.5 mM of $MgCl_2$, 200 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 2.5 % of DMSO, 0.4 µM of a RACE gene specific primer (gsp), 2.0 unit of Advantage® 2 Polymerase Mix (Clontech) or *Taq* DNA polymerase, 5 µl of 10x UPM and 2.5 µl of 5' RACE-Ready cDNA template. PCR conditions were as follows: five cycles consisting of 94 ^{o}C for 30 sec, 72 ^{o}C for 2 min; 5 cycles consisting of 94 ^{o}C for 30 sec, 65 ^{o}C for 30 sec and 72 ^{o}C for 2 min and 25 cycles consisting of 94 ^{o}C for 30 sec, 60 (Advantage® 2 Polymerase Mix) or 55 (*Taq* DNA polymerase) ^{o}C for 30 sec and 72 ^{o}C for 2 min. The nested PCR strategy was employed to increase specificity using NUP and nested gsp primers (Nested 5' gsp14-3-3BRACE primers for the second round of 5' gsp14-3-3BRACE1-2, respectively) as described in Table 2.2. Five microliters of the 50 fold diluted PCR product was used as the template for nested PCR under the conditions were as follows: 30 cycles consisting of 94 ^{o}C for 30 sec, 50 ^{o}C for 45 sec and 72 ^{o}C for 1 min (Fig. 2.3). The PCR products of the first and nested PCR were assessed by electrophoresis using 1x TBE buffer.

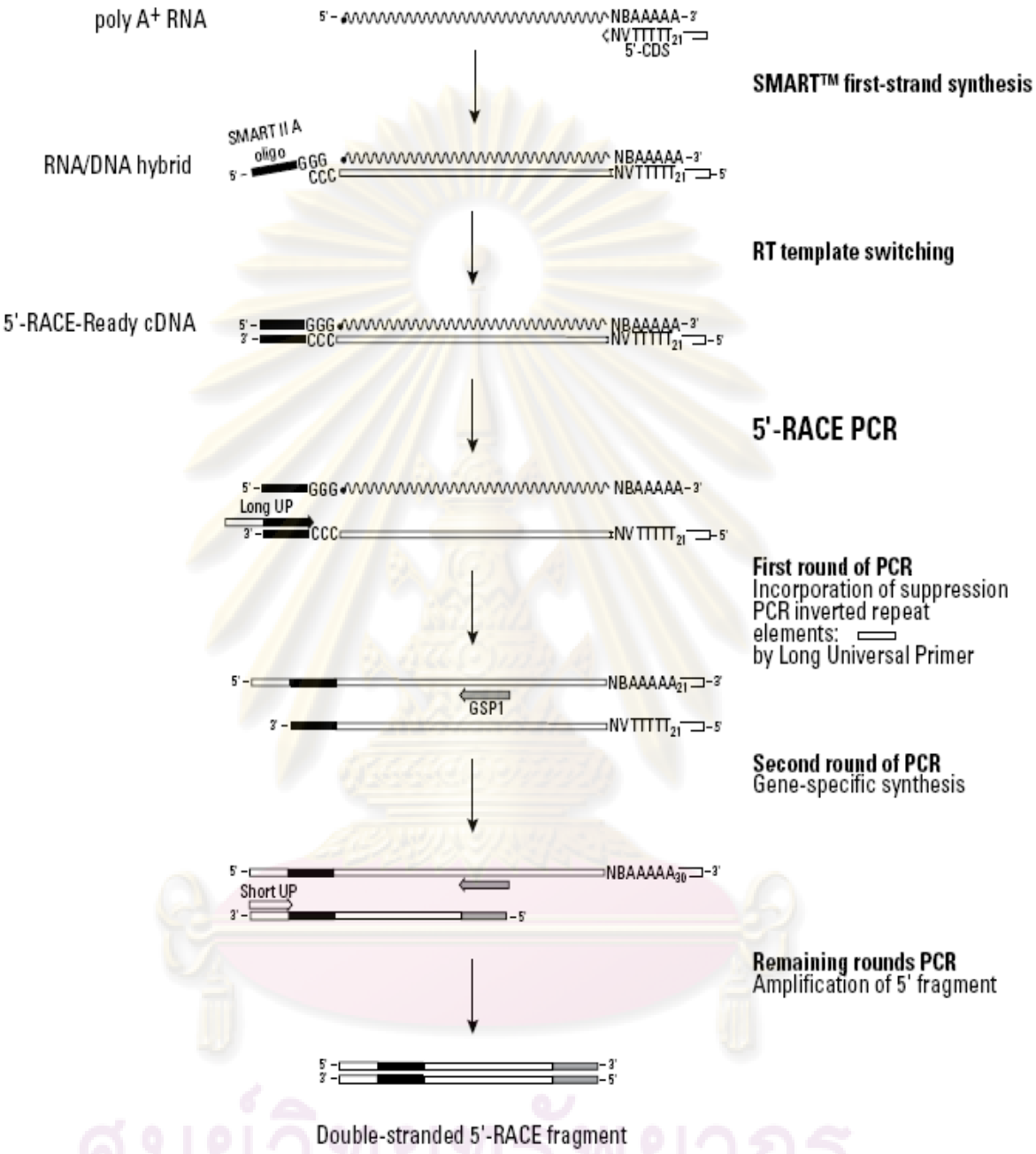


Figure 2.3 Overview of the BD SMART™ RACE procedure (Clontech) represents the mechanism of the 5'-RACE reactions.

(Source: SMART™ RACE cDNA Amplification kit User Manual)

2.9.4 Identification of the completed cDNA of 14-3-3B by using specific primers

2.9.4.1 First-stranded 14-3-3B specific cDNA synthesis

The first stranded cDNA was synthesized from 1 μg of total RNA using an ImProm-II™ Reverse Transcription system kit (Promega). Total RNA was combined with 0.5 μg of specific 14-3-3B primer (3' 14-3-3B-A) and appropriate DECP-treated water in final volume of 0.5 μl. The reaction was incubated at 70 °C for 5 min and immediately placed on ice for 5 min. After that, 4 μl of 5x reaction buffer, 2.6 μl of 25 mM $MgCl_2$, 1 μl of dNTP Mix (10 mM each), 20 units of Ribonuclease inhibitor and 1 μl of ImProm-II reverse transcriptase were added and gently mixed. The reaction mixture was incubated at 25 °C for 5 min and at 42 °C for 90 min. Then, the reaction was incubated at 70 °C for 15 min to terminate reverse transcriptase activity. All cDNA samples were stored at -20 °C until ready for use.

2.9.4.2 Primer designation

For this method, the 5' specific primer of 14-3-3B was A_{17} with adapter sequences and the 3' specific primer was designed from EST sequence of a 14-3-3B from the gill-epipodite cDNA library of *P. monodon* (http://pmonodon.biotec.or.th) using SECentral program (Scientific & Educational Software). *P. monodon* 14-3-3B deduced amino acid sequences showed the highest similarity to *Tetrahymena thermophila* B210 14-3-3 protein. Due to abundant A/T tracts at 5' UTR of *Tetrahymena thermophila* B210, A_{17} was used to amplify the *P. monodon* 14-3-3B at 5' UTR. Primer sequences for completed cDNA synthesis were listed in Table 2.3.

2.9.4.3 Amplification of 14-3-3B specific cDNA by PCR

The 5' UTR+Adaptor and 3' 14-3-3B-1 primers were used for PCR reaction. Amplification reactions were performed in a 50 μl total volume containing 4 μl cDNA sample, 1x PCR buffer, 0.2 μM each dNTP, 0.8 μM each primer and 0.025 units *Taq* DNA polymerase (Fermentus). Cycle conditions were as follows: 30 cycles

consisting of 94 ^{o}C for 45 sec, 50 ^{o}C for 45 sec and 72 ^{o}C for 1.5 min. The final extension was carried out at 72 ^{o}C for 10 min. The nested PCR strategy was employed to increase specificity using 5' Adaptor and 3' 14-3-3B-2 primers as described in Table 2.3. Five microliters of the 50 fold diluted PCR product was used as the template for nested PCR under the condition similar to the first PCR. The PCR products of the first and nested PCR were assessed by electrophoresis using 1x TBE buffer.

Table 2.3 Primer sequences for completed cDNA synthesis

Primer name	Sequence	Tm (°C)
5'specific primer		
5'UTR+Adaptor	5'GCCAGCCATCCAATAGTCAAAAAAAAAAAAAAAA3'	67
5'Adaptor	5' GCCAGCCATCCAATAGTCA 3'	52
3'specific 14-3-3B primer		
3'14-3-3B-1	5' GAGGTCAGCATTAG 3'	46
3'14-3-3B-2	5' TGGCATCGAGCATGTCCT 3'	55

2.9.4.4 Purification of PCR product from agarose gel

The expected bands were purified from agarose gel by NucleoSpin® Extract II Kits (MACHEREY-NAGEL) as describe below: The expected product was excised from the gel using a clean sharp scalpel, and then the weight of the gel slice was determined. The gel slice was completely dissolved in three volumes of NT buffer containing chaotropic salt at 60 °C. The sample was then loaded into the column and centrifuged at 12,000x g for 1 min to remove the supernatant. The column was washed with 500 µl of NT2 buffer and centrifuged as described above. Six hundred microliters of NT3 buffer were added into the column and centrifuged. The additional centrifugation was used for completely removal of the NT3 buffer containing ethanol. The column was placed into a clean 1.5 ml microcentrifuge tube. The DNA was eluted with 40 µl of elution NE buffer (5 mM Tris-Cl, pH 8.5) and stood at room temperature for 1 min before centrifugation. The eluted DNA was then stored at -20 °C until used.

2.9.4.5 Cloning of DNA fragment into T&A Cloning

The DNA fragment was ligated into T&A Cloning vector (Fig. 2.4). The T&A Cloning vector was 2,728 bp in length and had unique restriction sites in the multiple cloning region flanked by T7 RNA promoter, therefore M13 forward and reverse primers can be used to identify the recombinant clone. The reaction was composed of 5 µl of 1x Rapid A and B ligation buffer, 2 µl of T&A Cloning Vector (50 ng), proper amount of PCR product, 1 µl of T4 DNA ligase (3 Weiss units/µl), and deionized water to a final volume of 10 µl. The reactions were mixed by pipetting, briefly spun and incubated overnight at 4 °C. The appropriate amount of insert in the ligation reaction was calculated following equation:

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

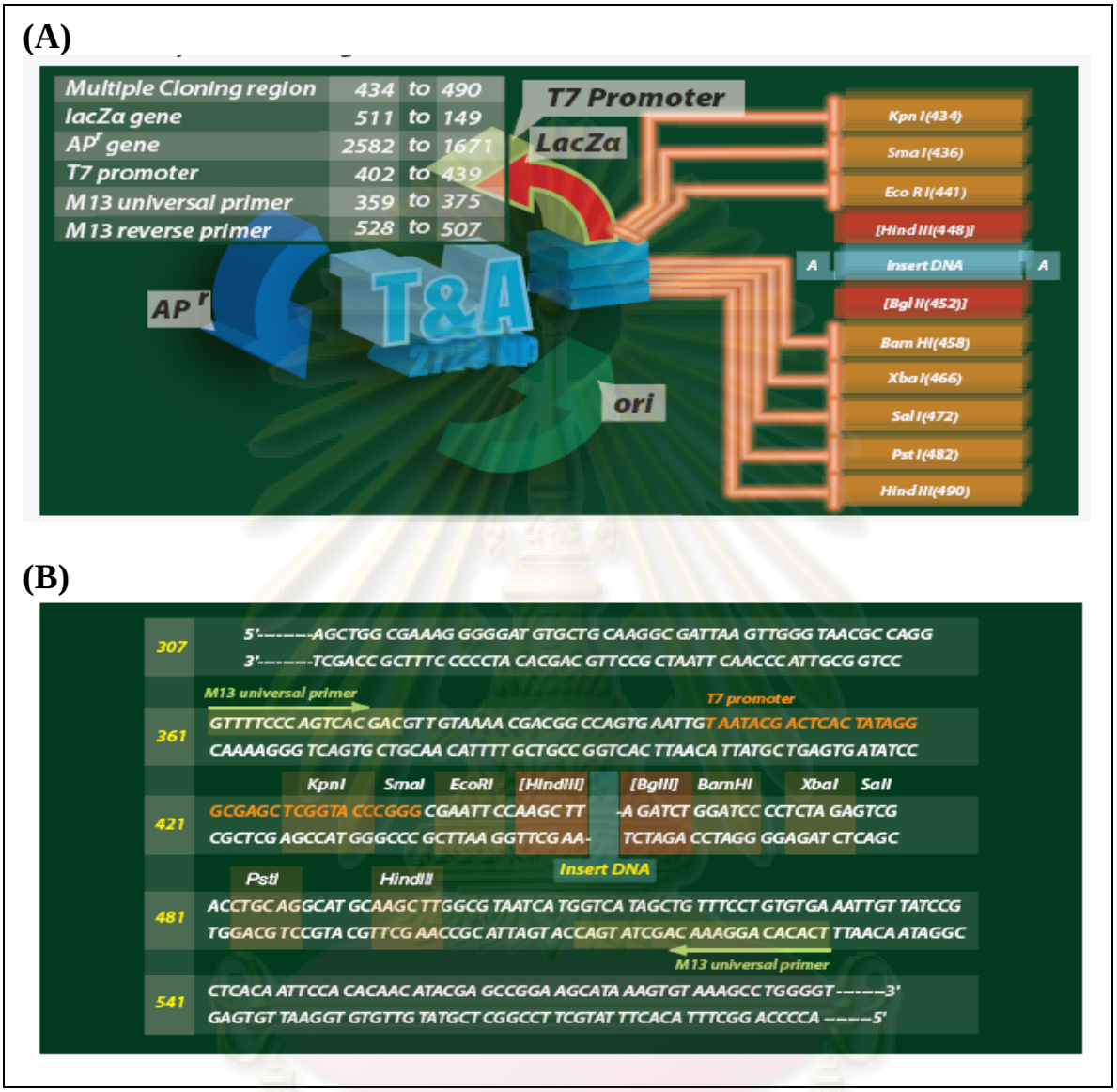


Figure 2.4 T&A Cloning vector map (A) and multiple cloning site sequences (B) (RBC)

(Source: T&A Cloning vector kit User Manaul)

2.9.4.6 Competent cells preparation

The starter of *E. coli* strain XL-1 blue was prepared from a single colony cultured in 10 ml of LB broth (1 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, and 1 % (w/v) NaCl) and cultured at 37 ^{o}C with shaking at 250 rpm overnight.

One percent of starter was inoculated into 1 L of LB broth and incubated at 37 ^{o}C with vigorous shaking for 3 to 5 hr until OD_{600} of the cells reached 0.5 to 0.7 Cells were then chilled on ice for 15 to 30 min and harvested by centrifugation at 5,000xg for 10 min at 4 ^{o}C. The supernatant was removed as much as possible. The cell pellet was washed twice time with cold sterilized water and followed by cold sterilized 10 % (v/v) glycerol with gently mixing and centrifugation. The pellet was suspended in cold sterilized 10 % (v/v) glycerol to a final volume of 2 to 3 ml. This cell suspension was divided into 40 µl aliquots and stored at -80 °C until used.

2.9.4.7 Electrotransformation

The ligation reaction was transformed to *Escherichia coli* XL-1 Blue. The competent cells were gently thawed on ice, mixed with 1 µl of ligation mixture and then placed on ice for 1 min. The mixture was transformed by electroporation in a cold 0.2 cm cuvette with setting the apparatus as follows: 25 µF of the Gene pulser, 200 Ω of the pulse controller unit, and 2.50 kV of the Gene pulser apparatus (Bio-RAD). After electroporation, SOC medium (2 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose) was immediately added to the cuvette and quickly resuspended cells. The cell suspension was transferred to a new tube and incubated at 37 °C with shaking at 250 rpm for an hour. One hundred microliters of transformant was then spread onto a LB agar plate containing 100 µg/ml of amplicillin, 20 µg/ml of X-gal and 30 µg/ml of IPTG and then incubated at 37 ^{o}C for overnight. After incubation, the recombinant clone was identified by colony PCR using universal M13 forward and reverse primers.

2.9.4.8 Screening of transformant by colony PCR

White colonies were picked and screened for the inserts by colony PCR. The amplification was carried out in a 20 µl reaction volume containing 1x PCR buffer, 200 µM of dNTP mix, 0.25 µM of M13 forward and reverses primers, and 2.5 units of *Taq* DNA polymerase (Fermentus). The single colony was diluted in 10 µl sterilized water. One microliter of colony suspension was employed as the template in

the PCR reaction. The PCR profile was performed at 94 °C for 3 min, 30 cycles at 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis.

2.9.4.9 Plasmid DNA extraction using QIAprep® miniprep kit

The plasmid was isolated from the positive clones by a QIAprep® Miniprep kits described in Qiagen's handbook. The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane under high salt condition. Firstly, bacterial cells were harvested by centrifugation and resuspend in 250 µl P1 buffer containing RNase A. Next, the 250 µl P2 buffer was added and mix thoroughly by inverting the tube 4–6 times for cell lyses. The cell lysate was neutralized by adding 350 µl N3 buffer. After maximum speed centrifugation for 10 min, the supernatant containing the plasmid was applied to column by pipetting. The column was centrifuged for 30–60 sec, and then the flowthrough was discarded. The QIAprep spin column was washed twice by adding 0.5 ml Buffer PB and 0.75 ml Buffer PE, respectively, and then centrifuged to remove residual ethanol from PE Buffer. Finally, the QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 µl EB buffer (10 mM Tris-HCl, pH 8.5) to the center of each column. After incubation at room temperature for 1 min, the eluted fraction was collected by centrifugation for 1 min.

2.9.4.10 Detection of the recombinant plasmid

The recombinant plasmid contained interested gene was examined with restriction enzyme digestion using *Eco*RI and *Bam*HI. The digested plasmid was analyzed by agarose gel electrophoresis. The size of DNA fragment was compared with standard DNA ladder (100 bp and 1kb ladder marker). The recombinant plasmid was sequenced by an automatic DNA sequencer at the Macrogen Inc.

2.9.4.11 DNA sequence analysis

DNA sequences were edited and translated using the GENETYX software program (Software Development Inc.). The sequences were further compared with data in the GenBank (http://www.ncbi.nlm.nih.gov) using the BlastX program (Altschul et al., 1997). The significant probabilities and identity were considered from E-values < 10^{-4} and the match included > 10 amino acid residues for BlastX (Supungul et al., 2004). Putative motifs and domains were investigated using SMART program. Related sequences were searched in GenBank and aligned using Clustal X program (Thompson et al., 1997). Aligned sequences were bootstrapped 1000 times using Seqboot. Sequence divergence between different 14-3-3 genes was calculated based on the two-parameter method using PRODIST (Kimura, 1980). Boostrapped neighbour-joining trees were constructed using Neighbour and Consense. All phylogenetic reconstruction programs are routine in PHYLIP (Felsenstein, 1993). Trees were appropriately illustrated using TreeView (http://taxonomy.zoology.gla.ac.uk/rod.html).

2.10 RNA interference

Oligonucleotide primers of *P. monodon* 14-3-3B gene were incorporated with T7 promoter sequences (italic) at the 5′ ends. To generate dsRNA, in vitro transcription was performed by T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega) (Fig. 2.5).

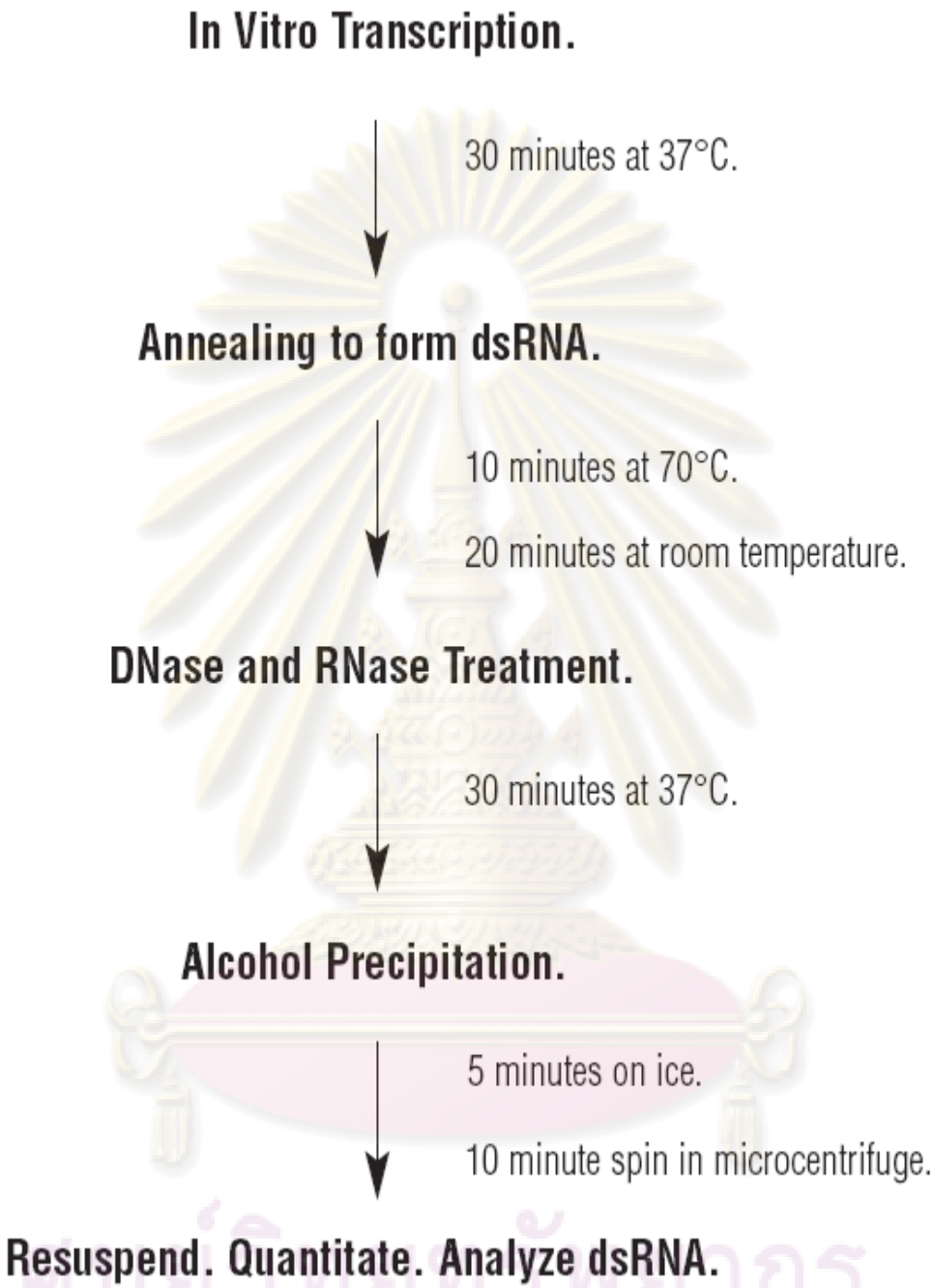


Figure 2.5 Outline of procedure for the production and purification of dsRNA using the T7 RiboMAX Express RNAi System.

