โปรทีโอมและลักษณะสมบัติของจีนและโปรตีนที่มีหน้าที่เกี่ยวข้องกับการสร้างไข่ของกุ้งกุลาดำ *Penaeus monodon*

นางสาววิชชุลดา ตาลาคุณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

PROTPROTEOME AND CHARACTERIZATION OF GENES AND PROTEINS FUNCTIONALLY INVOLVED IN OOGENESIS OF THE BLACK TIGER SHRIMP *Penaeus monodon*

Miss Witchulada Talakhun

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2013

Thesis Title	PROTPROTEOME AND CHARACTERIZATION OF GENES AND PROTEINS FUNCTIONALLY INVOLVED IN OOGENESIS OF THE BLACK TIGER SHRIMP *Penaeus monodon*
By	Miss Witchulada Talakhun
Field of Study	Biotechnology
Thesis Advisor	Professor Piamsak Menasveta, Ph.D.
Thesis Co-Advisor	Bavornlak Khamnamtong, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Thaithaworn Lirdwitayaprasit, Ph.D.)

.....Thesis Advisor
(Professor Piamsak Menasveta, Ph.D.)

.....Thesis Co-Advisor
(Bavornlak Khamnamtong, Ph.D.)

.....Examiner
(Assistant Professor Sanit Piyapattanakorn, Ph.D.)

.....Examiner
(Kittinan Komolpis, Ph.D.)

.....External Examiner
(Sirawut Klinbunga, Ph.D.)

.....External Examiner
(Sittiruk Roytrakul, Ph.D.)

วิชชุลดา ตาลาคุณ : โปรทีโอมและลักษณะสมบัติของจีนและโปรตีนที่มีหน้าที่เกี่ยวข้องกับการสร้างไข่ของกุ้งกุลาดำ *Penaeus monodon*. (PROTPROTEOME AND CHARACTERIZATION OF GENES AND PROTEINS FUNCTIONALLY INVOLVED IN OOGENESIS OF THE BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. เปี่ยมศักดิ์ เมนะเศวต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. บวรลักษณ์ คำน้ำทอง, 248 หน้า.

ศึกษาโปรติโอมิกส์ของโปรตีนในรังไข่ของกุ้งกุลาดำที่ปรับปรุงพันธุ์ และกุ้งจากธรรมชาติด้วยวิธี GeLC-MS/MS พบจำนวนโปรตีนที่เหมือนกับโปรตีนจากฐานข้อมูลจำนวน 1638 โปรตีน โดย 1253 โปรตีน (76.50%) จัดเป็นโปรตีนที่ทราบหน้าที่ และพบว่ามีโปรตีนจำนวน 514 โปรตีน ที่มีระดับการแสดงออกที่แตกต่างกันระหว่างกลุ่มตัวอย่าง นอกจากนี้ศึกษาโปรตีนที่เกี่ยวข้องกับการสลายเยื่อหุ้มนิวเคลียสด้วยวิธีการเดียวกัน พบโปรตีนที่เหมือนกับฐานข้อมูลจำนวนทั้งหมด 724 โปรตีน เมื่อวิเคราะห์ตำแหน่งที่แสดงออกจากการสืบค้นจากฐานข้อมูล พบว่ามีโปรตีนจำนวน 89 โปรตีนที่มีตำแหน่งการแสดงออกที่เยื่อหุ้มนิวเคลียส และพบ 99 โปรตีนที่มีตำแหน่งการแสดงออกในนิวเคลียส ในวิทยานิพนธ์นี้สนใจโปรตีนที่เกี่ยวข้องกับกระบวนการส่งสัญญาณได้แก่ protein kinase C และ cyclic AMP regulated-protein like protein โปรตีนที่เกี่ยวข้องกับกระบวนการขนส่งโปรตีนคือ valosin-containing protein , thymosin beta และ Rac GTPase-activating protein 1 และโปรตีนที่เกี่ยวข้องกับการสลายของเยื่อหุ้มนิวเคลียสคือ nuclear pore complex protein NUP133 และ semaphorin-2a ดังนั้นจึงหาลำดับนิวคลีโอไทด์ที่สมบูรณ์ของจีน *Valosin containing protein* (*PmVCP*) พบว่ามีความยาว 2724 คู่เบสมี ORF เท่ากับ 2481 เบส แปรรหัสเป็น 826 อะมิโน, *Thymosin beta* (*PmTmsb*) พบว่ามีความยาว 1084 คู่เบส มี ORF เท่ากับ 387 เบส แปรรหัสเป็น 128 กรดอะมิโน, *Protein kinase C* (*PmPKC*) พบว่ามีความยาว 3404 คู่เบส มี ORF เท่ากับ 2235 เบส แปรรหัสเป็น 744 กรดอะมิโน, *cyclic AMP-regulated protein like protein* (*PmcAMP-RLP*) พบว่ามีความยาว 1272 คู่เบส มี ORF เท่ากับ 435 เบส แปรรหัสเป็น 114 กรดอะมิโน และ *Nuclear pore complex protein NUP133* (*PmNUP133*) พบว่ามีความยาว 4130 คู่เบส มี ORF เท่ากับ 3228 เบส แปรรหัสเป็น 1085 กรดอะมิโน นอกจากนี้สามารถแยกส่วน ORF ของจีน *Rac GTPase activating protein 1* (*PmRacgap1*) พบว่ามี ORF ยาว 1881 คู่เบส แปรรหัสเป็น 626 กรดอะมิโน