(Source: T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega))

2.10.1. Producing PCR product templates

The approximate size of dsRNA recommended for RNAi analysis is 400 bp. In this experiment, dsRNA production requires a T7 RNA polymerase promoter at the 5´-ends of both DNA target sequence strands. The minimal T7 RNA polymerase promoter sequence requirement is: *5´-TAATACGACTCACTATAGGN* (17 to 22)-3´. Oligonucleotide primers of *P. monodon* 14-3-3B gene were incorporated with T7 promoter sequences (italic) at the 5′ ends. Generating the necessary two DNA templates requires four PCR primers were components 5′-T7 promoter sequence -CGAAGAACTGCCTGGAGAG-3′ with 5′-AACTCCGGGGTCCTCATTGACT-3′ ; 5′-T7 promoter sequence AACTCCGGGGTCCTCATTGACT-3′ with 5′-CGAAGAACTGCCTGGAGAG-3′ and two PCR amplifications (Fig. 2.6). Amplification reactions were performed in a 25 µl total volume containing 1 µl cDNA sample, 1x PCR buffer, 0.2 µM each dNTP, 0.8 µM each primer and 0.025 units *Taq* DNA polymerase (Fermentus). Cycle profile was a denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 sec, 62 °C for 30 sec, 72 °C for 45 sec with a final extension phase of 72 °C for 5 min. PCR products were examined by agarose gel electrophoresis before transcription to verify that a single PCR product of the expected size is generated. A single PCR product was purified by using NucleoSpin® Extract II Kits (MACHEREY-NAGEL) as describe below: The expected product was excised from the gel using a clean sharp scalpel, and then the weight of the gel slice was determined. The gel slice was completely dissolved in three volumes of NT buffer containing chaotropic salt at 60 °C. The sample was then loaded into the column and centrifuged at 12,000x g for 1 min to remove the supernatant. The column was washed with 500 µl of NT2 buffer and centrifuged as described above. Six hundred microliters of NT3 buffer were added into the column and centrifuged. The additional centrifugation was used for completely removal of the NT3 buffer containing ethanol. The column was placed into a clean 1.5 ml microcentrifuge tube. The DNA was eluted with 40 µl of elution NE buffer (5 mM Tris-Cl, pH 8.5) and stood at room temperature for 1 min before centrifugation. The concentration of eluted DNA was determined by measuring absorption at 260nm.

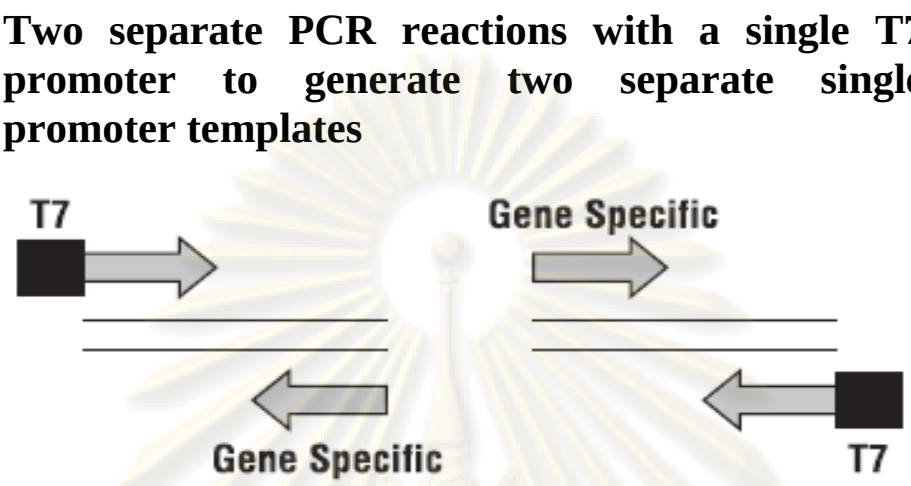


Figure 2.6 Strategy for adding T7 promoters to DNA templates by PCR.

(Source: T7 RiboMAX$^{(TM)}$ Express RNAi System (Promega))

2.10.2. Synthesizing large quantities of ssRNA

The DNA derived from 2.10.1 was used as template for synthesis of large quantites of ssRNA. The reaction contained 10 µl at the components of RiboMAX express T7 2x buffer, 1 to 8 µl of linear DNA template (~1 µg total), 2 µl of enzyme mix-T7 Express and nuclease-free water was added to the final volume of 20 µl. The reaction was stood at room temperature for 1 h.

2.10.3. Annealing dsRNA

To anneal the RNA strands, equal volumes of complementary RNA reactions were mixed together and incubated at 70 °C for 10 minutes, and then the reaction was slowly cooled to room temperature (~20 min). This allows annealing of the double-stranded RNA. The supplied RNase solution was diluted 1:200 by adding 1 µl RNase solution to 199 µl Nuclease-Free Water. Freshly diluted RNase solution (1 µl) and 1 µl RQ1 RNase-Free DNase per 20 µl reaction volume were mixed, and incubated for 30 min at 37 °C. This will remove any remaining single-stranded RNA and the template DNA, leaving dsRNA.

2.10.4. Purifying dsRNA

The dsRNA derived from 2.10.4 was added with 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol or 2.5 volumes of 95 % ethanol. The reaction was mixed and placed on ice for 5 min, Spun at top speed in a microcentrifuge for 10 min. A white pellet was washed with 0.5 ml of cold 70 % ethanol, air-dried and resuspended in nuclease-free water. The dsRNA was stored at 20 °C or 70 °C.

2.10.5. Determining RNA concentration and visualizing by gel ectrophoresis

The dsRNA concentration was quantitated by ultraviolet light absorbance at a wavelength of 260 nanometers. The DNase-treated dsRNA transcript was examined by native gel electrophoresis (1.2 %) using 1x bromophenol blue loading dye to determine the accuracy of the A_{260} quantitation and the integrity of the dsRNA. The separated dsRNA was visualized by staining the gel in 0.5 mg/ml ethidium bromide.

2.11 Shrimp injection

Small intermolt shrimps (3 to 5 g of body weight) were used for *in vivo* RNAi experiments. The experimental shrimps were injected with 10 µg of 14-3-3 dsRNA whilst the control shrimp received dsRNA Poly CG (Sigma) or 150 mM NaCl. Poly CG was double-stranded homopolymer used for *in vivo* internal control. Gills of experimental and control shrimps were excised and collected 1 to 7 days after dsRNA injection for determination of gene silencing efficiency and total ATPase activity. The 14-3-3 gene silencing efficiency was determined by RT-PCR with the same condition for semi-quantification of 14-3-3 mRNA except that the amplification cycle was repeated 40 times.

2.12 Enzymatic activity determination of ATPase

Gills tissue was homogenized in 5 volumes of buffer (20 mmol L^{-1} imidazole buffer, pH 6.8, containing 250 mmol L^{-1} sucrose, 6 mmol L^{-1} EDTA and the protease inhibitor cocktail). The homogenates were centrifuged at 10,000×*g* for 35 min at 4 °C and keep on the supernatant. Protein content was estimated by Bradford's procedure (BioRad, catalog no. 500–0002), using BSA as a reference.

ATPase activity was assayed at 25 ^{o}C using the PK/LDH linked system in which the hydrolysis of ATP is coupled to the oxidation of NADH. The oxidation of NADH was monitored at 340 nm. Standard conditions were: 50 mM Hepes buffer, pH 7.5, containing 2 mM ATP, 3 mM $MgCl_2$, 10 mM KCl, 100 mM NaCl, 0.14 mM NADH, 2.0 mM PEP, 82 µg PK (49 U) and 110 ug LDH (94 U) in a final volume of 1 ml. Controls without added enzyme were included in each experiment to quantify the non-enzymatic hydrolysis of substrate. The specific activity *A* (international units IU per mg of protein) was calculated according to $A=\Delta A_{340}/6.2m_{prot}$, where ΔA_{340} is the change in NADH absorbance at 340 nm per min and m_{prot} is the weight of protein in mg in the assay volume. The values of *A* was the mean values from triplicate experiments. The data were then subjected to statistical analysis for comparison between groups**.**

2.13 Survival of salinity stressed shrimp

The overall objective of this test was to compare the mortality rate of shrimps that expressed and not expressed of 14-3-3B mRNAs when they were stressed by low salinity water. Shrimps were reared at salinity of 40 ppt for two weeks in the laboratory and then transferred abruptly to low salinity at 3 ppt. The mortality rate was observed for 4 days following the transfer.

2.14 Statistical analysis

The significance of the difference between the two sample groups was determined using a two-tailed, independent sample t-test**.** The significance of the difference between 3 or more group samples was determined by one-way ANOVA followed by a post hoc test (Duncan's new multiple range test). Statistical package, SPSS-PC^{+} (SPSS Inc) was used for statistical analyzes. A P value of less than 0.05 was considered statistically significant. Values were expressed as means±S.D.

CHAPTER III

RESULTS

3.1 Tissues and total RNA preparation

Gill, epipodite, antennal gland, heart, hemocytes, hepatopancreas, intestine, lymphoid gland and eye stalk of shrimp were collected for total RNA isolation. The A_{260}/A_{280} ratio of total RNA samples were 1.5 to 1.8 which indicated acceptable quality of total RNA. The average quantity of total RNA obtained from different tissues ranged from 24 to 90 µg per individual shrimp (depending on tissue). The quality of total RNA was also monitored by running on a denaturing formaldehyde/agarose/EtBr gel. Each tissue revealed a predominant band of 18S rRNA (1.9 kb) as shown in Fig. 3.1.

3.2 Identification and characterization of the full-length cDNA of 14-3-3

Two isoforms of 14-3-3, namely 14-3-3A and 14-3-3B, were identified from the *Penaeus monodon* EST database (http://pmonodon.or.th). The full length sequence of 14-3-3A was already obtained from the database whereas that of 14-3-3B was a partial cDNA sequence. The nucleotide and deduced amino acid sequences of 14-3-3A and 14-3-3B are shown in Figs. 3.2 and 3.4 respectively. Sequence analysis of 14-3-3A and 14-3-3B using BLAST X program is shown in Figs. 3.3 and 3.5 respectively. The full length cDNA of 14-3-3A is 2,866 bp. Eighty-one of which are 5' UTR, while the coding sequence (CDS), including stop codon, is 741 nucleotides long, and the 3' UTR is 2,044 bp with a poly (A) tail (Fig. 3.2). The CDS encodes a protein of 246 amino acid residues. Sequence comparison showed that the deduced amino acids of *P. monodon* 14-3-3A had 100 % identity to *P. monodon* 14-3-3 protein previously submitted to the GenBank (acc. no. AAY56092.1)(Fig. 3.3).

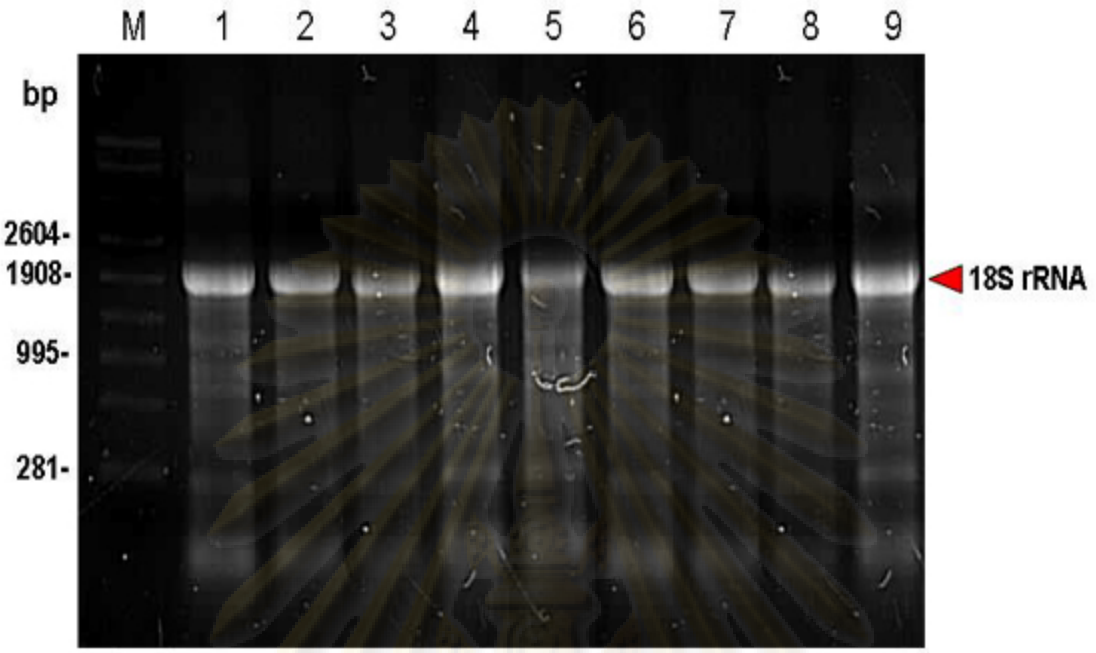


Figure 3.1 Total RNA isolated from various tissues of *P. monodon*

Approximately 15 µg of total RNA from different shrimp tissues were electrophoresed on a 1.2% formaldehyde agarose gel.

Lane M : RNA marker

Lane 1 : Total RNA from gill

Lane 2 : Total RNA from epipodite

Lane 3 : Total RNA from antennal gland

Lane 4 : Total RNA from heart

Lane 5 : Total RNA from hemocytes

Lane 6 : Total RNA from hepatopancreas

Lane 7 : Total RNA from intestine

Lane 8 : Total RNA from lymphoid gland

Lane 9 : Total RNA from eye stalk

```
1     GTTGGTCGAGTGAGGGTCGCTCTGCCCTGGGATATACCTTTATATTTGTGTAAATAGCAC
61    CTATTTTATAAAACAGACACAATGTCGGACAAGGAAGAACAAGTACAGCGGGCCAAGCTT
                          M  S  D  K  E  E  Q  V  Q  R  A  K  L
121   GCTGAGCAGGCAGAGAGGTACGATGATATGGCAGCAGCCATGAAACAGGTCACAGAAACT
      A  E  Q  A  E  R  Y  D  D  M  A  A  A  M  K  Q  V  T  E  T
181   GGCGTCGAGCTGTCAAATGAGGAACGTAATCTTTTGTCAGTAGCCTACAAGAATGTCGTG
      G  V  E  L  S  N  E  E  R  N  L  L  S  V  A  Y  K  N  V  V
241   GGAGCTCGGAGAAGTTCCTGGCGAGTAATTTCATCCATAGAACAGAAGACAGAAGGTTCA
      G  A  R  R  S  S  W  R  V  I  S  S  I  E  Q  K  T  E  G  S
301   GAGCGAAAACAACAAATGGCAAAGGAATACAGAGAGAAGGTCGAAACAGAACTTAGGGAA
      E  R  K  Q  Q  M  A  K  E  Y  R  E  K  V  E  T  E  L  R  E
361   ATCTGCCAGGATGTATTGGGTCTCCTCGACAAGTTCCTTATTCCCAAAGCCTCCAACCCT
      I  C  Q  D  V  L  G  L  L  D  K  F  L  I  P  K  A  S  N  P
421   GAATCTAAGGTCTTCTACCTGAAGATGAAGGGAGATTATTACAGGTACCTTGCTGAAGTC
      E  S  K  V  F  Y  L  K  M  K  G  D  Y  Y  R  Y  L  A  E  V
481   GCCACCGGTGATGCGCGAGCGGGCGTAGTGGACGACTCTCAGAAGTCATACCAGGAGGCT
      A  T  G  D  A  R  A  G  V  V  D  D  S  Q  K  S  Y  Q  E  A
541   TTCGACATCGCCAAGGCAGAGATGCAGCCCACCCACCCCATCAGGCTGGGTCTGGCGCTC
      F  D  I  A  K  A  E  M  Q  P  T  H  P  I  R  L  G  L  A  L
601   AACTTCTCCGTCTTCTTCTACGAGATCCTCAACTCGCCTGATAAGGCTTGTCAGCTAGCG
      N  F  S  V  F  F  Y  E  I  L  N  S  P  D  K  A  C  Q  L  A
661   AAACAGGCATTCGACGACGCTATTGCGGAGTTGGATACGCTAAATGAAGACTCGTACAAG
      K  Q  A  F  D  D  A  I  A  E  L  D  T  L  N  E  D  S  Y  K
721   GATTCTACGCTCATAATGCAGCTCTTGCGAGATAACTTGACACTTTGGACGAGTGACACG
      D  S  T  L  I  M  Q  L  L  R  D  N  L  T  L  W  T  S  D  T
781   CAGGGCGAAGGAGACGAAGCTAACGAGGGCGACCAAAACTGATGAACGCAACCCCTTTTA
      Q  G  E  G  D  E  A  N  E  G  D  Q  N
841   GCAGGCTCCACTCGACCCTCCACACTCCTACCTACCAGTCATGATGCCTGAAGGGTCGAC
901   CATTCCTCACAGCTCACTCTTCACACCGCCAGCGTGGCTACAGTAGTCGAGGCTACGGCT
961   GGGCGCTCCTCAGTGACGCTACGCTCACCACCTCGATACTCCCGGCCGCCTCACCACCGC
1021  ATCAACGGTCGTCCCTCGGCCTCTCTGCTGCCTCAAATGAACATCACCTCGAGGAAGTCA
1081  AAGCTCGAGTAGACATCAATTCTTTCCCAGTGCATTTGTGACTCACCATTGAGGAGCAGT
1141  CCCGGCTGTTAGGTTAACAATCGTGCACAGGCTACCCGGCGAGTGTGATTGTCGCAGCTC
1201  TTTGCCACCATCTCGAAAGAGACTGTGAAGTGAATATGACAGTGGGATCTTCTCTCTGGA
1261  ACAACATTGAGAGACAATTTTGATAGTGTGAAATCAACGCTTGGGTCGGCTGGTCGTTTC
1321  TGTTGGGGCGGCCAGCCAGACTGCATTACTTATTACTGCTAAATGAACCACTTAGCATTT
1381  TGTACTTCCCATGGGGTCGGGCTCCCTCGGTCCCGCCTCTTTGTATGTAACGTTGCGTAC
1441  CGACAGTCAAGAGGACAAACCAACAAGTATGAAGAGATTGTTACTGTATTAACCGGTGGA
1501  TCATGTGGTTGTAGCAAGCAAGATTTGTCTCCTTGATTTGGATAAAATTTGACCCCCAGA
1561  GCTAAATCGATAAGAAATTGTTTTTGAAGTTGGTAAATAAGATATTCGCCTTTAATGTTA
1621  CAGCCCATGAACACACTGTACGTTTAAGATTAATACTGTATTACTTATTGGTTTATTAGA
1681  TGTGACCCCTTCAAAATTAAGCTGATTTTCTGTTATGTCGTGGCCAGTTGGTGTCTACAA
1741  GATCAATAATAATGCGGTAGATATTGACTTTGTTGGATGTAAGCATGGAGTAATGATGGA
1801  AAATGATTACTTTATTTTCATTTTTTTCACGTCGCTTTTGAGAATGATCGGCGGTCAGGT
1861  GAGTCAGAACAGGCATGGGAAGCAATATTGATTAGAATAACTTTCACCACAAGGGCGGGA
1921  TTTAATCGGCTACTCATTTTACCATCTTCAAATAGACCCCATTGGCCAAAAGAGATCCGA
1981  AATTGGACTACCATCACCAACAGGGGAGAGCGAGATCAAAGGTACTAAGCGGAACACTCA
2041  CGCCAGTCCTCCCACCTCTCCTGACGCCGAGAGTTCTCATTTGCTTTATTTGAAGACCAG
2101  TGAACTGAGGAGCTTTGTTTTCTCTGGGTTTGTACTTATAATCACGATGATTTGTAATAG
2161  GCAGAGCACCCAGCGAGGGAGGGAGAAGTACTATCAGTGCGTCTCTCTATTGCCTAGCCA
2221  ACGGTATTCAGTAAAATAAAATACAAAAAAAACAATGAATGGTATTAGATATTTCTCATT
2281  CATTTTGTCATCAGCATTAATGGTAACCAAACACCTCCCCCGTAACAATTAATATTGGTC
2341  AAATCTAAAACAATAATAGTCACAACCATCTGTGCTTGAAATTGACTGCCAAATGTGAAA
2401  TGTATTAAGAAAGCGTAAAAATGTTTTGGTGGTAGTTTAGATCGTCAAAATTTAACTTTA
2461  AAAAAAAACTCTCGCCCCTTTGTCAAAATTGCTTTCCTAAATCAACGTAATAAACGTTTC
2521  ATATATATGTTGTTGTTTCCACAAACCTTTTTGTCTGTACTGTTTCAAATAAAACTTCAA
2581  GATAGTGCAGCATAACACTACTTTTACATCGCCTGAGAAATCGTCACACTTGCAGACCCC
2641  ACACACAGTAAGTGAAGGAAAATATATAGTATTAGTGTCAAGGTATAAAAAAGAGCACAA
2701  GTTTATAATTTGTGCTAATTAAGAGGCTTAAAAAATACTGTACAAACTACTTTGGAAGTA
2761  AACACCTTGTTTTCATAATACAAATGTTATATCTGATTGTAGCCGATCCCATGAAGGCAA
2801  TAGAAAATAATAAATTTAAACTGATAAATAAGAAAAAAAAAAAAAA
```

Figure 3.2 The full length nucleotide (above) and deduced amino acid (below) sequences *P. monodon* 14-3-3A. The stop codon, putative polyadenylation signal (AATAAA) are in bold and underlined.

```
>   gb|AAY56092.1|  14-3-3-like protein [Penaeus monodon]
Length=246

 Score =  507 bits (1305),  Expect = 3e-142, Method: Compositional matrix
adjust.
 Identities = 246/246 (100 %), Positives = 246/246 (100 %), Gaps = 0/246 (0 %)

Query  1    MSDKEEQVQRAKLAEQAEGYDDMAAAMRQVTETGVELSNEERNLLSVAYKNVVGARRSSW  60
            MSDKEEQVQRAKLAEQAEGYDDMAAAMRQVTETGVELSNEERNLLSVAYKNVVGARRSSW
Sbjct  1    MSDKEEQVQRAKLAEQAEGYDDMAAAMRQVTETGVELSNEERNLLSVAYKNVVGARRSSW  60

Query  61   RVISSIEQKTEGSERKQQMAKEYREKVETELREICQDVLGLLDKFLIPKASNPESKVFYL  120
            RVISSIEQKTEGSERKQQMAKEYREKVETELREICQDVLGLLDKFLIPKASNPESKVFYL
Sbjct  61   RVISSIEQKTEGSERKQQMAKEYREKVETELREICQDVLGLLDKFLIPKASNPESKVFYL  120

Query  121  KMKGDYYRYLAEVATGDARAGVVDDSQKSYQEAFDIAKAEMQPTHPIRLGLALNFSVFFY  180
            KMKGDYYRYLAEVATGDARAGVVDDSQKSYQEAFDIAKAEMQPTHPIRLGLALNFSVFFY
Sbjct  121  KMKGDYYRYLAEVATGDARAGVVDDSQKSYQEAFDIAKAEMQPTHPIRLGLALNFSVFFY  180

Query  181  EILNSPDKACQLAKQAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDTQGEGDEA  240
            EILNSPDKACQLAKQAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDTQGEGDEA
Sbjct  181  EILNSPDKACQLAKQAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDTQGEGDEA  240

Query  241  NEGDQN  246
            NEGDQN
Sbjct  241  NEGDQN  246
```

Figure 3.3 Sequence analysis of 14-3-3A from *Penaeus monodon*. The BLAST X resulted showed the highest sequence identity (100 %) of *P. monodon* 14-3-3A protein with the 14-3-3-like protein from *P. monodon* previously reported (acc. no. AAY56092.1).

A partial cDNA sequence of *P. monodon* 14-3-3B contains 766 bp including stop codon but missing start codon at the N-terminus. The 14-3-3B partail cDNA encodes a novel 14-3-3B protein which showed 71 % identity to 14-3-3 protein from *Tetrahymena thermophila* SB210 (Figs. 3.4 and 3.5). In order to obtain the full-length cDNA of 14-3-3B a Rapid Amplification of cDNA ENDs (RACE) technique and amplification of *P. monodon* 14-3-3 with specific primers were carried out.

```
1    GGCACGAGGGACGACTTCATCTACATGGCCAAGATCACTGAGCAGACTGAGAGGTTTGAG
     G  T  R  D  D  F  I  Y  M  A  K  I  T  E  Q  T  E  R  F  E
61   GACATGCTCGATGCCATGAACAAGGTTGTCAACGCTAATGCTGACCTCAGCGTTGAAGAA
     D  M  L  D  A  M  N  K  V  V  N  A  N  A  D  L  S  V  E  E
121  AGAAACTTATTGTCGGTGGCCTATAAAAACACTATTGGCTCCCGAAGAACTGCCTGGAGA
     R  N  L  L  S  V  A  Y  K  N  T  I  G  S  R  R  T  A  W  R
181  GCCCTCAGCTCCATTGAGAAGAAGGAGGAACAAAAGGGATCCAAGAACCTCCCTCTCCTC
     A  L  S  S  I  E  K  K  E  E  Q  K  G  S  K  N  L  P  L  L
241  AAGGGATACAAGAGCAAGATCGAAGGTGAGCTCAACAGATACTGCAACGAGATCCTCAAT
     K  G  Y  K  S  K  I  E  G  E  L  N  R  Y  C  N  E  I  L  N
301  CTCATTGACTCTCAGCTTATCGGAAAAGCCTCCAACGATGAAGCCAAGGTCTTCTATCAC
     L  I  D  S  Q  L  I  G  K  A  S  N  D  E  A  K  V  F  Y  H
361 AAGATGAAGGGAGATTACTACCGCTATATTTCTGAATACACCTCCGGTGAAGCCAGAAAG
     K  M  K  G  D  Y  Y  R  Y  I  S  E  Y  T  S  G  E  A  R  K
421  AAAGCTGGTGAAGATGCTCACGGTGCCTACAAAGCTGCCACCGCCATCGCCGAAGAAAAG
     K  A  G  E  D  A  H  G  A  Y  K  A  A  T  A  I  A  E  E  K
481  CTGAAGACCACCCACCCAATCAGGCTCGGCCTCGCCCTCAACTACTCCGTCTTCTATTAC
     L  K  T  T  H  P  I  R  L  G  L  A  L  N  Y  S  V  F  Y  Y
541  GAAGTCAATGAGGACCCCGGAGTTGCCTGCCAACTTGCCAAAAAGGCCTTCGATGATGCC
     E  V  N  E  D  P  G  V  A  C  Q  L  A  K  K  A  F  D  D  A
601  ATTGCTGACATTGATCAAATCGAAGAAGATCAATACAAGGATGCCACCACCATTATGCAG
     I  A  D  I  D  Q  I  E  E  D  Q  Y  K  D  A  T  T  I  M  Q
661  CTCATTCGAGACAACTTGACTCTCTGGACTTCTGAGCTCGAAGAAGGAGGTGACGAATGA
     L  I  R  D  N  L  T  L  W  T  S  E  L  E  E  G  G  D  E
721  TCGTCTTTATAAGGCATATATATATATAAAAAAAAAAAAAAAAAAT
```

Figure 3.4 The partial nucleotide (above) and deduced amino acid (below) sequences *P. monodon* 14-3-3B. The stop codon is in bold and underlined.

```
gb|EAR89562.2|  14-3-3 protein [Tetrahymena thermophila SB210]
Length=250

 Score =  301 bits (772),  Expect = 3e-80
 Identities = 163/229 (71 %), Positives = 193/229 (84 %), Gaps = 0/229 (0 %)
 Frame = +1

Query  4    TRDDFIYMAKITEQTERFEDMLDAMNKVVNANADLSVEERNLLSVAYKNTIGSRRTAWRA  183
            TR++ IY+AKI+EQTERFEDML+ M +VV    +LSVEERNLLSVAYKNT+GSRRTAWRA
Sbjct  5    TREELIYLAKISEQTERFEDMLENMKQVVKIEQELSVEERNLLSVAYKNTVGSRRTAWRA  64

Query  184  LSSIEKKEEQKGSKNLPLLKGYKSKIEGELNRYCNEILNLIDSQLIGKASNDEAKVFYHK  363
            +SSIE+KEE KGSK++ LLK YK KIEGEL+ +CN+IL L+D+ LI K++++EAKVF+ K
Sbjct  65   ISSIEQKEESKGSKHISLLKDYKKKIEGELSNFCNDILELLDNHLISKSNSNEAKVFFLK  124

Query  364  MKGDYYRYISEYTSGEARKKAGEDAHGAYKAATAIAEEKLKTTHPIRLGLALNYSVFYYE  543
            MKGDYYRYI+EY SG    +A   A  AYK A+ IA ++L TTHPIRLGLALN+SVFYYE
Sbjct  125  MKGDYYRYIAEYASGNTHDEAANGALQAYKQASDIATKELNTTHPIRLGLALNFSVFYYE  184

Query  544  VNEDPGVACQLAKKAFddaiadidqieedqYKDATTIMQLIRDNLTLWT  690
            V  DP  AC LAK+AFDDAIADI+ IEEDQYKDATTIMQLIRDNLTLWT
Sbjct  185  VMNDPTKACNLAKQAFDDAIADIEHIEEDQYKDATTIMQLIRDNLTLWT  233
```

Figure 3.5 Sequence analysis of *P. monodon* 14-3-3B. The BLAST X resulted showed the highest sequence identity (71 %) of *P. monodon* 14-3-3B protein with the sequence of 14-3-3 protein from *Tetrahymena thermophila* SB210 (acc. no. XP_001009807.2).

3.2.1 Rapid Amplification of cDNA ENDs (RACE)

A partial sequence of 14-3-3B cDNA from EST library already contains the stop codon. Thus, the 5' RACE-PCR was performed to obtain the complete CDS including a start codon. However, 5' RACE-PCR using 5' gsp14-3-3BRACE-2 and UMP primers failed to amplify the 5' end cDNA. We modified condition of RACE-PCR by synthesis new first-stranded RACE-cDNAs and used them as templates for amplification of 14-3-3 gene by RT-PCR before used in 5' RACE-PCR. The result shows that the 14-3-3B PCR product was detected from the new first-stranded RACE-cDNA (Fig. 3.6). 5' RACE-PCR conditions were then adjusted by increasing the template cDNA from 50 ng to 100 ng and decreasing the annealing temperature from 65 ^{o}C to 55 ^{o}C. A single band was achieved in the amplification reaction of the 14-3-3B using Nested 5' gsp14-3-3BRACE and NUP primers. The size of the amplicon was approximately 200 bp (Fig. 3.7). The amplified product was then cloned and sequenced. Although a partial cDNA sequence of 14-3-3B was obtained from 5' RACE, the obtained cDNA sequence did not extend to start site further than the sequence previously identified from the *P. monodon* database (Fig. 3.7). Thus, the complete cDNA sequence of 14-3-3B was alternatively isolated by amplification of the first-stranded cDNA with specific primers.

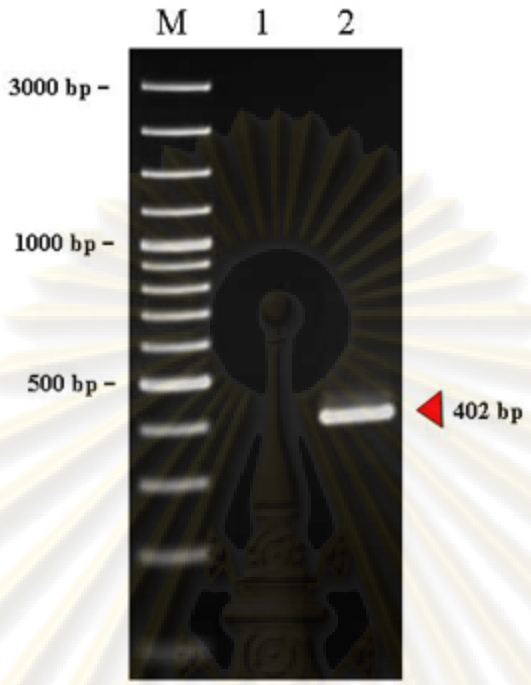


Figure 3.6 Detection of 14-3-3B gene from the first-stranded RACE-cDNA. The amplification product was electrophoresed on a 1.2 % agarose gel. Lane M; 100 bp DNA marker, lane 1; negative control with no cDNA template, lane 2; PCR product from amplification of the first-stranded RACE-cDNA template

Figure 3.7 The 5' RACE-PCR of *P. monodon* 14-3-3B. PCR products were electrophoresed on a 1.2 % agorose gel. Lane 1; Nested PCR product from amplification of primary PCR product template using Nested 5' gsp14-3-3BRACE and NUP primers, lane M; 100 bp DNA marker

3.2.2 Amplification of *P. monodon* 14-3-3B using specific primers

Deduced amino acid sequence of *P. monodon* 14-3-3B showed the highest sequence similarity to *Tetrahymena thermophila* B210 14-3-3 protein. Due to abundant A/T tracts at 5' UTR of *Tetrahymena thermophila* B210, A_{17} was used to amplify the *P. monodon* 14-3-3B at 5' UTR. The first amplification of 14-3-3B using A_{17} with adaptor and 3' 14-3-3B-1 primer derived from the 14-3-3B cDNA sequence of *P. monodon* showed smear pattern. Then the PCR product from the first amplification was used as template for the second amplification using the nested primers of 3' 14-3-3B-2. Two PCR bands with the size of about 130 and 200 bp were observed from the second amplification.

These expected bands were purified and transformed to T&A cloning vector kit (RBC)(Fig 3.8). Comparison of PCR sequences obtained from the second amplification showed that the sequences of PCR bands with size 130 and 200 bp were unmatched with any sequence in the GenBank database using the BlastX program (data not shown). Thus a full length sequence of *P. monodon* 14-3-3B cDNA was unsuccessfully isolated, although, several conditions were tried. Nevertheless, bassed on the sequence of 14-3-3 protein of the closest species (*Tetrahymena thermophila* SB210), probably only a few amino acid residues were missing (see Fig. 3.5)

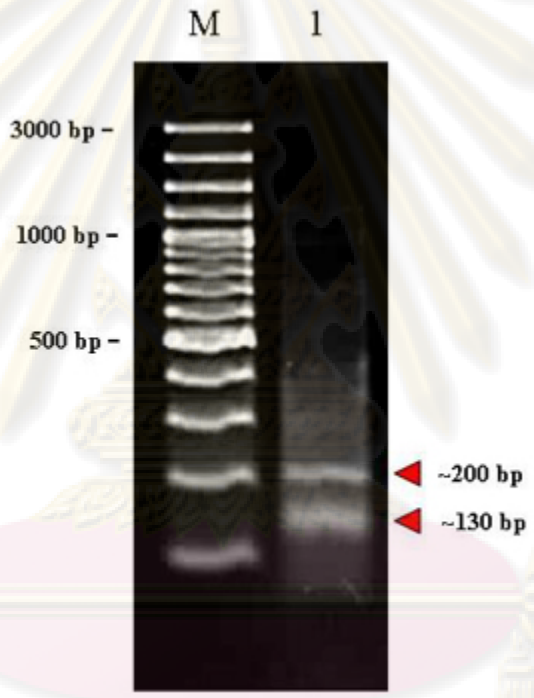


Figure 3.8 Amplification of 14-3-3B cDNA using specific primers designed from the sequence of 14-3-3 of *Tetrahymena thermophila* and *P. monodon*. PCR product from the second amplification was electrophoresed on a 1.2 % agarose gel (lane 1). The second amplification yielded 2 products with sizes ~130 and ~200 bp. Lane M; 100 bp marker

3.2.3 Sequence comparison of 14-3-3A

Homology search of the deduced amino acid sequence of 14-3-3A against the GenBank database using the BLASTX search program showed high identity to 14-3-3 like protein of *Penaeus monodon* (100 %), 14-3-3zeta of *Apis mellifera* (90 %), 14-3-3 protein sigma, gamma, zeta, beta/alpha of *Aedes aegypti* (90 %), 14-3-3zeta of *Bombyx mori* (86 %), 14-3-3zeta of *Drosophila melanogaster* (87 %), putative 14-3-3 protein of *Maconellicoccus hirsutus* (87 %), 14-3-3 protein of *Caenorhabditis brenneri* (86 %), 14-3-3b protein of *Meloidogyne incognita* (86 %), 14-3-3 family of *Caenorhabditis elegans* (86 %) and 14-3-3 protein of sheep brain (81 %). The BLASTX results are shown in Table 3.1.

Table 3.1 The BLASTX results and percentages of identity of 14-3-3A sequence to homologue sequences in the GenBank database

Sequence homology	Closest species	% Identity to 14-3-3A	Expect values	Score (bits)	Accession no.
14-3-3 like protein	*Penaeus monodon*	246/246 (100%)	3e-115	484	AAY56092.1
14-3-3 isoform c	*Apis mellifera*	221/244 (90%)	5e-121	437	XP_623183.1
14-3-3 zeta	*Bombyx. mori*	220/244 (90%)	2e-120	434	BAG38533.1
14-3-3 protein	*Aedes aegypti*	212/244 (86%)	7e-117	423	AAEL006885
14-3-3zeta	*Drosophila melanogaster*	213/244 (87%)	2e-116	421	CAA73153.1
14-3-3 protein	*Maconellicoccus hirsutus*	213/244 (87%)	3e-115	417	ABM55627.1
14-3-3 protein	*Caenorhabditis brenneri*	210/243 (86%)	9e-112	406	ACE74683.1
14-3-3b protein	*Meloidogyne incognita*	206/237 (86%)	9e-112	406	AAR85527.1
14-3-3 family	*Caenorhabditis elegans*	210/243 (86%)	9e-112	406	NP_509939.1
14-3-3 protein	sheep brain	199/244 (81%),	8e-111	403	AAB22282.1

3.2.4 Sequence comparison of 14-3-3B

Homology search of the deduced amino acid sequence of 14-3-3B against the GenBank database using the BLASTX search program showed high identity to 14-3-3 protein of *Tetrahymena thermophila* SB210 (71 %), 14-3-3 protein of *Tetrahymena pyriformis* (66 %), 14-3-3 protein of *Arabidopsis thaliana* (57%), 14-3-3 protein homolog of *Maackia amurensis* (56 %), similar to putative 14-3-3 protein epsilon of *Acyrthosiphon pisum* (57 %), 14-3-3 protein of *Schizophyllum commune* (56 %), 14-3-3 protein GF14epsilon of *Arabidopsis thaliana* (57 %), 14-3-3 of *Lentinula edodes* (56 %), 14-3-3-like protein of *Pneumocystis carinii* (56 %) and 14-3-3 protein of *Nicotiana tabacum* (58 %). The BLASTX results are shown in Table 3.2.

Table 3.2 The BLASTX results and percentages of identity of 14-3-3B sequence to homologue sequences in the GenBank database

Sequence homology	Closest species	% Identity to 14-3-3B	Expect values	Score (bits)	Accession no.
14-3-3 protein	*Tetrahymena hermophila*	163/229 (71%)	3e-80	301	XP_001009807.2
14-3-3 protein	*Tetrahymena pyriformis*	153/229 (66%)	3e-75	285	XP_001020471.1
14-3-3 protein	*Arabidopsis thaliana*	131/229 (57%)	3e-67	258	AAF98570.1
14-3-3 protein homolog	*Maackia amurensis*	130/229 (56%)	3e-67	258	AAC15418.1
14-3-3 protein epsilon	*Acyrthosiphon pisum*	131/229 (57%)	7e-67	257	XP_001952295.1
14-3-3 protein	*Schizophyllum commune*	129/230 (56%)	7e-67	257	AAK33011.1
14-3-3 protein GF14epsilon	*Arabidopsis thaliana*	132/229 (57%)	7e-67	257	AAG50088.1
14-3-3 protein	*Lentinula edodes*	130/230 (56%)	7e-67	257	BAA89421.1
14-3-3 like protein	*Pneumocystis carinii*	133/230 (57%)	1e-66	256	AAK53389.1
14-3-3 protein	*Nicotiana tabacum*	136/233 (58%)	1e-66	256	AAC49895.1

3.3 Analysis of 14-3-3 cDNAs

The deduced amino acid sequence of *P. monodon* 14-3-3A and 14-3-3B were aligned with the other 14-3-3 proteins of known structure, human 14-3-3 isotypes and fish 14-3-3a. Of possible importance for the regulation of its activity, *P. monodon* 14-3-3A contains five important domains of protein kinase A phosphorylation site, protein kinase C phosphorylayion site, casein kinase II phosphorylation site, tyrosine kinase phosphorylation site and Asn glycosylation site. Whilst *P. monodon* 14-3-3B contains only three domains of casein kinase II phosphorylation site, tyrosine kinase phosphorylation site and Asn glycosylation site (Fig. 3.9). The helices 3, 5, 7 and 9 which form the amphipathic substrate binding groove and the residues within the binding cleft, are highly conserved in their amino acid sequences. Among the high conserve regions, *P. monodon* 14-3-3B amino acid sequence was less similar to 14-3-3 of human and fish than 14-3-3A. Based on these results, it is possible that 14-3-3A interacts with the same substrates as the other 14-3-3 proteins, whereas 14-3-3B may have some function different from *P. monodon* 14-3-3A and 14-3-3 of human and fish.