ศึกษาระดับการแสดงออกของจีนระหว่างการพัฒนารังไข่ของกุ้งกุลาดำพบว่าจีน *PmVCP* มีการแสดงออกที่ไม่แตกต่างกันระหว่างการพัฒนารังไข่ของกุ้งเต็มวัยปกติจากธรรมชาติ แต่การแสดงออกของ *PmVCP* สูงขึ้นในระยะไวเทลโลเจเนซิส และระยะรังไข่ที่สมบูรณ์ในกุ้งธรรมชาติที่ตัดก้านตา โดยการตัดก้านตาส่งผลให้การแสดงออกของจีนนี้ในรังไข่ระยะที่ 4 สูงกว่าในกุ้งธรรมชาติปกติ ส่วนจีน *PmTmsb* มีการแสดงออกที่ไม่แตกต่างกันระหว่างการพัฒนารังไข่ของกุ้งเต็มวัยปกติจากธรรมชาติ แต่การแสดงออกของ *PmTmsb* สูงขึ้นในระยะที่ 2 และ 4 ในกุ้งธรรมชาติที่ตัดก้านตา โดยการตัดก้านตาส่งผลให้การแสดงออกของจีนนี้ในรังไข่ระยะที่ 3 ต่ำกว่าในกุ้งธรรมชาติปกติ สำหรับจีน *PmcAMP-RPL* และ *PmPKC* นั้นมีการแสดงออกที่ไม่แตกต่างกันระหว่างการพัฒนารังไข่ของกุ้งเต็มวัยธรรมชาติปกติและกุ้งที่ตัดก้านตา อย่างไรก็ดีการตัดก้านตาส่งผลให้การแสดงออกของจีน *PmPKC* ในรังไข่ระยะที่ 1-4 ต่ำกว่าในกุ้งธรรมชาติปกติ โดยจีน *PmcRacgap1* มีการแสดงออกที่ไม่แตกต่างกันระหว่างการพัฒนารังไข่ของกุ้งเต็มวัยธรรมชาติปกติและกุ้งที่ตัดก้านตา เมื่อศึกษาผลของการฉีดโปรเจสเตอโรนและซีโรโตนินต่อการแสดงออกของจีนต่างๆในในกุ้งเลี้ยงขนาดแม่พันธุ์ พบว่าโปรเจสเตอโรนไม่ส่งผลต่อการแสดงออกของจีน *PmVCP* แต่ซีโรโตนินกระตุ้นการแสดงออกของจีน *PmVCP*, *PmRacgap1*, *PmcAMP-RPL* และ *PmPKC* แต่ไม่มีผลต่อการแสดงออกของ *PmTmsb*