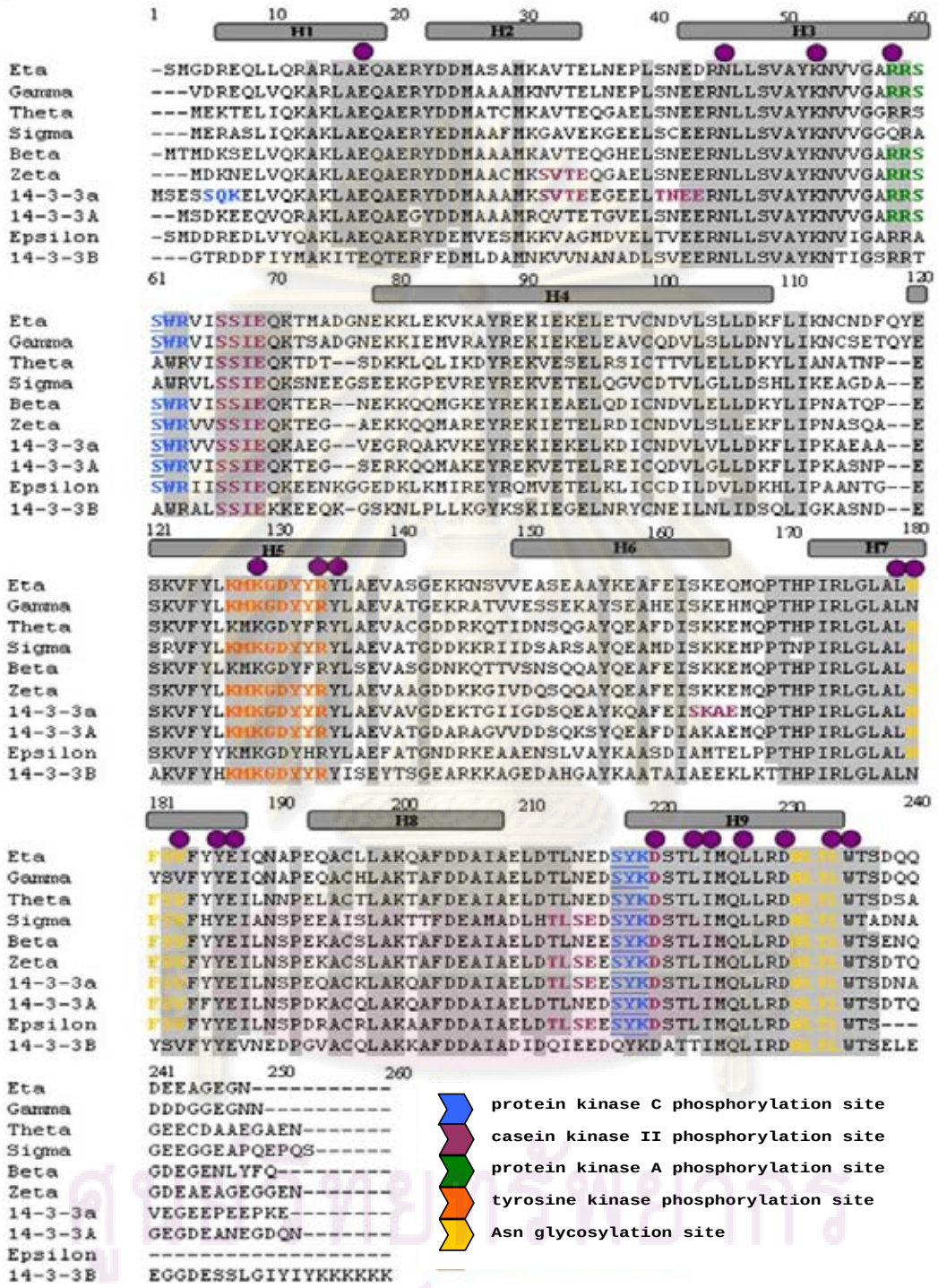


Figure 3.9 Amino acid sequence alignments of *P. monodon* 14-3-3 (14-3-3A and 14-3-3B) with other 14-3-3 proteins. The other 14-3-3 proteins include human of

Figure 3.9 (continued)

Eta isotype (acc. no. NP_003396.1), Gamma isotype (acc. no. XP_519163.2), Theta isotype (acc. no. XP_525684.2), Sigma isotype (acc. no. NP_006133.1), Beta isotype (acc. no. NP_003395.1), Zeta isotype (acc. no. XP_001098275.1), Epsilon isotype (acc. no. AAP36544.1) and fish 14-3-3a (acc. no. AAG22081.1). The locations of helices are indicated by black bars above the corresponding residues. Residues within the binding cleft that interact with peptide ligands or with the serotonin N-acetyl transferase molecule are indicated by filled circles. Important domains are indicated by colored letter: blue, protein kinase C phosphorylation site; purple, casein kinase II phosphorylation site; green, protein kinase A phosphorylation site; orange, tyrosine kinase phosphorylation site; and yellow, Asn glycosylation site. High light indicates complete conservation.

3.4 Phylogenetic analysis

A phylogenetic analysis of the *P. monodon* 14-3-3 (14-3-3A and 14-3-3B) with 14-3-3 of arthropods, humans and fishes was performed at the amino acid level using PHYLIP by neighbour-joining (NJ) method (Fig. 3.10). Based on NJ analysis, the other 14-3-3 proteins can be classified into three major groups: A, B and C. Group A can be classified into three subgroups; one subgroup containing the humans 14-3-3 (Sigma, Theta, Zeta and Beta) and fish 14-3-3 (*Oncorhynchus mykiss*); the second subgroup consisting of the humans 14-3-3 (Gamma and Eta), the third subgroup containing fishes 14-3-3 (*Fundulus heteroclitus* and *Danio rerio*). Group B can be classified into two subgroups; one and second subgroups containing the arthropods 14-3-3 (14-3-3A, *Drosophila melanogaster*, Zeta *Artemia mellifera*, Zeta *Manduca sexta*, *Litopenaeus setiferus*, *Marsupenaus japonicus*, Zeta *Rhagoletis suavis* and *Trichoplusia ni*). Group C can be classified into two subgroups; one subgroup containing the Epsilon 14-3-3 of the human and *Drosophila melanogaster*; the second subgroup containing the arthropods 14-3-3 (14-3-3B, *Callinectes sapidus* and *Wasmannia auropunctata*). This result classified the *P. monodon* 14-3-3A into 14-3-3 Zeta isotype while *P. monodon* 14-3-3B can be classified into 14-3-3 Epsilon isotype.

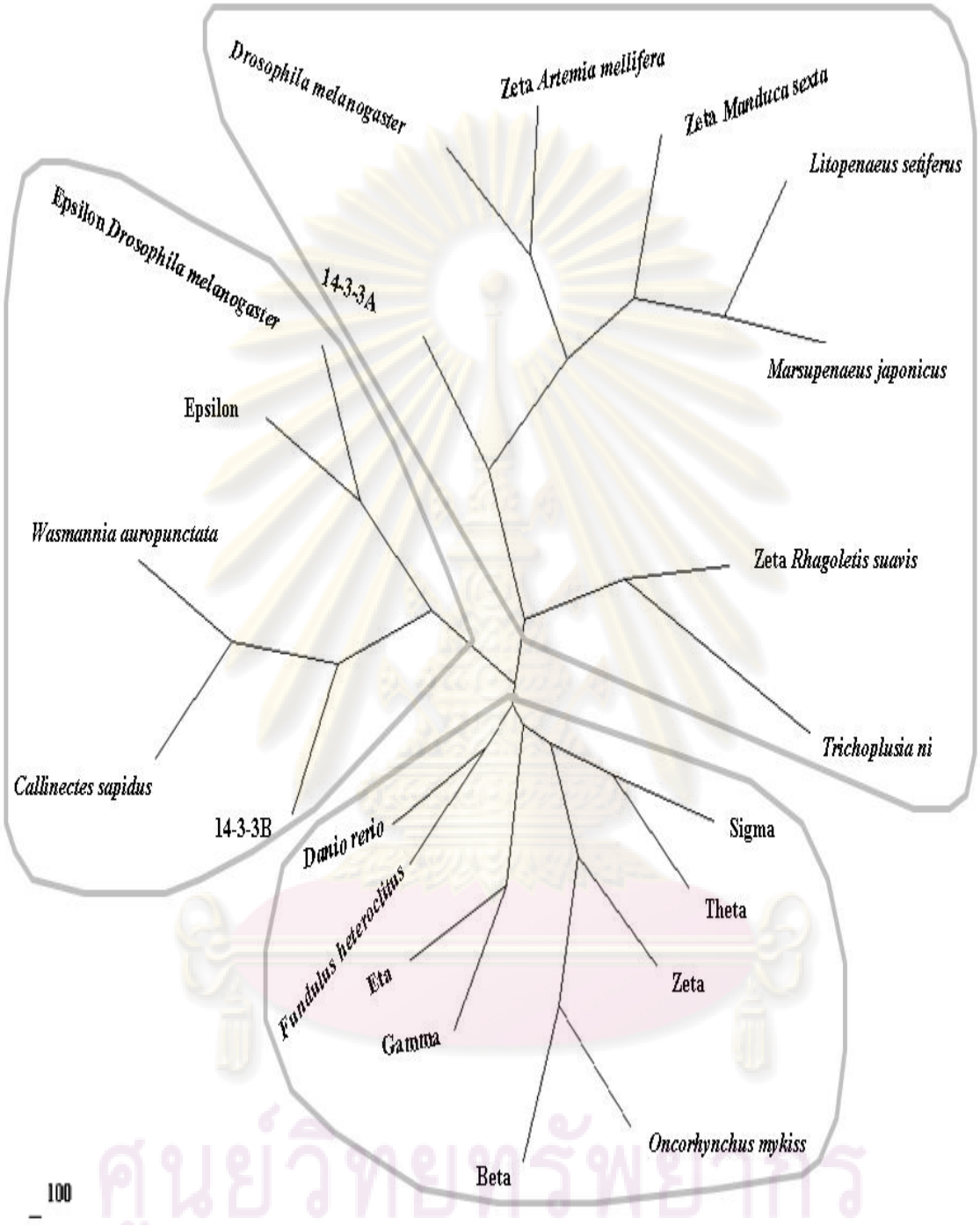


Figure 3.10 A bootstrapped neighbour-joining tree illustrating relationships between different families of 14-3-3 proteins in humans, arthropods and fishes The 14-3-3 proteins include human Theta (acc. no. XP_525684.2), human Sigma -

Figure 3.10 (continued)

(acc. no. NP_006133.1), human Zeta (acc. no. XP_001098275.1), human Beta (acc. no. NP_003395.1), human Eta (acc. no. NP_003396.1), human Gamma (acc. no. XP_519163.2), human Epsilon (acc. no. AAP36544.1), *Drosophila melanogaster* (acc. no. AI542081.1), Zeta *Artemia mellifera* (acc. no. FC860610.1), Zeta *Manduca sexta* (acc. no. AI187543.1), *Litopenaeus setiferus* (acc. no. BE846610.1), *Marsupenaeus japonicus* (acc. no. AU176065.1), Zeta *Rhagoletis suavis* (acc. no. EX453993.1), *Trichoplusia ni* (acc. no. CF258245.1), Epsilon *Drosophila melanogaster* (acc. no. XM_002133540), *Wasmannia auropunctata* (acc. no. EH413075.1), *Callinectes sapidus* (acc. no. CV162094.1), *Oncorhyncus mykiss* (NM_001124475), *Fundulus heteroclitus* (acc. no. AAG22081.1) and *Danio rerio* (acc. no. AAI52270.1). Values at the node indicate the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original deduced protein sequences.

3.5 Two isoforms of 14-3-3 and their expression in various shrimp tissues

Using RT-PCR, it was found that not all groups of shrimps expressed 14-3-3B mRNA which was in contrast to 14-3-3A mRNA, that was found in all individual shrimps tested. Therefore, 10 individual shrimps from each group were acclimatized at 3 ppt for 1 day and then investigated for the presence or absence of 14-3-3B mRNA by RT-PCR prior to conducting the experiments. Shrimp expressing 14-3-3B mRNA showed a band of PCR product with size about 402 bp (Fig. 3.11A) where as those not expressing 14-3-3B mRNA showed a primer-dimer band (Fig. 3.11B). Tissue-specific expression of the two 14-3-3 isoforms was then examined in various shrimp tissues by RT-PCR analysis, of which total RNA was pooled from 5 individual shrimps. The PCR primers (Table 2.1) were designed from cDNA sequences obtained previously from the *P. monodon* EST project. Distribution of 14-3-3A mRNA were found in all analyzed tissues including gill, epipodite, antennal gland, heart, hemocytes, hepatopancreas, intestine, lymphoid gland, eye stalk whilst another isoform, 14-3-3B mRNA was observed only in gill and epipodite (Fig. 3.12).

Figure 3.11 Expression of 14-3-3B mRNA in gill of shrimp determined by RT-PCR. The amplification product was electrophoresed on a 1.2 % agorose gel. (A) Group of shrimps expressing 14-3-3B mRNA (B) Group of shrimps not expressing 14-3-3B mRNA

Lane M	:	100bp marker
Land N	:	Negative control
Lane 1 to 10	:	10 individual shrimps for each group

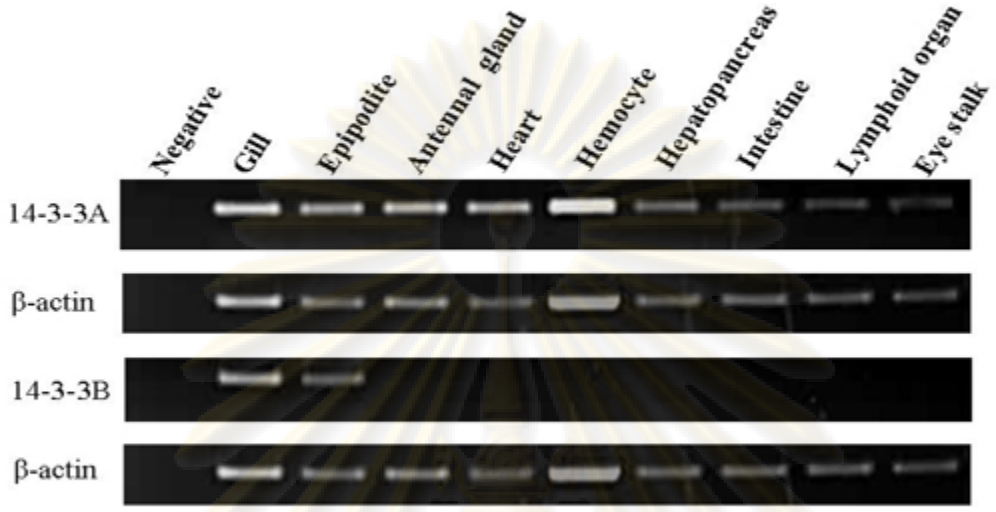


Figure 3.12 Expression of the two isoforms of 14-3-3 in various shrimp tissues. RT-PCR was performed using primer pairs, specific to 14-3-3A and 14-3-3B. The amplification was performed on a cDNA pool obtained from 5 individual shrimps. β-actin serves as an internal control.

3.6 Expression analysis of 14-3-3 mRNAs in response to salinity stress

Salinity stress response of 14-3-3A and 14-3-3B mRNAs was determined by semi-quantitative RT-PCR in gills of shrimp using isoform specific primer pairs (Table 2.1). The shrimp samples were first checked for the presence of 14-3-3B mRNA in gill before used in the experiments. Then, they were transferred from 3 ppt salinity to either 25 or 40 ppt salinity for a period of 3 weeks. The temporal expression patterns of the 14-3-3 mRNAs after transfer of shrimp are shown in Figs. 3.13 and 3.14. Of interest, the expression levels of 14-3-3B were markly decreased when shrimp were transferred from 3 to 25 and 40 ppt salinity levels. In contrast, the expression levels of 14-3-3A mRNA

remained unchanged or slightly differ after transfer of shrimp to higher salinity (25 and 40 ppt), A reduction of 14-3-3B mRNA, to 7.5-fold in shrimp group I and 2.3-fold in shrimp group II were observed after transfer of shrimp from 3 to 40 ppt salinity. Similarly, transfer of shrimp from 3 ppt to 25 ppt salinity, a salinity nearly iso-osmotic condition, resulted in a 26-fold decrease of 14-3-3B transcript in shrimp group I and 43.5-fold decrease in shrimp group II. The decrease in expression of 14-3-3B was observed as early as day1 of transfer. To confirm the high abundant of 14-3-3B mRNA in the lower-salinity waters, shrimps were reared at 40 ppt salinity for two weeks and then transferred abruptly to 3 ppt salinity. As can be seen in Fig. 3.15, the expression level of 14-3-3B mRNA was increased for up to 43.6-fold in shrimp group II at a period of 1 week after transfer of shrimp from 40 to 3 ppt salinity. In contrast, the expression levels of 14-3-3A mRNA remained unchanged or slightly differ after transfer of shrimp to lower salinity (3 ppt).

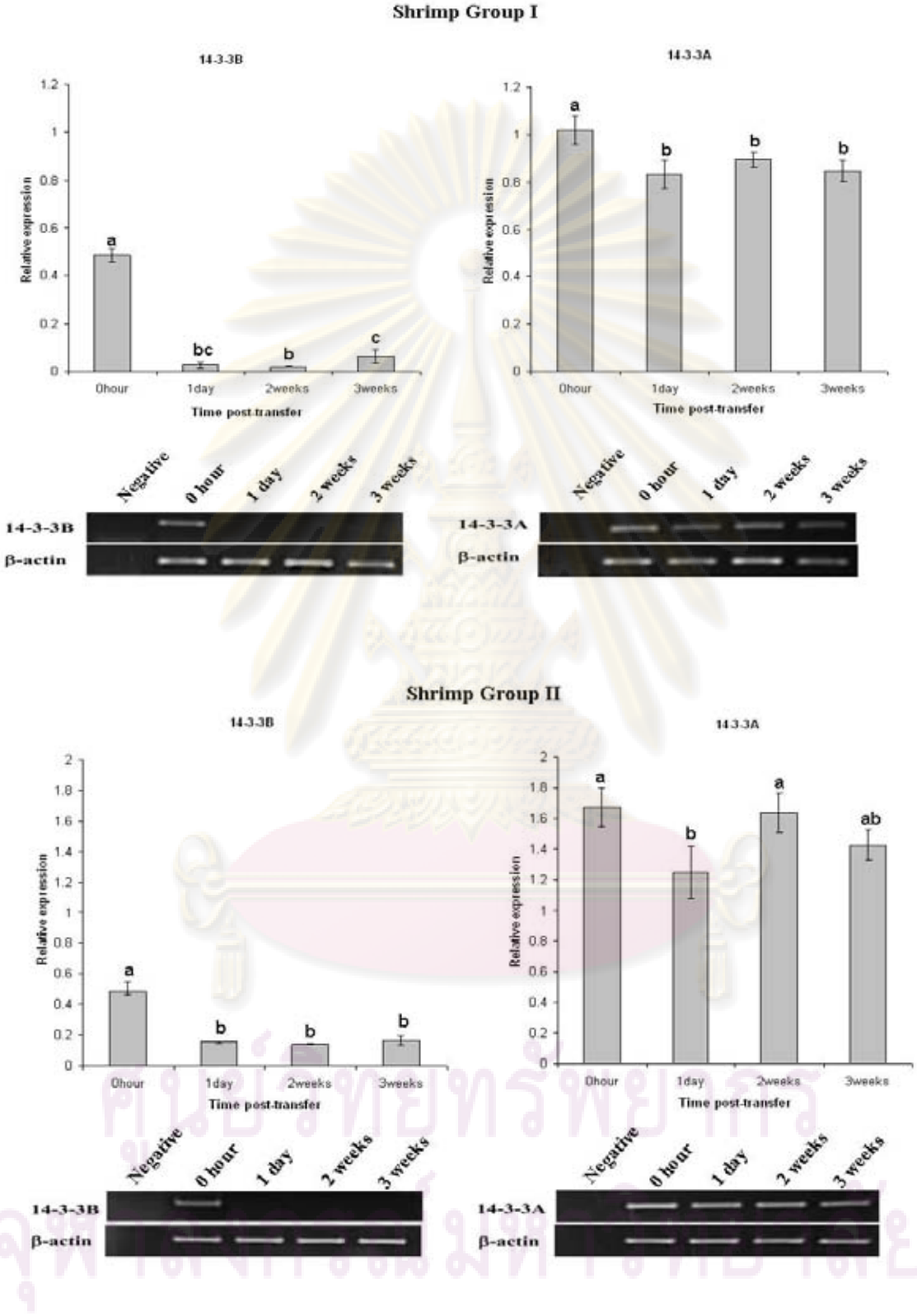


Figure 3.13 Relative expression levels of 14-3-3 mRNAs in shrimp gills after-

Figure 3.13 (continued)

transfer of shrimp from 3 to 40 ppt. The relative expression was determined by semi-quantitative RT-PCR. Two different groups of shrimp were used for analysis of each isoform of 14-3-3 mRNA. mRNA levels of 14-3-3 were normalized using β-actin as the reference gene. Data represent the average of three individual shrimps ±S.D. The same letters are not significantly different at $P<0.05$ (Appendix A).