ศึกษาการแสดงออกของโปรตีน PmVCP, PmRacgap1 และ PmTmsb ด้วยวิธี Western blot พบว่า PmVCP มีการแสดงออกในรังไข่ทุกระยะการพัฒนา โดยการแสดงออกของโปรตีน PmVCP นั้นไม่แตกต่างกันในรังไข่ระยะต่างๆ สำหรับ PmRacgap1 พบแถบโปรตีนจำนวนสองแถบ ที่ขนาดน้ำหนักโมเลกุลประมาณ 34 และ 100 kDa โดยพบว่าแถบโปรตีนขนาด 34 kDa มีการแสดงออกลดลงในรังไข่ระยะที่ 3 และ 4 ทั้งในรังไข่ของกุ้งกุลาดำปกติและกุ้งกุลาดำตัดตา ในขณะที่โปรตีน PmTmsb มีแถบโปรตีนที่ให้ผลบวกจำนวนสองแถบ ที่ขนาดน้ำหนักโมเลกุลประมาณ 22 และ 28 kDa โดยการแสดงออกของโปรตีนขนาด 28 kDa (thymosin-β-repeated protein 2) มีระดับลดลงในรังไข่ระยะที่ 4 ของกุ้งธรรมชาติเต็มวัยปกติ โดยไม่พบการแสดงออกของโปรตีนนี้ในรังไข่ระยะที่ 3 และ 4 ในกุ้งธรรมชาติที่ตัดก้านตา ศึกษาตำแหน่งการแสดงออกของโปรตีน PmVCP และ PmRacgap1 พบว่าโปรตีน VCP มีตำแหน่งการแสดงออกที่ ooplasm ในระยะ previtellogenic และมีการโยกย้ายเข้าสู่นิวเคลียส ในระยะ vitellogenic และนอกจากนั้นยังพบที่ nucleo-cytoplasm, cytoskelatal architechture และ plasma membrane ในระยะไข่ที่สมบูรณ์ (mature oocytes) ของกุ้งธรรมชาติปกติและกุ้งที่ตัดก้านตา ในขณะที่โปรตีน Racgap1 มีการแสดงออกที่ ooplasm ของไข่ทุกระยะ นอกจากนี้ยังพบการแสดงออกของ PmRacgap1ใน nucleo-cytoplasm, cytoskeletal architechture และใน cortical rod ของไข่ระยะสมบูรณ์ในกุ้งธรรมชาติปกติและกุ้งที่ตัดก้านตา

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5273852023 : MAJOR BIOTECHNOLOGY
KEYWORDS: PENAEUS MONODON / BLACK TIGER SHRIMP / ONE-DIMENSIONAL GEL ELECTROPHORESIS / MASS SPECTROMETRY

WITCHULADA TALAKHUN: PROTPROTEOME AND CHARACTERIZATION OF GENES AND PROTEINS FUNCTIONALLY INVOLVED IN OOGENESIS OF THE BLACK TIGER SHRIMP *Penaeus monodon*. ADVISOR: PROF. PIAMSAK MENASVETA, Ph.D., CO-ADVISOR: BAVORNLAK KHAMNAMTONG, Ph.D., 248 pp.