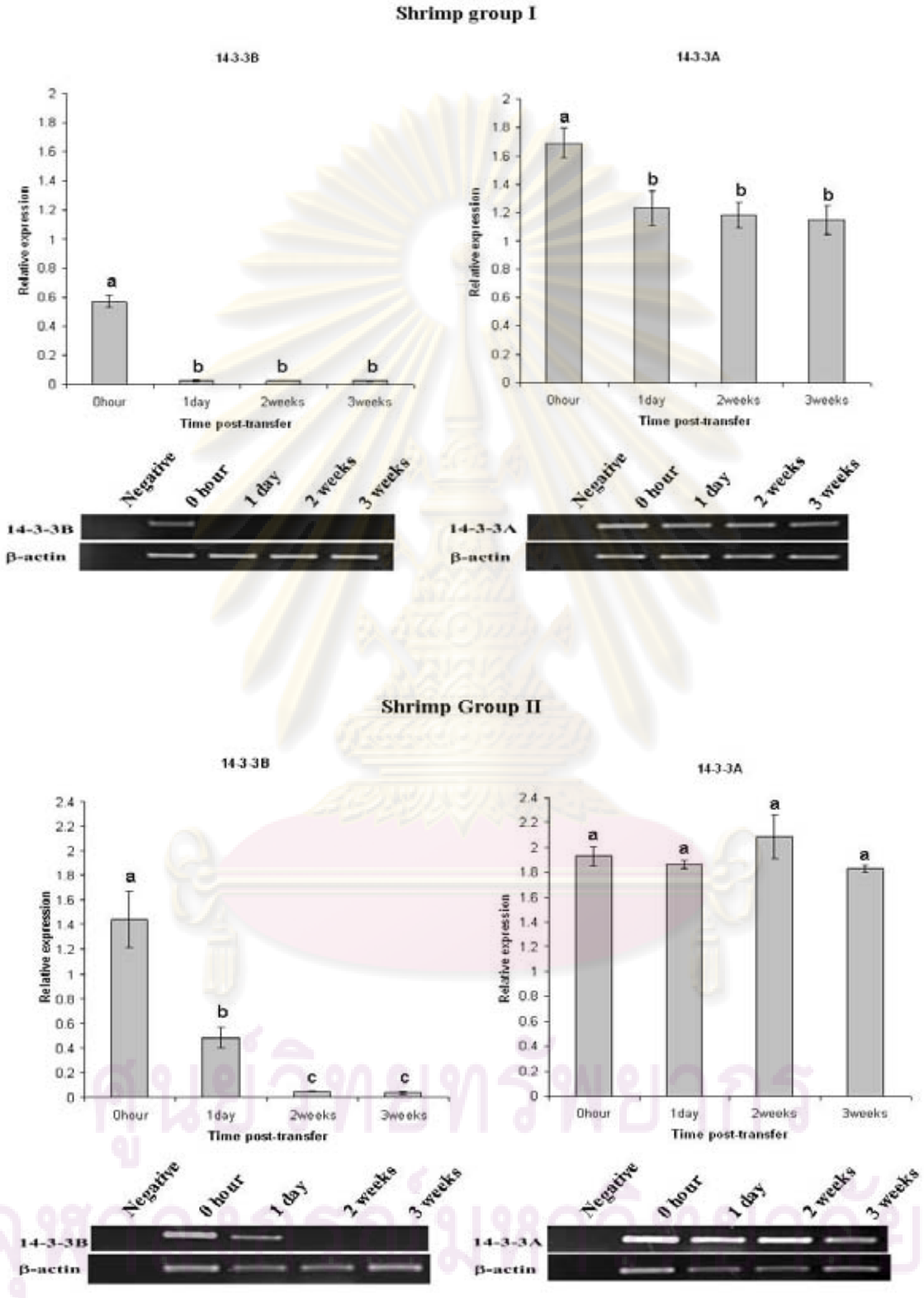


Figure 3.14 Relative expression level of 14-3-3 mRNAs in shrimp gills after-

Figure 3.14 (continued)

transfer of shrimp from 3 to 25 ppt. The relative expression was determined by semi-quantitative RT-PCR Two different groups of shrimp were used for analysis of each isoform of 14-3-3 mRNA. mRNA levels of 14-3-3 were normalized using β-actin as the reference gene. Data represent the average of three individual shrimps ±S.D. The same letters are not significantly different at P<0.05 (Appendix A).

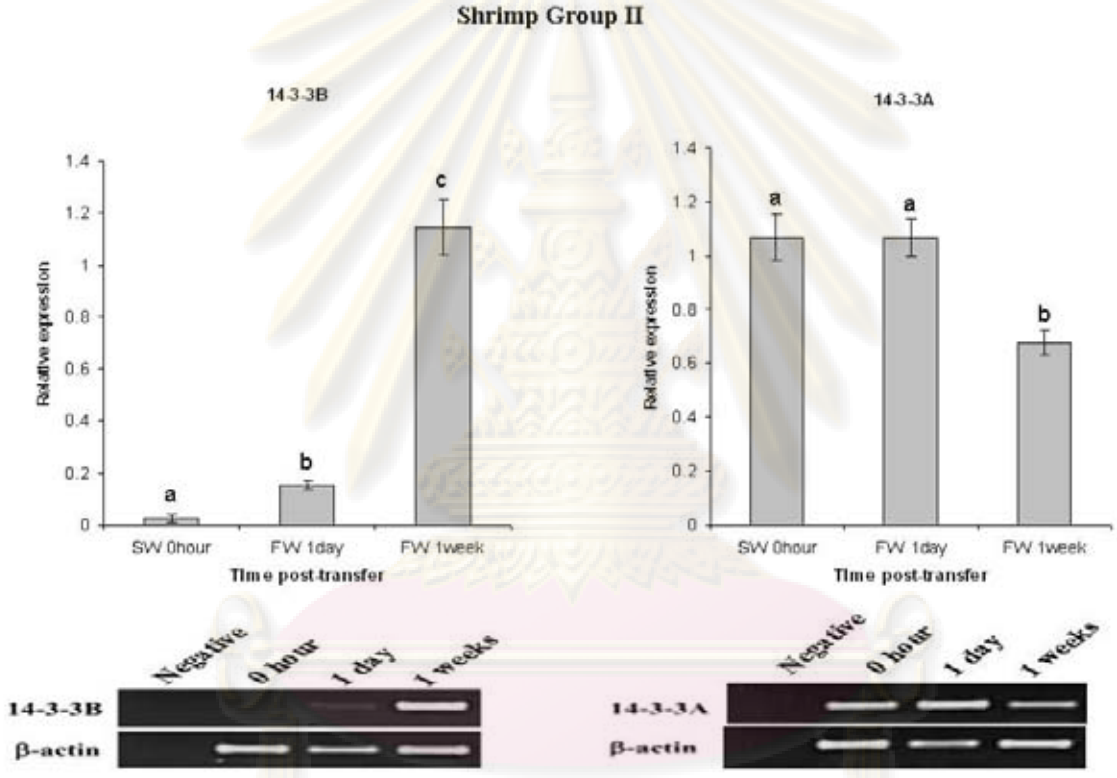


Figure 3.15 Relative expression level of 14-3-3B mRNAs in shrimp gills after transfer of shrimp from 40 to 3 ppt. The relative expression was determined by semi-quantitative RT-PCR. 14-3-3 mRNA levels were normalized using β-actin as the reference gene. Data represent the average of three individual shrimps ±S.D. The same letters are not significantly different at P<0.05 (Appendix A).

3.7 Survival of salinity stressed shrimp

The induction of 14-3-3B isoform in gill of shrimp stressed by low salinity water made it attractive candidate for investigation the influence of 14-3-3B isoform on adaptation of shrimp in low salinity stress. In this experiment, two shrimp farms from Chuntaburi province each composed of two groups of shrimps, expressing 14-3-3B mRNA group from farm I (Tamai district) and not expressing 14-3-3B mRNA group from fram II (Lamsing district) were used for determining survival at salinity stress. Shrimps group I were small juveniles (approximately 1 to 1.5 months old, 3 to 5 g of body weight) and shrimp group II were juveniles (approximately 3 months old, 15 to 20 g of body weight). For the shrimps expressing 14-3-3B mRNA, it was found that all 10 individuals of shrimp group I from fram I (Tamai district) showed a band of 14-3-3B transcript whereas 8 from those in the shrimp group II from fram I (Tamai district) expressed 14-3-3B mRNA. For shrimps not expressing 14-3-3B of group I from farm II (Lamsing district), a band of 14-3-3B mRNA could not detect in all shrimps except the one from shrimp group II from farm II (Lamsing district)(Figs. 3.16 A and 3.17 A). After abrupt transfer from 40 to 3 ppt salinity, the cumulative mortality in shrimp expressing 14-3-3B mRNA of group I from farm I (Tamai district) was about 23 % and that of group II from farm I (Tamai district) was about 57 % in day4 after low salinity stress while those of shrimp not expressing 14-3-3B mRNA in the two groups from farm II were 100 % (Figs. 3.16 B and 3.17 B).

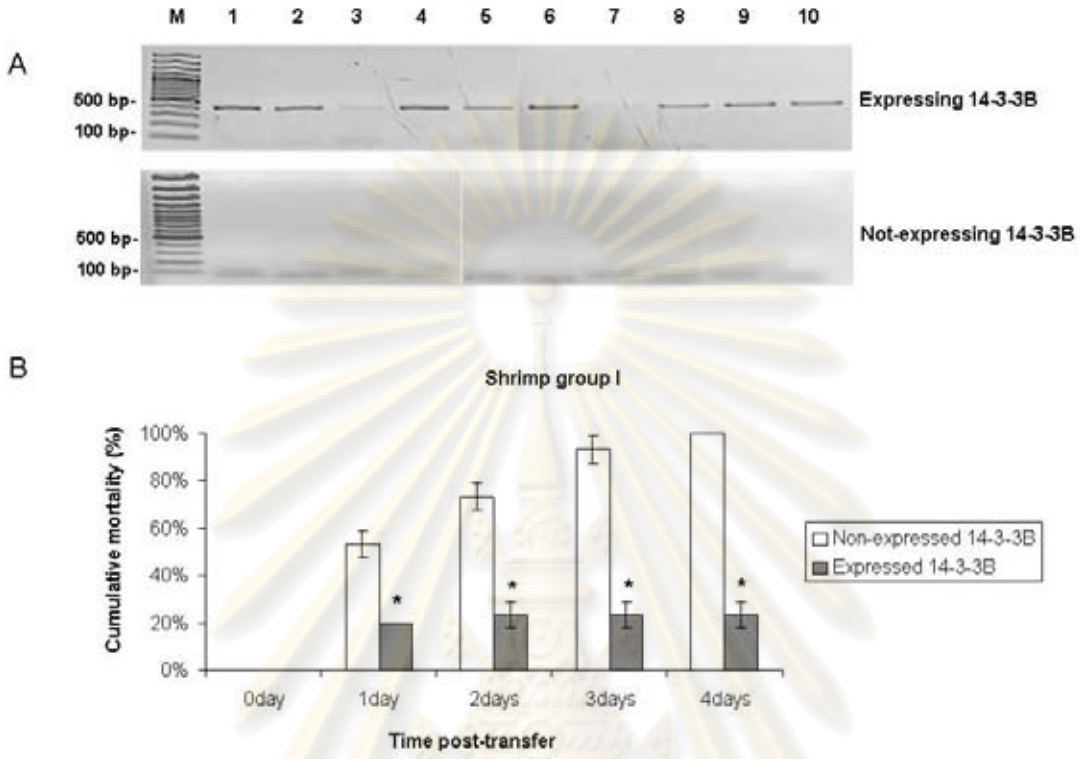


Figure 3.16 Cumulative mortality of shrimp from Farm I under low salinity stressed (40 to 3 ppt).

(A) RT-PCR analysis of 14-3-3B mRNA in the shrimp groups expressing and not expressing 14-3-3B gene. PCR products of 14-3-3B amplification were electrophoresed on 1.2 % agarose gel.

Lane M: 100bp Marker

Lane 1 to 10: 10 individual shrimps

(B) Percentage of cumulative mortality of the shrimp groups expressing and not-expressing 14-3-3B gene after abrupt transfer from 40 to 3 ppt salinity. Three replicated experiments were performed and the number of shrimp per group was 10. Asterisks designate significantly different at $P<0.05$ (Appendix D).

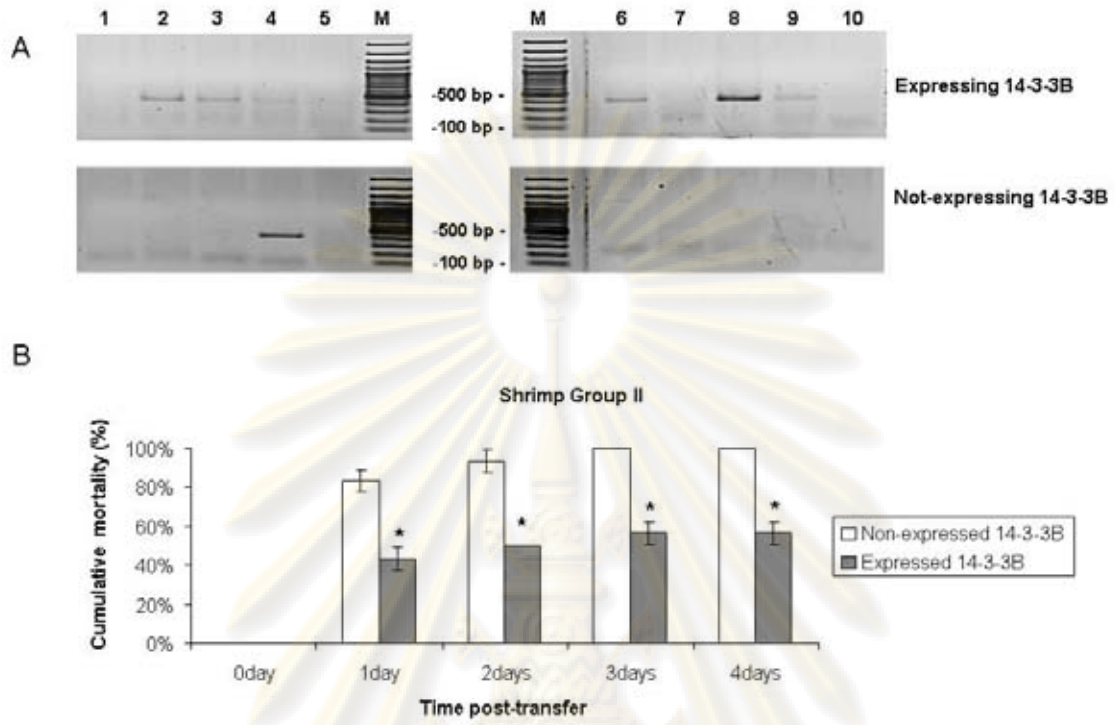


Figure 3.17 Cumulative mortality of shrimp from Farm II under low salinity stressed (40 to 3 ppt).

(A) RT-PCR analysis of 14-3-3B mRNA in the shrimp groups expressing and not expressing 14-3-3B gene. PCR products of 14-3-3B amplification were electrophoresed on 1.2 % agarose gel.

Lane M: 100bp Marker

Lane 1 to 10: 10 individual shrimps

(B) Percentage of cumulative mortality of the shrimp groups expressing and not-expressing 14-3-3B gene after abrupt transfer from 40 to 3 ppt salinity. Three replicated experiments were performed and the number of shrimp per group was 10. Asterisks designate significantly different at $P<0.05$ (Appendix D).

3.8 Suppression of 14-3-3B by RNA interference (RNAi) and ATPase activity in gill of 14-3-3B knocked-down shrimp

Previously, 14-3-3 proteins were shown to regulate some transporters such as H^+-ATPase. Therefore, knock down of 14-3-3B may affect the ATPase activity. In this study, shrimps stressed with low salinity water at 3 ppt were injected with dsRNA for 14-3-3B, Poly CG (an unrelated dsRNA) or 150 mM NaCl. Shrimp gill was collected at days 1 to 7 after dsRNA injection to investigate the silencing effect of 14-3-3B on total ATPase activity. The expression of 14-3-3B mRNA in the knock down shrimps maintained at a relatively low level from day1 to day3 as compared with the control (Fig. 3.18 A). The 14-3-3B mRNA was almost completely suppressed on day 2. The expression of 14-3-3B mRNA remained unchanged after injection with Poly CG or 150 mM NaCl. The 14-3-3B silenced shrimps were then assayed for the total ATPase activity in gill. The result showed a significant decrease in total ATPase activity in the gill of knockdown shrimps from day1 to day3 after dsRNA injection as compared to controls (Poly CG and NaCl), (Fig. 3.18 B). On the contrary, injection of poly CG or NaCl solution did not affect total ATPase activity. These data suggest that 14-3-3B in some way either directly or indirectly affects the ATPase activity.

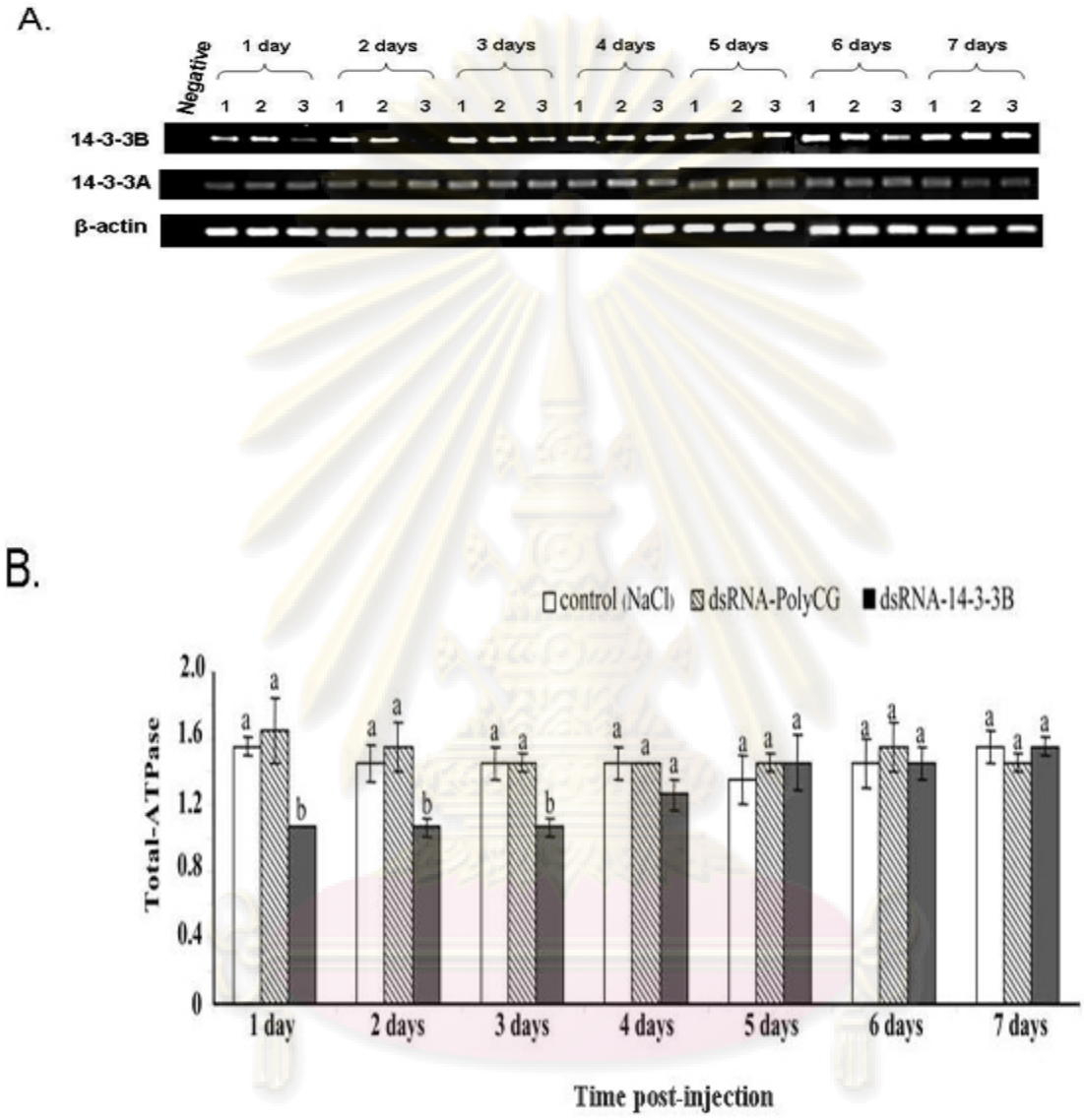


Figure 3.18 Suppression of 14-3-3B in shrimp by injection of dsRNA for 14-3-3B gene.