Proteomics of ovarian proteins in domesticated and wild broodstock of the giant tiger shrimp *Penaeus monodon* were studied using GeLC-MS/MS. In total, 1638 proteins were identified and 1253 (76.50%) proteins matched known proteins. Of these, 514 proteins were differential expressed between groups of samples. To characterized proteins involved in GVBD, nuclear membrane and nuclear proteins were identified. In total, 724 proteins were identified and matched known proteins. Localization of these proteins were searched and 89 and 99 proteins were classified as those integrated to membrane and nuclear proteins. In this thesis, proteins involved in signal transduction pathways and cytoskeletal reorganization during GVBD were characterized. The full-length cDNA of *Valosin-containing protein* (*PmVCP1*, 2724 bp with an ORF of 2481 deducing to 826 amino acids), *Thymosin beta* (*PmTmsb*; 1084 bp, an ORF = 387 bp, 128 aa), *Protein kinase C* (*PmPKC*; 3404 bp, an ORF = 2235 bp, 744 aa), *cyclic AMP-regulated protein like protein* (*PmcAMP-RLP*; 1272 bp, an ORF = 435 bp, 144 aa), *Nuclear pore complex protein NUP133* (*PmNup133*; 4130 bp, an ORF = 3228 bp, 1085 aa) were successfully characterized by RACE-PCR. In addition, the complete ORF of *Rac GTPase activating protein 1* (*PmRacgap1*; 1881 bp, 626 aa) was also successfully isolated.

The expression levels of *PmVCP* were not significant different throughout ovarian development of intact broodstock ($P > 0.05$). However, *PmVCP* was up-regulated during vitellogenesis and final maturation of eyestalk-ablated broodstock ($P < 0.05$) and its expression in stage IV ovaries was greater than that of the same stage in intact broodstock ($P < 0.05$). The expression level of *PmTmsb* was not differential expressed in ovaries of wild intact broodstock but it was up-regulated in stages II and IV ovaries in eyestalk-ablated broodstock ($P < 0.05$). Eyestalk ablation resulted in the reduction of this transcript in stage III ovaries ($P < 0.05$). The expression of *PmcAMP-RPL* was not different during ovarian development of intact and eyestalk-ablated broodstock. Similarly, the expression of *PmPKC* was not significantly expressed during ovarian development of intact and eyestalk-ablated brooodstock. Nevertheless, eyestalk ablation resulted in the reduction of this transcript in stages I-IV ovaries. The expression level of *PmRacgap1* was not differentially expressed during ovarian development of intact and eyestalk ablated broodstock ($P > 0.05$). In addition, effects of progesterone and serotonin (5-HT) administration in domesticated *P. monodon* were evaluated Results indicated that progesterone did not induce the expression of *PmVCP*. In contrast, the expression level of *PmVCP*, *PmRacgap1*, *PmcAMP-RPL* and *PmPKC* but not *PmTmsb* was stimulated by serotonin administration.

The expression profiles of ovarian PmVCP, PmTmsb and PmRacgap1 proteins were examined. PmVCP were observed in juvenile ovaries and at all stages of ovarian development in both intact and eyestalk-ablated broodstock of wild *P. monodon*. It seemed to be expressed at comparable levels for all stages of ovarian development in shrimp broodstock. Two immunoreactive bands (34 and 100 kDa) of PmRacgap1 was observed in ovarian membrane proteins. The expression level of PmRacgap1 reflected from a 34 kDa band seemed to be decreased in late stages of ovarian development (stages III and IV ovaries) in both intact and eyestalk-ablated broodstock. Anti-rPmTmsb PAb gave positive the immunoreactive signals of 22 and 28 kDa, respectively. The expression level of PmTmsb reflected from a positive 28 kDa band (thymosin-β-repeated protein 2) seemed to be decreased in mature (IV) ovaries in intact broodstock. In eyestalk-ablated broodstock, it was not expressed in late vitellogenic (III) and mature ovaries. Localization of PmVCP protein was observed in the ooplasm of previtellogenic oocytes and it was translocated into the nucleus of vitellogenic oocytes. Interestingly, it was found in nucleo-cytoplasmic compartments, the cytoskeletal architecture and the plasma membrane in mature oocytes of both intact and eyestalk-ablated broodstock. PmRacgap1 was observed in oogonia and ooplasm of all developmental stages of oocytes in both intact and eyestalk-ablated broodstock. During vitellogenesis, it was also observed in the nucleus of vitellogenic oocytes and subsequently, in nucleo-cytoplasmic compartments, the cytoskeletal architecture and in cortical rods of more mature oocytes of both intact and eyestalk-ablated broodstock.

Field of Study: Biotechnology
Academic Year: 2013

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest sense of gratitude to my advisor Professor Dr. Piamsak Menasveta and my co-advisor, Dr Bavornlak Khamnamtong, Dr. Sirawut Klinbunga and Dr. Sittiruk Roytrakul for their great helps, guidance, encouragement, valuable suggestion and supports throughout my study.

My gratitude is also extended to Associate Professor Dr. Thaithaworn Lirdwitayaprasit, Dr. Kittinan Komolpis and Assistant Professor Dr. Sanit Piyapattanakorn who serve as the committee of my thesis.

I would particularly like to thank the Center of Excellence for Marine Biotechnology, National Center for Genetic Engineering and Biotechnology (BIOTEC), Faculty of Science, Chulalongkorn University, National Science and Technology Development Agency (NSTDA) for providing facilities and a student grant was supported by the Thailand Graduate Institute of Science and Technology (TGIST), NSTDA award.

I would like to extend my special thank to all of every one in AAMG laboratory and the Proteomic laboratory, Genome Institute for valuable suggestions. In addition, many thanks are also expressed to all of every one in our laboratory for best friendship, their help and friendly assistance.

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

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

Page

Page

Page

Page

LIST OF ABBREVIATIONS

bp	base pair
°C	degree celcius
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
Kb	kilobase
kDa	kilo daltan
M	molar
$MgCl_2$	magnesium chloride
mg	mlligram

ml	mllilitre
mM	mllimolar
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
pI	isoelectric point
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolution per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
Tris	tris (hydroxyl methyl) aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet

CHAPTER I
INTRODUCTION

1.1 Background information

The giant tiger shrimp, *P. monodon* has dominated production of farmed shrimp along with the Pacific white shrimp (*Litopenaeus vannamei*) and is one of the most economically important penaeid species in South East Asia (Sambrook and Russell, 2001). Reduced reproductive maturation of captive *P. monodon* females is found (Kenway et al. (2006)); (Preechaphol et al., 2007). Accordingly, breeding of pond-reared *P. monodon* is extremely difficult and rarely produced enough quality of larvae required by the industry. In Thailand, farming of *P. monodon* in Thailand relies almost entirely on wild-caught broodstock for supply of juveniles (Sambrook and Russell, 2001). The lack of high quality wild and domesticated broodstock has probably caused the reduction of aquacultural production of *P. monodon* since the last several years.

The domestication and selective breeding programs of penaeid shrimp would provide a more reliable supply of seed stock and the improvement of their production efficiency. The use of selectively bred stocks having improved culture performance on commercially desired traits rather than the reliance on wild-caught stocks is a major mean of sustainability of the shrimp industry (Clifford and Preston, 2006) ; (Coman et al., 2006).

Several neurohormones are known to stimulate or inhibit ovarian maturation in crustaceans. These include the gonad inhibiting hormone (GIH) secreted by the X-organ sinus gland complex (XO-SG), the gonad stimulating hormone (GSH) secreted

from brain and thoracic ganglion as well as other steroids and terpenoids (Huberman, 2000). The removal of the sources of gonad inhibiting hormone by unilateral eyestalk ablation has commonly been used to induce maturation of the female of closed thelycum shrimp species, like *P. monodon*. However, the technique leads to an eventual loss in egg quality and death of the spawner (Benzie, 1998). Therefore, predictable maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is an ultimate goal for the industry (Quackenbush, 2001).