(A) RT-PCR analysis of 14-3-3B, 14-3-3A and β-actin mRNAs from shrimp gill injected with 100 µl of 150 mM NaCl (lane 1) 10 µg of Poly CG (lane 2) and 10 µg 14-3-3B dsRNA (lane 3). Each lane represents an individual shrimp (weigh about 3.5 g).

Figure 3.18 (continued)

(B) Analysis of total ATPase activity of protein in shrimp gill using the PK/LDH linked system. Data represent the average of three individual shrimps ±S.D. The same letters are not significantly different at $P<0.05$ (Appendix C).

CHAPTER IV

DISCUSSION

Osmotic regulation is one of the most important adaptive physiological processes permitting the successful establishment of a species in a given habitat (Haond et al., 1998). 14-3-3 proteins represent a novel type of adapter/scaffold protein that works as a molecular switch and modulates interactions between components involved in many signal transduction pathways to induce a rapid change from one type of metabolism to another in response to a change in environment (Aitken, 1995; Baldin, 2000; Van der Hoevenet al., 2000). They sequester other proteins that are phosphorylated on serine (and sometimes threonine). In addition, they promote subcellular translocation of phosphoproteins (e.g. from nucleus into cytosol) and thereby affect the function of phosphoproteins (Fiol and Kültz, 2007). 14-3-3 proteins have been reported to be a critical element for cellular hypertonic stress response pathways (Fiol and Kültz, 2007).

Although 14-3-3 proteins are present in all eukaryotes and represent a large and highly evolutionarily conserved protein family, the number of genes contained in a genome differs greatly depending on species. For example, yeast, *Caenorhabditis elegans* and *Drosophila melanogaster* have only two 14-3-3 isoforms, while mammals have seven, and the plant *Arabidopsis thaliana* has ten isoforms of 14-3-3 (Wang and Shakes, 1996; Fu et al., 2000). For *P. monodon*, two isoforms of 14-3-3 cDNAs, namely 14-3-3A and 14-3-3B, were identified. The 14-3-3A sequence was a full-length cDNA containing a complete coding sequence (CDS) whereas the 14-3-3B sequence was partial cDNA with incomplete CDS. In this study, isolation of a full-length 14-3-3B cDNA was performed. However, the complete cDNA sequence of 14-3-3B was not successfully obtained although several conditions or techniques were applied. The 14-3-3B cDNA sequence probably lacking only 3 amino acids at the 5' end of the open reading frame as compared with the sequence from *Tetrahymena thermophila SB210* which showed the highest identity. This trouble was perhaps due to the structure of 14-3-3B mRNA. The 5' end of the mRNA may form strong secondary structure due to, for example, a GC-rich

region, complementary of long nucleotides, etc. Most cellular RNA molecules are single stranded and they may form secondary structures such as stem-loop and hairpin.

The CDS of 14-3-3A isoform encodes a protein consisting of 246 amino acid residues. Partial cDNA sequence of *P. monodon* 14-3-3B had a total length of 766 bp including TGA (stop codon) but missing ATG (start codon). Duduced amino acids of *P. monodon* 14-3-3A and B showed the highest similarity scores to those of 14-3-3 Zeta of *Apis mellifera* and *Tetrahymena thermophila* 14-3-3 with identities of 90 % and 71 %, respectively.

Phylogenetic analysis indicates separation of *P. monodon* 14-3-3 (14-3-3A and 14-3-3B) from other 14-3-3 proteins in arthropods, humans and fishes. Based on the phylogenetic analysis, the *P. monodon* 14-3-3A was more closely related to arthropod 14-3-3 Zeta isotype whereas the *P. monodon* 14-3-3B was more closely related to 14-3-3 Epsilon isotype. Moreover, the 14-3-3A showed in the same branch pattern indicated that 14-3-3A was the closest to Zeta *Manduca sexta* (91 % identity) and the 14-3-3B was the closest to Epsilon human (55 % identity). It should be noted that the sequence similarity of 14-3-3 proteins depends on the isotype more than the closest species.

In addition, primary structure analysis of deduced 14-3-3 proteins of *P. monodon* was determined by comparing the amino acid sequences with the other 14-3-3 proteins of known structure. The very high degree of structural conservation of the substrate-binding groove of *P. monodon* 14-3-3A and 14-3-3 of human and fish provides strong evidence that this 14-3-3A protein probaly binds the same substrates as eukaryote 14-3-3 isoforms, i.e. proteins phosphorylated on Ser and Thr in a sequence-specific manner (Fu et al., 2000). In this conserved region, *P. monodon* 14-3-3B mRNA was less similar to the other 14-3-3 isoforms. In addition, 14-3-3A and 14-3-3 of human and fish have 5-12 motif binding sites whereas *P. monodon* 14-3-3B protein has 3 motif binding sites. The 14-3-3B may have other motif binding site different from those reported in human and fish. Motif binding sites of 14-3-3 proteins in invertebrates have been poorly characterized. In multicellular eukaryotes, including higher animals and plants, many protein kinases and phosphatases are regulated during osmolality changes. Protein kinases whose activity is

regulated during osmolality changes include various protein kinase C isoforms, protein kinase A, various protein tyrosine kinases, Ca^{2+}/calmodulin-dependent kinase, myosin light chain kinase, and MAPKs (Shrode et al., 1998; Kultz and Burg, 1998). Many of the osmoregulated kinases, phosphatases, transcription factors, and some of the DNA repair proteins mentioned above are targets as seen in Fig 3.10. Amino acid sequence alignments of 14-3-3 human, shrimp and fish showed a putative binding motif of protein kinases. A feature of 14-3-3 proteins in their ability to bind a multitude of functionally diverse signaling proteins provides a clue for the role of shrimp 14-3-3 proteins in salinity adaptation.

14-3-3 proteins are found in association with key control enzymes of primary metabolism, regulation of which could rapidly alter metabolic flux in response to signals such as water, osmotic, or salt stress. The 14-3-3 proteins are known to be osmotically regulated and they are expected to play a significant role for cellular osmoregulation (Finnie et al., 1999). Tissue-specific expression study of the present study suggested that the 14-3-3B may be important for salinity adaptation of *P. monodon* since distribution of 14-3-3B mRNA was observed in gills and epipodites only whereas 14-3-3A mRNA was found in all analyzed tissues. Surprisingly, it was found that not all of the shrimp samples collected from different shrimp farms showed the expression of 14-3-3B isoform and it appeared to depend on the group of shrimp samples not on individuals. On the contrary, the other isoform, 14-3-3A was found to be expressed in all shrimps. Actually, 14-3-3B gene may exist in all shrimps but in some shrimps, the 14-3-3B gene is not expressed. This might occur from the mutation in 14-3-3B gene, such as a promoter region leading to loss of gene expression. This speculation awaits for further investigation.

Expression analysis of *P. monodon* 14-3-3 mRNAs in response to salinity stress revealed that the levels of 14-3-3B were markly decreased when shrimp were transferred from low (3 ppt) to higher (25 and 40 ppt) salinity levels and increased when shrimp were transferred from high (40 ppt) to low (3ppt) salinity. In contrast, the expression levels of 14-3-3A mRNA remained unchanged or slightly differ after transfer of shrimps. Thus, the 14-3-3B gene may be important for low salinity adaptation in *P. monodon*.

In the previously study, euryhaline fish surrounded by freshwater need to actively absorb salt from the environment to account for passive losses that occur as a result of diffusion along a concentration gradient. According to the prevalent model of branchial Na^+ absorption in freshwater fish, Na^+ enters gill epithelial cells via an apical Na^+ channel that is electrically coupled to a plasma membrane H^+-ATPase (Avella and Bornancin, 1989). Freshwater adaptation of euryhaline fish is mainly energized by the H^+-ATPase, while the Na^+/K^+-ATPase mainly energizes NaCl absorption across the gills of fish in seawater. Na^+/K^+-ATPase activity is downregulated and H^+-ATPase activity is upregulated in gill epithelium of fish transferred from SW to FW (Kültz and Somero, 1995). Activation of H^+-ATPase, inhibition of Na^+/K^+-ATPase, and regulation of ion channels are all important functions of 14-3-3 proteins.

In plant cells an increase in osmolality activates the H^+-ATPase fivefold and the 14-3-3 protein abundance in the plasma membrane two to three fold, suggesting that 14-3-3 proteins are involved in the osmotic regulation of H^+-ATPase (Babakov et al., 2000). Indeed, virtually any 14-3-3 protein binds to phosphorylated Thr in the last 98 C-terminal amino acid residues (motif YTV) of plasma membrane H^+-ATPase and stimulates its activity in the presence of fusicoccin (Fuglsang et al., 1999; Baunsgaard et al., 1998). In addition, several 14-3-3 isoforms bind to and activate protein kinase C (PKC) (Isobe et al., 1992; Van der Hoeven et al., 2000), which in turn, inhibits Na^+/K^+-ATPase activity in gill epithelium of teleosts, e.g. Atlantic cod (Crombie et al., 1996). 14-3-3 proteins not only affect the activity of ATPases, but are also very potent modulators of many ion channels.

The induction of 14-3-3B isoform in gills of shrimp stressed by low salinity water suggests a possible role of 14-3-3B isoform on adaptation of shrimp in low salinity stress. The cumulative mortality of shrimp expressing 14-3-3B mRNA was about 23 % (group I from farm I) and 57 % (group II from farm I) in day 4 after low salinity stress while that of shrimp not expressing 14-3-3B mRNA was 100 % in both groups from farm II. Higher survival rate after abrupt transfer to low salinity was observed in both groups of shrimp expressing 14-3-3B. However, the cumulative mortality of shrimp expressing 14-3-3B mRNA from group II was higher than that from group I. This observation may be resulted from the differential expression of 14-3-3B mRNA between the groups of

shrimps expressing 14-3-3B from both groups. Higher survival rate after abrupt transfer to low salinity of the group of shrimp expressing 14-3-3B mRNA from group I may be due to higher expression level of 14-3-3B mRNA than the shrimp from group II. Another reason for higher survival rate of shrimp group I may be resulted from the stage of shrimp. Shrimp from group II was juvenile stage but shrimp from group I was younger than those from group II. In general, younger juvenile shrimp seem to be more tolerance to low salinity than those with older age. Juveniles at late stage have a natural tendency to migrate to deeper water offshore where water salinity is high. When the juvenile shrimps grow up, they prefer to live in high salinity environment (Rosenberry, 1997). The results from salinity stress test revealed that the group of shrimp that expressed 14-3-3B mRNA appears more tolerant to low salinity stress than those that did not expressed 14-3-3B mRNA. This study emphasized the important of the 14-3-3B in low salinity adaptation of *P. monodon.*

14-3-3 proteins are known to regulate activities of plasma membrane H^+-ATPase and Na^+/K^+-ATPase as discussed above (Borch et al. 2002). In addition, α- subunit Na^+/K^+-ATPase N termini containing a putative binding site for 14-3-3 are found in many other species, including the honeybee *Apis mellifera* (acc. no. XP_623072), sea urchin *Strongylocentrotus purpuratus* (acc. no. XP_795226), zebrafish *Danio rerio* and shore crab *Pachygrapsus marmoratus* (acc.no. AF375957) (Jayasundara et al., 2007). In the case of mammalian α-subunit Na^+/K^+-ATPase, 14-3-3 was shown to be essential for the dopamine-induced endocytosis of subunit protein in opossum kidney, apparently by binding directly to the N terminus of the protein (Efendiev et al., 2005). Therefore, knock down of 14-3-3B gene may affect the ATPase activity. In this study, shrimps with reduce expression of a 14-3-3B gene by RNAi technology exhibited decrease in total ATPase activity suggesting that the shrimp 14-3-3B protein might be involved in the regulation of ATPase activity. However, 14-3-3 also participates in several pathway of stress.

In conclusion, high-level of expression of 14-3-3B gene and higher survival rate of shrimp expressing 14-3-3B under low salinity condition and, taken together with tissue specific expression in osmoregulatory tissues such as gills and epipodites suggest that shrimp 14-3-3B is important for hypo-osmotic regulation of *P. monodon*. In addition, the

14-3-3B gene appears to regulate ATPase function since knockdown of 14-3-3B gene by dsRNA resulted in a significant decrease in total ATPase activity. Further study of 14-3-3B isoform and identification of its binding partners during salinity acclimation will also provide useful information on understanding of low salinity adaptation in shrimp and also other marine species.

CHAPTER V

CONCLUSIONS

Two isoforms of *P. monodon* 14-3-3 proteins, namely 14-3-3A and 14-3-3B were found. A full length cDNA of *P. monodon* 14-3-3A encodes a 246 amino acids protein which showed 100 % identity to *P. monodon* 14-3-3 protein previously reported in the GenBank. Whilst a nearly complete coding sequence of *P. monodon* 14-3-3B was obtained and exhibited the highest sequence identity to *Tetrahymena thermophila SB210* 14-3-3 protein. A phylogenetic analysis of *P. monodon*, human, fish and arthropod 14-3-3s revealed that 14-3-3A could be classified into a Zeta isotype whereas 14-3-3B was classifiled as Epsilon isotype.

The primary structure of deduced 14-3-3A and 14-3-3B proteins from *P. monodon* contains important motif binding sites similar to other 14-3-3 proteins. However, the 14-3-3A protein shows higher degree of structural conservation of the substrate binding groove than 14-3-3B, which may imply different substrate binding between the two isoforms of *P. monodon* 14-3-3 protein.

Expression analysis showed that 14-3-3A mRNA was found in all shrimp tissues examined while 14-3-3B mRNA was found only in gill and epipodite. Interestingly, not all groups of shrimps expressed 14-3-3B isoform. Moreover, it was shown that salinity stress affects the mRNA level of 14-3-3B but not that of 14-3-3A. Transfer of shrimps from 3 ppt to 25 or 40 ppt resulted in a reduction of 14-3-3B mRNA level whereas that of 14-3-3A remained unchanged or slightly changed. In addition, shrimps that expressed 14-3-3B showed higher survival rate than those that did not suggested an important role of 14-3-3B in hypo-osmotic adaptation. Gene silencing of 14-3-3B also suggested that 14-3-3B protein might be involved in the regulation of ATPase activity in shrimp.

REFERENCES

Aducci, P., Ballio, A., Nasta, D., Fogliano, V., Fullone, M.R., and Marra, M. (1996). Fusicoccin and its receptors. *Plant Growth Regul*. **18**: 93–96.

Aducci, P., M. Marra, et al. (1995). "Fusicoccin receptors: Perception and transduction of the fusicoccin signal." *Journal of Experimental Botany* **46**(291): 1463-1478.

Aitken, A. (2006). "14-3-3 proteins: A historic overview." *Seminars in Cancer Biology* **16**(3): 162-172.

Aitken, A. (1995). "14-3-3 proteins on the MAP." *Trends in Biochemical Sciences* **20**(3): 95-97.

Allen, E. (1892). On the minute structure of the gills of Pa/uernonetes varians. *Quarterly Journal of Microscopical Science*. **34:** 75-84.

Anderson, I. (1993). The veterinary approach to marine prawns. In Brown (ed.), *Aquaculture for veterinations: Fish husbandry and medicine*, pp. 271-290. Amsterdam: Oxford Pergamon Press.

Athithan, S., Francis, T., Ramanathan, N., and Ramdhas, V., (2001). A note on monoculture of *Penaeus monodon* in a hardwater seasonal pond. Naga, *The ICLARM Quartely* **24** (3-4): 14-15.

Avella, M. and Bornancin, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater Rainbow trout (*Salmogairdneri*). *Journal of Experimental Biology*.**142**: 155–175.

Babakov, A. V., V. V. Chelysheva, et al. (2000). "Involvement of 14-3-3 proteins in the osmotic regulation of H^+-ATPase in plant plasma membranes." *Planta* **211**(3): 446-448.

Baily-Brook, J.H. and Moss, S.M. (1992). Penaeid taxonomy, biology and zoogeography. In A.W. Fast and L.J. Lester (eds.), *Marine shrimp culture: Principles and practices*, Amsterdam:Elsrvier Science Publishers.

Baldin, V. (2000). "14-3-3 proteins and growth control." *Progress in cell cycle research* **4**: 49-60.

Barra, J. A., Pequeux, A., and Humbert, W. (1983) A morphological study on gills of a crab acclimated to freshwater. *Tissue and Cell*. **15**: 583-596.

Barrero-Gil, J., B. Garciadebla?s, et al. (2005). "Sodium, potassium-ATPases in algae and oomycetes." *Journal of Bioenergetics and Biomembranes* **37**(4): 269-278.

Baunsgaard, L., A. T. Fuglsang, et al. (1998). "The 14-3-3 proteins associate with the plant plasma membrane H^+-atpase to generate a fusicoccin binding complex and a fusicoccin responsive system." *Plant Journal* **13**(5): 661-671.

Bouaricha, N., P. Thuet, et al. (1991). "Na^+-K^+ ATPase and carbonic anhydrase activities in larvae, postlarvae and adults of the shrimp *Penaeus japonicus* (decapoda, penaeidea)." *Comparative Biochemistry and Physiology - A Physiology* **100**(2): 433-437.

Braselmann, S. and F. McCormick (1995). "BCR and RAF form a complex in vivo via 14-3-3 proteins." *EMBO Journal* **14**(19): 4839-4848.

Broadie, K., E. Rushton, et al. (1997). "Leonardo, a drosophila 14-3-3 protein involved in learning, regulates presynaptic function." *Neuron* **19**(2): 391-402.

Camoni, L., J. F. Harper, et al. (1998). "14-3-3 proteins activate a plant calcium-dependent protein kinase (CDPK)." *FEBS Letters* **430**(3): 381-384.

Campbell, P.J. and Jones, M.B. (1989). Osmoregulation of the estuarine prawn *Palaemon longirostris* (Caridea: Palaemonidea). *Journal - Marine Biological Association. U.K.* **69**: 261-272

Castille Jr, F. L. and A. L. Lawrence (1981). "The effect of salinity on the osmotic, sodium, and chloride concentrations in the hemolymph of the freshwater shrimps, *Macrobrachium ohione smith* and *Macrobrachium rosenbergii de man*." *Comparative Biochemistry and Physiology - Part A: Physiology* **70**(1): 47-52.

Chan, H. C., W. L. Wu, et al. (2000). "Modulation of the Ca2+-activated Cl- channel by 14-3-3?" *Biochemical and Biophysical Research Communications* **270**(2): 581-587.

Chanratchakool, P., (2003). Problems in *Penaeus monodon* culture in low salinity areas. *Aquaculture Asia*, **8** (3): 53-56.

Charmantier, G. and M. Charmantier-Daures (2001). "Ontogeny of osmoregulation in crustaceans: The embryonic phase1." *American Zoologist* **41**(5): 1078-1089.

Chen, Z., H. Fu, et al. (1994). "A NaCl-regulated plant gene encoding a brain protein homolog that activates ADP ribosyltransferase and inhibits protein kinase C." *Plant Journal* **6**(5): 729-740.

Cheng, J. H. and Liao, I. C. (1986). The effect of salinity on the osmotic and ionic concentration in the haemolymph of *Penaeus monodon* and *P. Penicillatus*. In: J. L. Macclean, L. B. Dizon and L. V. Hosillos (eds.). *The First Asian Fisheries Forum*, pp. 633-636. Asian Fisheries Society, Manila, Phillippines.

Crombie, H. J., Bell, M. V. and Tytler, P. (1996). Inhibition of sodium-pluspotassium-stimulated adenosine-triphosphatase (Na^+-K^+-ATPase) by protein kinase C activators in the gills of Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology, Part B* **113**: 765–772.

Dall, W. (1985). A review of penaeid prawn biological research in Australia. In: P. C. Rothlisbera, B. J. Hill and D. J. Staples (eds.), *Second Australian National Prawn Seminar*. **32**: 11-32. Cleveland, Australia.

de Boer, A.H., and Korthout, H.A. (1996). 14-3-3 protein homologues play a central role in the fusicoccin signal transduction pathway. *Plant Growth Regul*. **18**: 99–105.

Debaisieux, P. (1970). Appareil branchial de Crangon vulgaris (*Decapode nageur*). Anatomie et histologie. *La Cellule* **69**: 64-82.

Dellambra, E., M. Patrone, et al. (1995). "Stratifin, a keratinocyte specific 14-3-3 protein, harbors a pleckstrin homology (PH) domain and enhances protein kinase C activity." *Journal of Cell Science* **108**(11): 3569-3579.

Drach, P. (1930). Etude sur le systeme branchial des Crustaces Decapodes. *Archives d'Anatomie Microscopique et de Morphologie Experimentale* **26**: 83-133.

Du, X., J. E. Fox, et al. (1996). "Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib?" *Journal of Biological Chemistry* **271**(13): 7362-7367.

Efendiev, R., Z. Chen, et al. (2005). "The 14-3-3 protein translates the NA^+,K^+-ATPase α-subunit phosphorylation signal into binding and activation of phosphoinositide 3-kinase during endocytosis." *Journal of Biological Chemistry* **280**(16): 16272-16277.

Emi, T., T. Kinoshita, et al. (2001). "Specific binding of 14-3-3a isoform to the plasma membrane H^+-ATPase in response to blue light and fusicoccin in guard cells of broad bean." *Plant Physiology* **125**(2): 1115-1125.

Ferl, R. J., M. S. Manak, et al. (2002). "The 14-3-3s." *Genome Biology* **3**(7): 3010.1-3010.7.

Ferl, R. J., G. Lu, et al. (1994). "Evolutionary implications of the family of 14-3-3 brain protein homologs in *Arabidopsis thaliana*." *Genetica* **92**(2): 129-138.

Ferrais, R.P., Parado-Estepa, F.D., de Jesus, E.Q. and Ladja, J.M. (1987). Osmotic and chloride regulation in the haemolymph of the Tiger prawn *Penaeus monodon* during molting in various salinities. *Marine Biology* **95**:377-385.

Ferrais, R.P., Parado-Estepa, F.D., de Jesus, E.Q. and Ladja, J.M. (1986). Osmoregulation in *Penaeus monodon*: effects of molting and external salinity. In: J.L. Macclean, L.B. Dizon and L.V. Hosillos (eds.), *The First Asian isheries Forum*, pp. 637-640. Asian Fisheries Soceity, Manila, Philippines

Finnie, C., J. Borch, et al. (1999). "14-3-3 Proteins: Eukaryotic regulatory proteins with many functions." *Plant Molecular Biology* **40**(4): 545-554.

Foster, C. A. and H. D. Howse (1978). "A morphological study on gills of the brown shrimp, *Penaeus aztecus*." *Tissue and Cell* **10**(1): 77-92.

Freed, E., M. Symons, et al. (1994). "Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation." *Science* **265**(5179): 1713-1716.

Fu, H., J. Coburn, et al. (1993). "The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family." *Proceedings of the National Academy of Sciences of the United States of America* **90**(6): 2320-2324.

Fu, H., R. R. Subramanian, et al. (2000). 14-3-3 Proteins: Structure, function, and regulation. *Annual Review of Pharmacology and Toxicology* **40:** 617-647.

Fuglsang, A. T., S. Visconti, et al. (1999). "Binding of 14-3-3 protein to the plasma membrane H^+-ATPase AHA2 involves the three C-terminal residues Tyr946-Thr-Val and requires phosphorylation of Thr947. " *Journal of Biological Chemistry* **274**(51): 36774-36780.

Gilles, R. (1979). Intracellular organic osmotic effectors, In: R. Gilles (ed.), *Mechanisms of ion and osmoregulation.* pp. 111-154. Chichester: John Wiley & Sons.

Gilles, R. (1975). Mechanisms of ion and osmoregulation. In: O. Kinne(ed.), *Marine ecology, Vol. 2. Physiological mechanisms, Part 1.* pp. 259-348. London: Wiley Interscience.

Gilles, R. (1970). Mechanisms of ion and osmoregulation*. Marine Ecology* **2**: 259-347.

Goss, G. G., S. F. Perry, et al. (1992). "Mechanisms of ion and acid-base regulation at the gills of freshwater fish." *Journal of Experimental Zoology* **263**(2): 143-159.

Hwang, P. P. (1987). "Tolerance and ultrastructural responses of branchial chloride cells to salinity changes in the euryhaline teleost *Oreochromis mossambicus*." *Marine Biology* **94**(4): 643-649.

Irie, K., Y. Gotoh, et al. (1994). "Stimulatory effects of yeast and mammalian 14-3-3 proteins on the Raf protein kinase." *Science* **265**(5179): 1716-1719.

Isobe, T., Y. Hiyane, et al. (1992). "Activation of protein kinase C by the 14-3-3 proteins homologous with Exo 1 protein that stimulates calcium-dependent exocytosis." *FEBS Letters* **308**(2): 121-124.

Jarillo, J. A., J. Capel, et al. (1994). "Two related low-temperature-inducible genes of Arabidopsis encode proteins showing high homology to 14-3-3 proteins, a family of putative kinase regulators." *Plant Molecular Biology* **25**(4): 693-704.

Jayasundara, N., D. W. Towle, et al. (2007). "Gill-specific transcriptional regulation of $Na^{+/}K^{+}$-ATPase α-subunit in the euryhaline shore crab *Pachygrapsus marmoratus*: Sequence variants and promoter structure." *Journal of Experimental Biology* **210**(12): 2070-2081.

Kidou, S. i., M. Umeda, et al. (1993). "Isolation and characterization of a rice cDNA similar to the bovine brain-specific 14-3-3 protein gene." *Plant Molecular Biology* **21**(1): 191-194.

Kim, V. N. (2003). "RNA Interference in Functional Genomics and Medicine." *Journal of Korean Medical Science* **18**(3): 309-318.

Knut, S.N. (1983). *Animal physiology adaptation and environment.* U.S.: Cambridge University. 3 eds. pp. 309-364

Kohn, A., D. Chakravarty, et al. (2003). "Teleost Fish 14-3-3a Protein Protects Xenopus Oocytes from Hyperosmolality." *Journal of Experimental Zoology Part A: Comparative Experimental Biology* **299**(2): 103-109.

Kultz, D., D. Chakravarty, et al. (2001). "A novel 14-3-3 gene is osmoregulated in gill epithelium of the euryhaline teleost *Fundulus heteroclitus*." *Journal of Experimental Biology* **204**(17): 2975-2985.

Kültz, D. (2000). Osmotic regulation of DNA activity and the cell cycle. In: Cell and molceular responses to stress (K. Storey and J. Storey, eds.). *Elsevier, Amsterdam*, pp. 157–179.

Kultz, D., K. Jurss, et al. (1995). "Cellular and epithelial adjustments to altered salinity in the gill and opercular epithelium of a cichlid fish (*Oreochromis mossambicus*)." *Cell and Tissue Research* **279**(1): 65-73.

Kultz, D. and G. N. Somero (1995). "Osmotic and thermal effects on in situ ATPase activity in permeabilized gill epithelial cells of the fish *Gillichthys mirabilis*." *Journal of Experimental Biology* **198**(9): 1883-1894.

Laurent, P. and S. Dunel (1980). "Morphology of gill epithelia in fish." *The American journal of physiology* **238**(3): R147-159.

Laxminarayana, A., (2001). Improved shrimp farming techniques for environmental stability. *Proceedings of the international workshop on aquaculture and environment organized by CUSAT, and Wageningen Agricultural University, Netherlands, 13-14th July 2001*, 16-28.

Li, W., E. M. C. Skoulakis, et al. (1997). "The Drosophila 14-3-3 protein Leonardo enhances Torso signaling through D-Raf in a Ras1-dependent manner." *Development* **124**(20): 4163-4171.

Liao, J. and M. B. Omary (1996). "14-3-3 proteins associate with phosphorylated simple epithelial keratins during cell cycle progression and act as a solubility cofactor." *Journal of Cell Biology* **133**(2): 345-357.

Lin, H., Pfeiffer, D. C., Vogl, A. W., Pan, J. and Randall, D. J. (1994). Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *Journal of Experimental Biology.* **195**: 169–183.

Lin, H. and Randall, D. J. (1993). H^+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *Journal of Experimental Biology.* **180**: 163–174.

Lin, S. C., C. H. Liou and J. H. Cheng (2000). The role of the antennal glands in ion and body volume regulation of cannulated *Penaeus monodon* reared in various salinity conditions. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology.* **127**(2):121-9

Liu, Y. C., Y. Liu, et al. (1997). "Serine phosphorylation of Cbl induced by phorbol ester enhances its association with 14-3-3 proteins in T cells via a novel serine-rich 14-3-3- binding motif." *Journal of Biological Chemistry* **272**(15): 9979-9985.

Lockwood, A. P. M. (1968). Aspects of the Physiology of Crustacea. Oliver and Boyd, *Edinbrgh*. 328 pp.

Lockwood, A. P. M. (1962). The osmoregulation of Crustacea. *Journal of Experimental Biology*. **37**: 257-305.

Luquet, C. M., U. Postel, et al. (2002). "Transepithelial potential differences and Na^+ flux in isolated perfused gills of the crab *Chasmagnathus granulatus* (Grapsidae) acclimated to hyper- and hypo-salinity." *Journal of Experimental Biology* **205**(1): 71-77.

Mantel, L.H. and Farmer, L.L. (1983). Osmotic and ionic regulation. In: L. H. Mantel (ed.), *The biology of crustacean Internal anatomy and physiological regulation*, **5**: 53-161. U.S.: Academic Press.

Martinez, A. S., Charmantier, G., Compere, P. and Charmantier-Daures, M. (2005). Branchial chamber tissues in two caridean shrimps: The epibenthic *Palaemon adspersus* and the deep-sea hydrothermal *Rimicaris exoculata*. *Tissue and Cell* **37**(2): 153-165.

Maru, Y. and O. N. Witte (1991). "The BCR gene encodes a novel serine/threonine kinase activity within a single exon." *Cell* **67**(3): 459-468.

McKinsey, T. A., C. L. Zhang, et al. (2000). "Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation." *Nature* **408**(6808): 106-111.

Morrison, D. K. (1995). "Mechanisms regulating Raf-1 activity in signal transduction pathways." *Molecular Reproduction and Development* **42**(4): 507-514.

Morrison, D. (1994). "14-3-3: Modulators of signaling proteins?" *Science* **266**(5182): 56-57.

Motoh, H. (1981). Studies on the fisheries biology of the giant tiger prawn, *Penaeus monodon* in the Philippines. Tech. Report No. 7. *Philippines: SEAFDEC*. Aquaculture Department. 128 pp.

Oecking, C., Eckerskorn, C., and Weiler, E.W. (1994). The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins. *FEBS Lett*. **352**: 163–166.

Pequeux, A. (1995). Osmotic regulation in crustaceans. *Journal of Crustacean Biology* **15**(1): 1-60.

Pequeux, A., and Gilles, R. (1988) NaCl transport in gills and related structures. In: R. Greger, (ed.), Comparative and Environmental Physiology NaCl transport in Epithelia. *Spriger Verlag, New York*. pp. 2-47.

Pequeux, A., and Gilles, R. (1981) Na^+ fluxes across isolated perfused gills of the Chinese crab, *Eriochir sinensis*. *Journal of Experimental Biology*. **92**: 173-186.

Pietromonaco, S. F., G. A. Seluja, et al. (1996). "Association of 14-3-3 proteins with centrosomes." *Blood Cells, Molecules and Diseases* **22**(3): 225-237.

Potts, W.T.W. and Parry, G. (1964). Osmotic and ionic regulation in animals. *Series of Monographs on Pure and Applied Biology* **19**: 119-163. New York: Mac-Millan

Reuther, G. W., H. Fu, et al. (1994). "Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family." *Science* **266**(5182): 129-133.

Riegel, J.A., and Cook, M. A. (1975) Recent studies of excretion in Crustacea. In: Wessing, A (ed.). *Excretion Fortschritte der Zooogie 23 Gustav FisherVerlag*, Stuttgart, Germany, pp. 48-74.

Rittinger, K., J. Budman, et al. (1999). "Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding." *Molecular Cell* **4**(2): 153-166.

Robertson, J. D. (1960) Osmotic and ionic regulation. In: T. H. Waterman (ed.). *Physiology of Crustacea. Vol. 1 Academic Press*, New York.

Rosenberry, B. (2001). *World shrimp farming 2001*. Sandiego: Shrimp News International.

Rosenberry, B. (1997). *World shrimp farming 1997*. Sandiego: Shrimp News International.

Saha, S. B., Bhattacharya, S. B., and Choudhary, (1999). Preliminary observation on culture of *P. monodon* in low saline water. *Naga, ICLARM Quarterly* **22**(1) : 30-33.

Sehnke, P. C., J. M. DeLille, et al. (2002). "Consummating signal transduction: The role of 14-3-3 proteins in the completion of signal-induced transitions in protein activity." *Plant Cell* **14**(SUPPL.): S339-S354.

Schoffeniels, E., and Gilles, R. (1970). Osmoregulation in aquatic arthropods. In: M. Florkin and B. T. (eds.), *Scheer Chemical zoology, Vol. 5. Part A.* pp. 255-286. New York: Academic Press.

Shrode, L. D., E. Krump and S. Grinstein. 1998. Activation of protein kinases upon volume changes: role in cellular homeostasis. Contrib. *Nephrol*. **123**: 79–93.

Skoulakis, E. M. C. and R. L. Davis (1996). "Olfactory learning deficits in mutants for leonardo, a Drosophila gene encoding a 14-3-3 protein." *Neuron* **17**(5): 931-944.

Smyth, J. D. (1942). A note on the morphology and cytology of the branchiae of *Carcinus maenas. Proceedings of the Royal Irish Academy* **B48**: 105-l 18.

Solis, N.B. (1988). Biology and ecology. In Taki, Y. Premavara, J.H., and Lobrera, J.(eds.), *Biology and culture of Penaeus monodon, Aquaculture Department, Southeast Asia Fisheries Development Center.*

Spanings-Pierrot, C., D. Soyez, et al. (2000). "Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*." *General and Comparative Endocrinology* **119**(3): 340-350.

Towle, D. W. (1984a) Regulation functions of Na^+,K^+-ATPase in marine and estuarine animals. In: Pequeux, A., Gilles, R., and Bolis, L. (eds.). *Osmoregulation in Estuarine and Marine Animals.* **9**: 157-170. Spinger, Berlin, New York.

Toyooka, K., Muratake, T., Tanaka, T., Igarashi, S., Watanabe, H., Takeuchi, H., Hayashi, S., Maeda, M., Takahashi, M., Tsuji, S., Kumanishi, T., and Takahashi, Y. (1999). 14-3-3 protein eta chain gene (YWHAH) polymorphism and its genetic association with schizophrenia. *American Journal of Medical Genetics.* **88**: 164–167.

Uchida, K. and T. Kaneko (1996). "Enhanced chloride cell turnover in the gills of chum salmon fry in seawater." *Zoological Science* **13**(5): 655-660.

Van Der Hoeven, P. C. J., J. C. M. Van Der Wal, et al. (2000). "Protein kinase C activation by acidic proteins including 14-3-3." *Biochemical Journal* **347**(3): 781-785.

Van Hemert, M. J., H. Yde Steensma, et al. (2001). "14-3-3 Proteins: Key regulators of cell division, signalling and apoptosis." *BioEssays* **23**(10): 936-946.

Van Heusden, G. P. H., A. L. Van Der Zanden, et al. (1996). "Four *Arabidopsis thaliana* 14-3-3 protein isoforms can complement the lethal yeast bmh1 bmh2 double disruption." *FEBS Letters* **391**(3): 252-256.

Van Heusden, G. P. H., D. J. F. Griffiths, et al. (1995). "The 14-3-3 proteins encoded by the BMH1 and BMH2 genes are essential in the yeast Saccharomyces cerevisiae and can be replaced by a plant homologue." *European Journal of Biochemistry* **229**(1): 45-53.

Wang, W. and D. C. Shakes (1996). "Molecular evolution of the 14-3-3 protein family." *Journal of Molecular Evolution* **43**(4): 384-398.

Wuthisuthimethavee, S., Lumubol, P., Vanavichit, A. and Tragoonrung, S. (2005). EST-based identification of genes expressed in the branchiae of black tiger shrimp (*Penaeus monodon Fabricius*). *Science Asia*. pp. 137-144.

Yaffe, M. B. (2002). "How do 14-3-3 proteins work? - Gatekeeper phosphorylation and the molecular anvil hypothesis." *FEBS Letters* **513**(1): 53-57.

APPENDICES

Appendix A

I. Anova test of 14-3-3B expression in shrimp group I transferred from 3ppt to 40ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.396	3	.132	331.273	.000
Within Groups	.003	8	.000		
Total	.399	11			

II. Dancan test of 14-3-3B expression in shrimp group I transferred from 3ppt to 40ppt.

14-3-3B

Duncan

VAR00001	N	Subset for alpha = .05		
		1	2	3
2.00	3	.0199		
1.00	3	.0284	.0284	
3.00	3		.0635	
.00	3			.4550
Sig.		.616	.063	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

III. Anova test of 14-3-3A expression in shrimp group I transferred from 3ppt to 40ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.063	3	.021	8.316	.008
Within Groups	.020	8	.003		
Total	.083	11			

IV. Dancan test of 14-3-3A expression in shrimp group I transferred from 3ppt to 40ppt.

14-3-3A

Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
1.0000	3	.8328	
3.0000	3	.8462	
2.0000	3	.8942	
.0000	3		1.0165
Sig.		.189	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

V. Anova test of 14-3-3B expression in shrimp group II transferred from 3ppt to 40ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.259	2	1.130	290.449	.000
Within Groups	.023	6	.004		
Total	2.282	8			

VI. Dancan test of 14-3-3B expression in shrimp group II transferred from 3ppt to 40ppt.

14-3-3B

Duncan

VAR00001	N	Subset for alpha = .05		
		1	2	3
.00	3	.0263		
1.00	3		.1528	
7.00	3			1.1467
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

VII. Anova test of 14-3-3A expression in shrimp group II transferred from 3ppt to 40ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.351	3	.117	6.636	.015
Within Groups	.141	8	.018		
Total	.493	11			

VIII. Dancan test of 14-3-3A expression in shrimp group II transferred from 3ppt to 40ppt.

14-3-3A

Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
1.00	3	1.2489	
3.00	3	1.4289	1.4289
2.00	3		1.6372
.00	3		1.6738
Sig.		.136	.062

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

IX. Anova test of 14-3-3B expression in shrimp group I transferred from 3ppt to 25ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.668	3	.223	480.470	.000
Within Groups	.004	8	.000		
Total	.672	11			

X. Dancan test of 14-3-3B expression in shrimp group I transferred from 3ppt to 25ppt.

14-3-3B

Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
3.00	3	.0208	
2.00	3	.0269	
1.00	3	.0274	
.00	3		.5698
Sig.		.726	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

XI. Anova test of 14-3-3A expression in shrimp group I transferred from 3ppt to 25ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.576	3	.192	16.930	.001
Within Groups	.091	8	.011		
Total	.667	11			

XII. Dancan test of 14-3-3A expression in shrimp group I transferred from 3ppt to 25ppt.

14-3-3A

Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
3.00	3	1.1482	
2.00	3	1.1804	
1.00	3	1.2334	
.00	3		1.6886
Sig.		.375	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

XIII. Anova test of 14-3-3B expression in shrimp group II transferred from 3ppt to 25ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.905	3	1.302	88.997	.000
Within Groups	.117	8	.015		
Total	4.022	11			

XIV. Dancan test of 14-3-3B expression in shrimp group II transferred from 3ppt to 25ppt.

14-3-3B

Duncan

VAR00001	N	Subset for alpha = .05		
		1	2	3
3.00	3	.0333		
2.00	3	.0456		
1.00	3		.4827	
.00	3			1.4364
Sig.		.904	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

XV. Anova test of 14-3-3A expression in shrimp group II transferred from 3ppt to 25ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.156	3	.052	.438	.732
Within Groups	.953	8	.119		
Total	1.109	11			

XVI. Dancan test of 14-3-3A expression in shrimp group II transferred from 3ppt to 25ppt.

14-3-3A

Duncan

VAR00001	N	Subset for alpha = .05
		1
3.00	3	1.8308
1.00	3	1.9566
.00	3	2.0687
2.00	3	2.1302
Sig.		.346

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

XVII. Anova test of 14-3-3B expression in shrimp group II transferred from 40ppt to 3ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.259	2	1.130	290.449	.000
Within Groups	.023	6	.004		
Total	2.282	8			

XVIII. Dancan test of 14-3-3B expression in shrimp group II transferred from 40ppt to 3ppt

14-3-3B

Duncan

VAR00001	N	Subset for alpha = .05		
		1	2	3
.00	3	.0263		
1.00	3		.1528	
7.00	3			1.1467
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

XIX. Anova test of 14-3-3A expression in shrimp group II transferred from 40ppt to 3ppt.

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.311	2	.156	33.999	.001
Within Groups	.027	6	.005		
Total	.339	8			

XX. Dancan test of 14-3-3A expression in shrimp group II transferred from 40ppt to 3ppt

14-3-3A

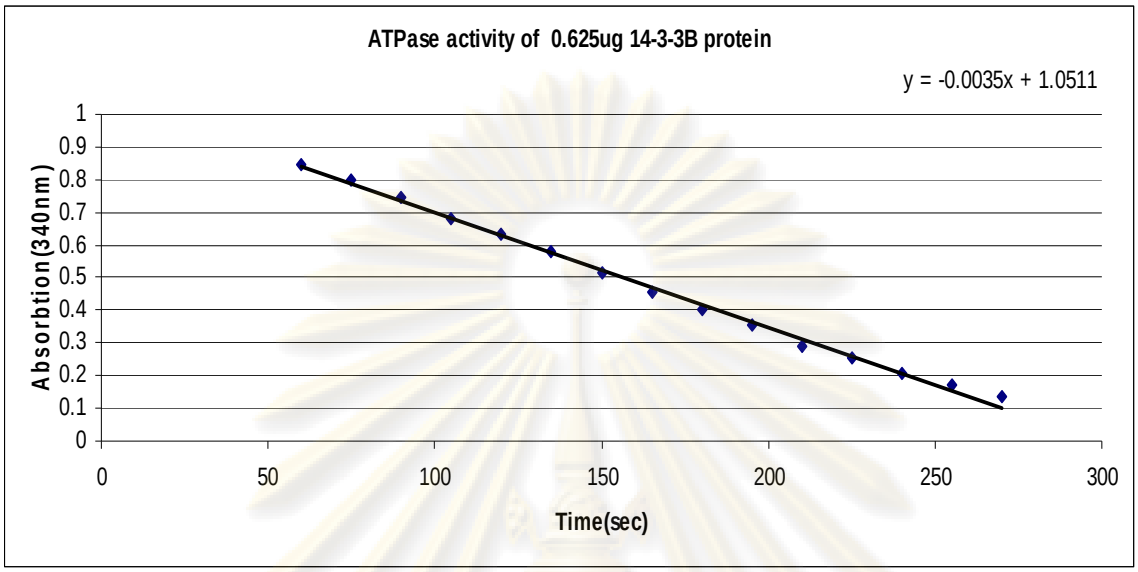
Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
7.00	3	.6741	
.00	3		1.0686
1.00	3		1.0688
Sig.		1.000	.997

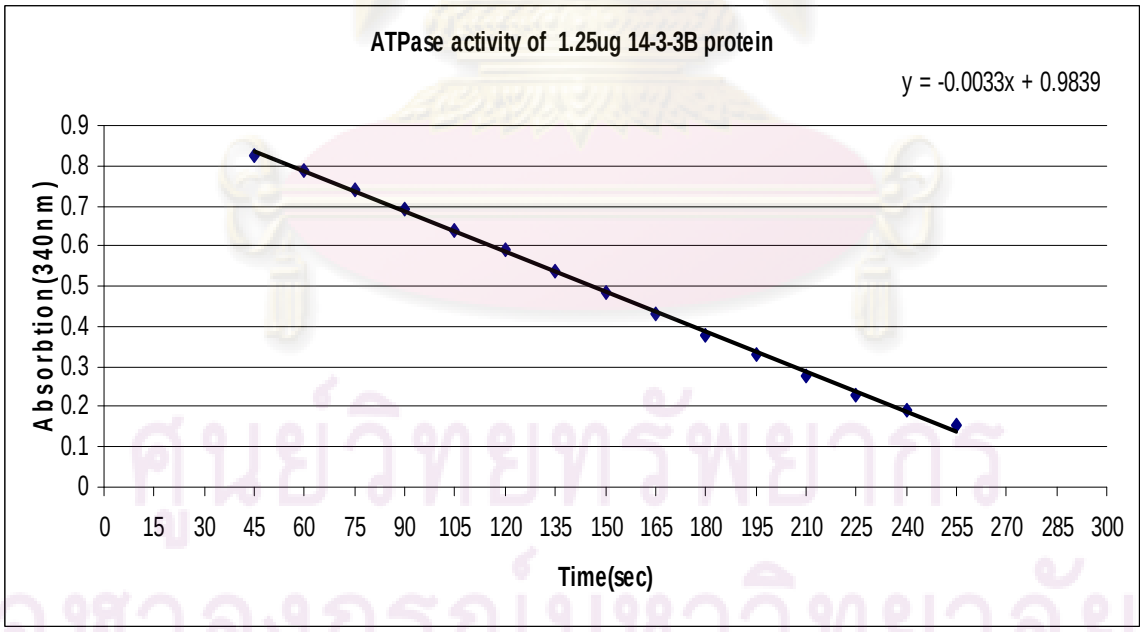
Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix B

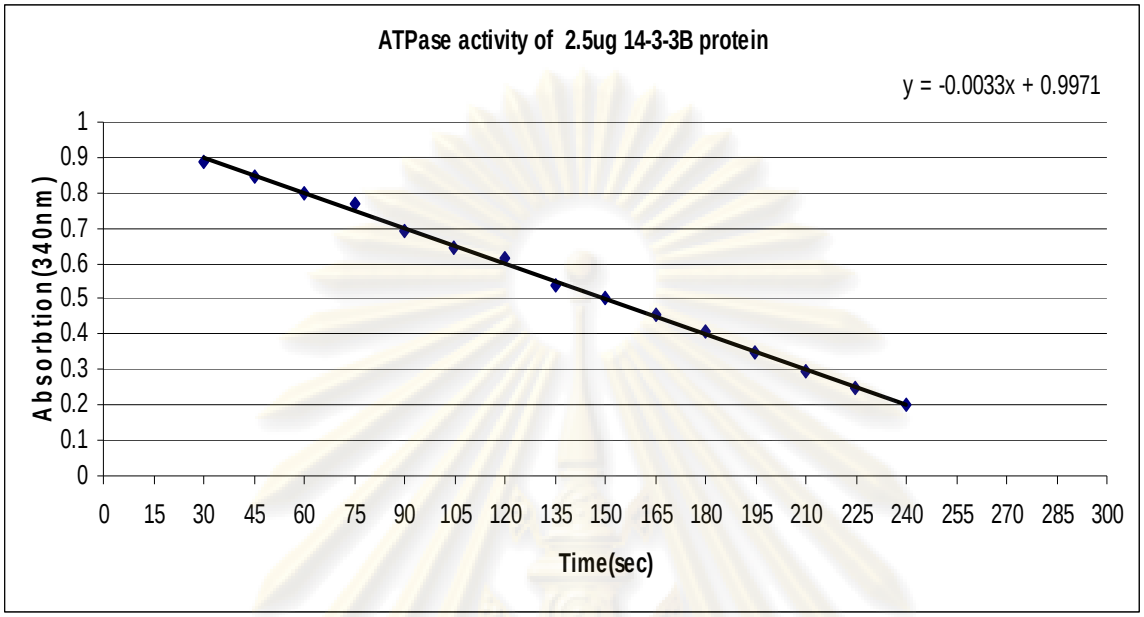
I. ATPase activity of 0.625ug 14-3-3B protein



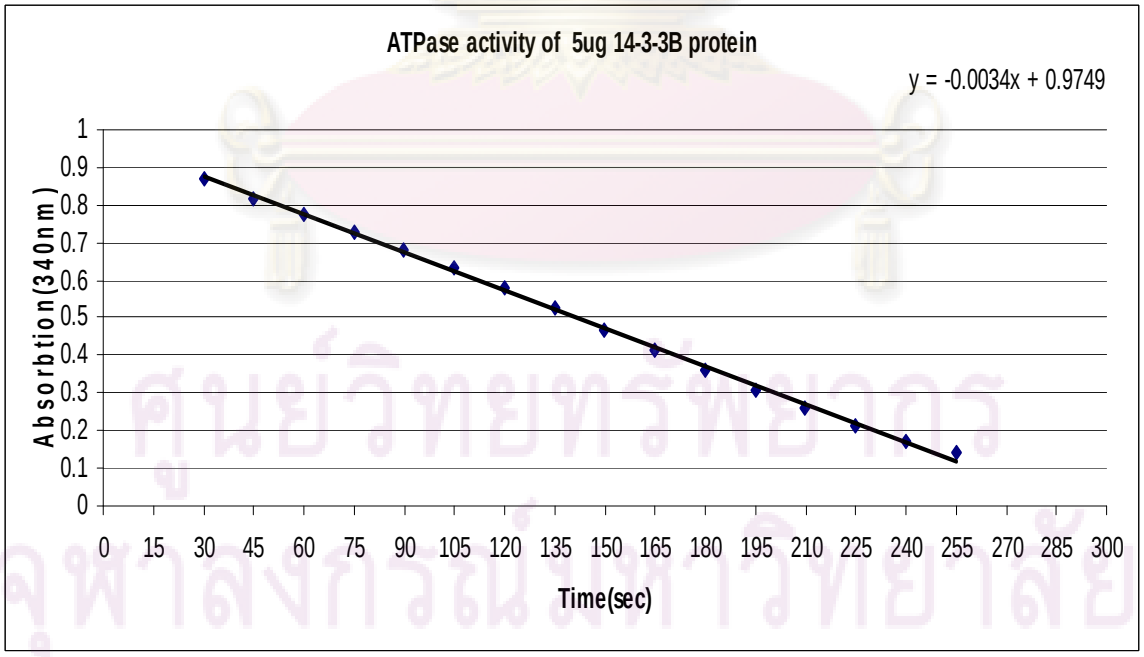
II. ATPase activity of 1.25ug 14-3-3B protein



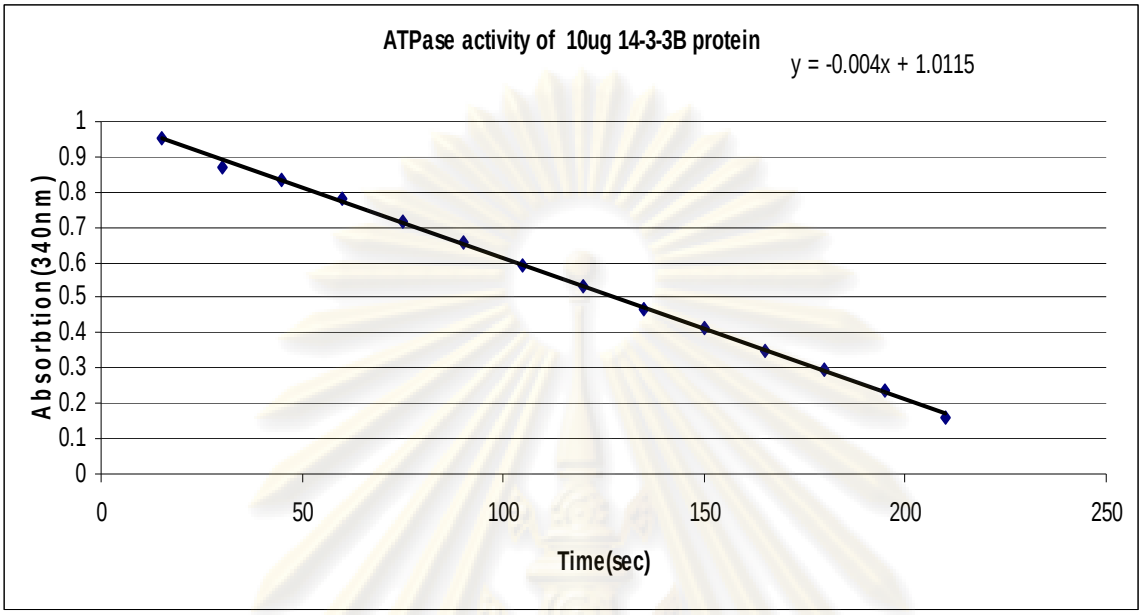
III. ATPase activity of 2.5ug 14-3-3B protein



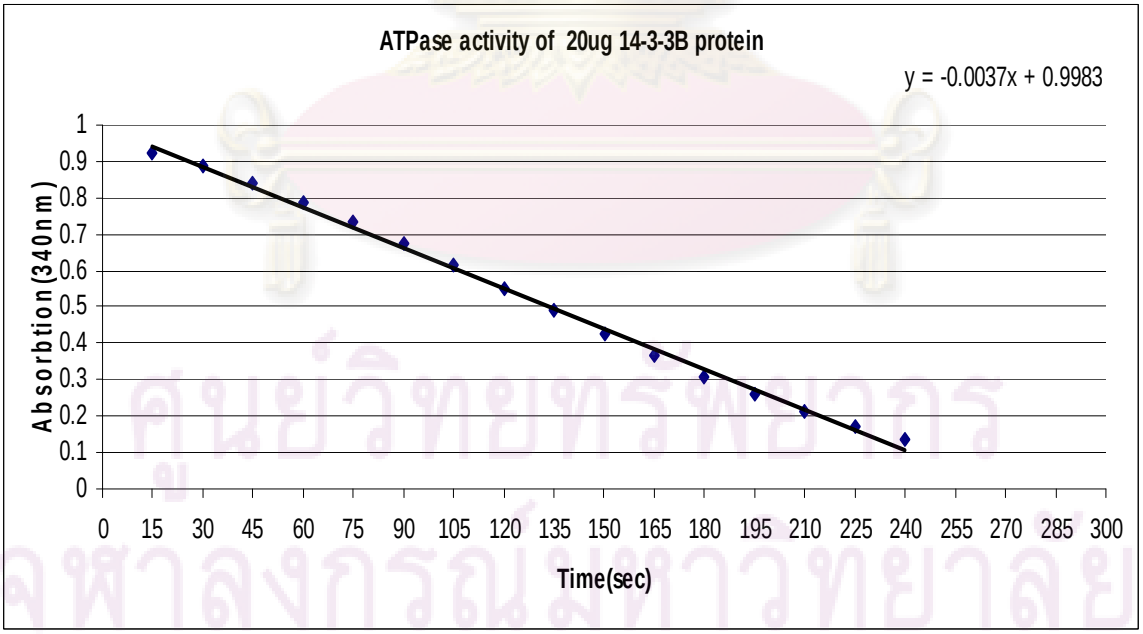
IV. ATPase activity of 5ug 14-3-3B protein



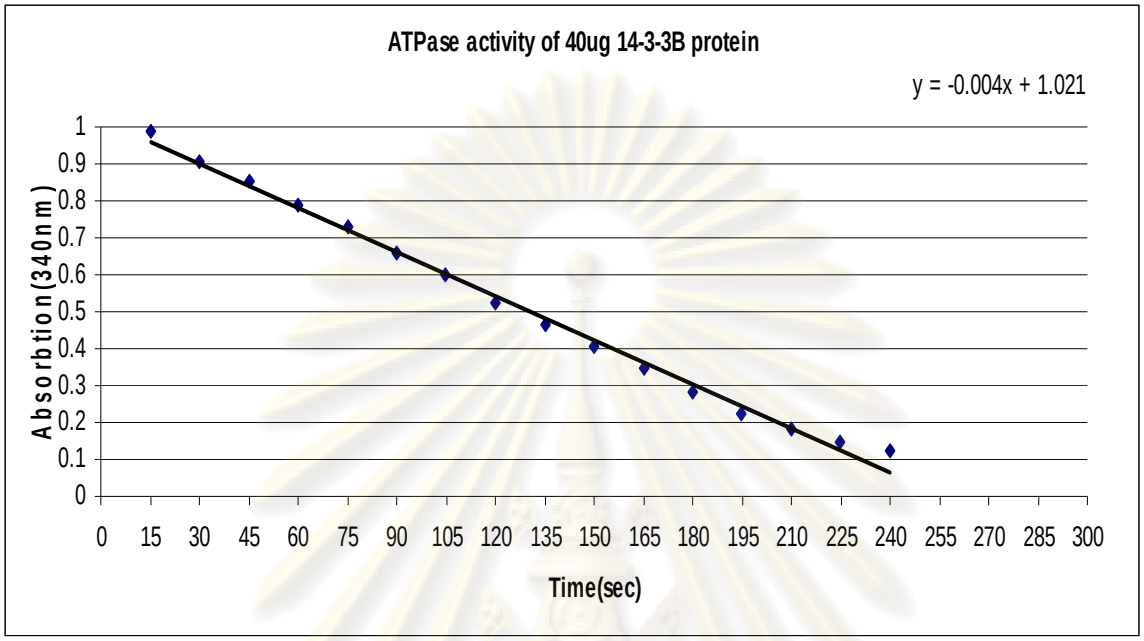
V. ATPase activity of 10ug 14-3-3B protein



VI. ATPase activity of 20ug 14-3-3B protein



VII. ATPase activity of 40ug 14-3-3B protein



Appendix C

I. Anova test of ATPase activity 1 day in shrimp injected dsRNA 14-3-3B

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.568	2	.284	19.500	.002
Within Groups	.087	6	.015		
Total	.656	8			

II. Dancan test of ATPase activity 1 day in shrimp injected dsRNA 14-3-3B

1 day

Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
3.0000	3	1.0645	
1.0000	3		1.5806
2.0000	3		1.6129
Sig.		1.000	.755

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

III. Anova test of ATPase activity 2 days in shrimp injected dsRNA 14-3-3B

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.539	2	.270	21.583	.002
Within Groups	.075	6	.012		
Total	.614	8			

IV. Dancan test of ATPase activity 2 days in shrimp injected dsRNA 14-3-3B

2 days

Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
3.0000	3	1.0323	
1.0000	3		1.5161
2.0000	3		1.5806
Sig.		1.000	.506

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

V. Anova test of ATPase activity 3 days in shrimp injected dsRNA 14-3-3B

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.475	2	.237	45.600	.000
Within Groups	.031	6	.005		
Total	.506	8			

VI. Dancan test of ATPase activity 3 days in shrimp injected dsRNA 14-3-3B

3days

Duncan

VAR00001	N	Subset for alpha = .05	
		1	2
3.0000	3	1.0323	
2.0000	3		1.4839
1.0000	3		1.5484
Sig.		1.000	.315

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

VII. Anova test of ATPase activity 4 days in shrimp injected dsRNA 14-3-3B

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.019	2	.009	1.500	.296
Within Groups	.037	6	.006		
Total	.056	8			

VIII. Dancan test of ATPase activity 4 days in shrimp injected dsRNA 14-3-3B

4 days

Duncan

VAR00001	N	Subset for alpha = .05
		1
3.0000	3	1.3548
1.0000	3	1.4516
2.0000	3	1.4516
Sig.		.197

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

IX. Anova test of ATPase activity 5 days in shrimp injected dsRNA 14-3-3B

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	2	.001	.059	.943
Within Groups	.106	6	.018		
Total	.108	8			

X. Dancan test of ATPase activity 5 days in shrimp injected dsRNA 14-3-3B

5 days

Duncan

VAR00001	N	Subset for alpha = .05
		1
1.0000	3	1.4194
2.0000	3	1.4194
3.0000	3	1.4516
Sig.		.783

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

XI. Anova test of ATPase activity 6 days in shrimp injected dsRNA 14-3-3B

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.027	2	.014	.765	.506
Within Groups	.106	6	.018		
Total	.133	8			

XII. Dancan test of ATPase activity 6 days in shrimp injected dsRNA 14-3-3B

6 days

Duncan

VAR00001	N	Subset for alpha = .05
		1
3.0000	3	1.4516
1.0000	3	1.4839
2.0000	3	1.5806
Sig.		.294

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

XIII. Anova test of ATPase activity 7 days in shrimp injected dsRNA 14-3-3B

ANOVA

VAR00002

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.006	2	.003	.600	.579
Within Groups	.031	6	.005		
Total	.037	8			

XIV. Dancan test of ATPase activity 7 days in shrimp injected dsRNA 14-3-3B

7 days

Duncan

VAR00001	N	Subset for alpha = .05
		1
2.0000	3	1.4839
3.0000	3	1.5161
1.0000	3	1.5484
Sig.		.330

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Appendix D

I. T-Test of mortality rates of farm I (day1) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water.

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002									
Equal variances assumed	16	0.0161	10	4	0	33.33	3	24.078516	43
Equal variances not assumed			10	2	0.01	33.33	3	18.991158	48

II. T-Test of mortality rates of farm I (day2) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water.

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002									
Equal variances assumed	0	1	10	4	0.000447	50	4.714045	36.91171	63.08829
Equal variances not assumed			10	4	0.000447	50	4.714045	36.91171	63.08829

III. T-Test of mortality rates of farm I (day3) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water.

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002 Equal variances assumed	0	1	14	4	0.00012	70	4.714045	56.91171	83.08829
Equal variances not assumed			14	4	0.00012	70	4.714045	56.91171	83.08829

IV. T-Test of mortality rates of farm I (day4) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002 Equal variances assumed	16	0.01	23	4	2.12E-05	76.66667	3.333333	67.41185	85.92148
Equal variances not assumed			23	2	0.001885	76.66667	3.333333	62.32449	91.00884

V. T-Test of mortality rates of farm II (day1) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water.

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002									
Equal variances assumed	0	1.000	8.485	4	0.001	0.4	0.04714	0.26912	0.53088
Equal variances not assumed			8.485	4	0.001	0.4	0.04714	0.26912	0.53088

VI. T-Test of mortality rates of farm II (day2) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water.

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002									
Equal variances assumed	16	.016	13	4	.000	0.4333	0.3333	0.34079	0.52588
Equal variances not assumed			13	2	.006	0.4333	0.3333	0.34079	0.52588

VII. T-Test of mortality rates of farm II (day3) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water.

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002 Equal variances assumed	16	.016	13	4	.000	0.4333	0.3333	0.34079	0.52588
Equal variances not assumed			13	2	.006	0.4333	0.3333	0.28991	0.57676

VIII. T-Test of mortality rates of farm II (day4) between the presence and absences the expressed 14-3-3B gene in salt stressed at low salinity water

Independent Samples Test									
	Levene's Test for Equality of Variances		t-test for Equality of Means		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	F	Sig.	t	df				Lower	Upper
VAR00002 Equal variances assumed	16	.016	13	4	.000	0.4333	0.3333	0.34079	0.52588
Equal variances not assumed			13	2	.006	0.4333	0.3333	0.28991	0.57676

BIOGRAPHY

Miss Montira Kaeodee was born on September 9, 1982 in Prae. She graduated with the degree of Bachelor of Science from the Department of Biotechnology, Faculty of Science, Ramkhumhang University in 2005. She has studied for the degree of Master of Science at the department of Biotechnology, Chulalongkorn University since 2005.