Several biotechnological areas including investigation of genetic variation (identification of stocks) and genome structure, controls of reproduction and growth, domestication of strains exhibiting required economically important traits (e.g. specific pathogen resistant, SPR and/or fast growing strains) are thought to have significant impact in the industry of this species. Moreover, identification and characterization of genes involving with reproductive maturation and mechanisms of sex differentiation and their expression patterns can be directly applied for selection of high quality pond-reared *P. monodon*.

Nevertheless, genetic improvement of *P. monodon* is slow owing to the lack of the basic information related with ovarian development and maturation in penaeid shrimp. An initial step toward understanding molecular mechanisms of ovarian and oocyte development in *P. monodon* is the identification and characterization of genes/proteins differentially expressed in different stages of ovaries in this economically important species (Preechaphol et al., 2007).

In penaeid shrimp, the mature oocyte arrests at the first meiotic prophase and oocytes reach complete metaphase I after ovulation (Yano, 1995). It remains unknown whether crustaceans possess a gonadotropin homologue that can trigger

the meiotic resumption during final oocyte maturation as those in most vertebrate animals such as mammals, fishes and amphibians. Additionally, there have been no reports associated with the characterization of a maturation promoting factor (MPF) and its regulatory mechanisms for final oocyte maturation, which is an important aspect towards artificial control of maturation of the commercially important species and will contribute to a sustainable production of the shrimp industry (Qiu and Yamano, 2005).

The development of oocytes consists of a series of complex cellular events, in which different genes express to ensure the proper development of oocytes and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu and Yamano, 2005). Different biotechnological approaches, for example; injection of vertebrate steroid hormones, neurotransmitters and ecdysteroids (Benzie, 1998); (Okumura, 2004) and the use of specially formulated feed (Harrison, 1990) have been applied to induce the ovarian maturation of female shrimp but results are inconsistent owing to limited knowledge on genetic and hormonal control of penaeid species (Meusy and Payen, 1988) ; (Okumura, 2004). An understanding of gene expression involving ovarian maturation between normal and eyestalk ablated *P. monodon* are useful for genetic improvement of this economically important species.

Discovery of genes expressed in ovaries of *P. monodon* has been reported based on EST (single-pass sequencing of randomly selected clones from cDNA libraries, (Preechaphol et al., 2007) and suppression subtractive hybridization (SSH) (Leelatanawit et al., 2004) ; (Preechaphol et al., 2010) analyses. Several reproduction-related genes (e.g. *anaphase promoting complex subunit 11*, *selenoprotein M*

precursor, *chromobox protein*, *ovarian lipoprotein receptor*, *progestin membrane receptor component 1* and *ubiquitin-specific proteinase 9, X chromosome*) were isolated. Nevertheless, cellular proteomic profiles of proteins expressed during ovarian development of *P. monodon* have not been reported.

Yamano et al. (2004a) illustrated that in most cases ovaries of the kuruma prawn (*Marsupenaeus japonicus*) start to develop in the reproductive season but fail to reach full grown requisite for the formation of cortical rods (CRs). Ovaries degenerate without spawning. This is also the major constraint in *P. monodon*. Reduced spawning potential and low degree of maturation of *P. monodon* in captivity crucially prohibits the efficiency of the genetic improvement through domestication and selective breeding programs in this species.

Understanding mechanisms and functions of genes and proteins involved in oogenesis would provide a tool applicable for understanding of their important biological and molecular processes and finally, for improving cognitive processes in oogenesis of *P. monodon*. Nevertheless, identification of proteins in ovaries during ovarian development of penaeid shrimp have not been performed and reported in any species.

Proteomic technique is a powerful and wildly used method for analysis of protein mapping and expression of interesting expressed proteins in various cells and tissues of organisms. Proteomic techniques provide the basic information on protein expression profiles and post-translational modification of interesting proteins. Molecular mechanisms and expression patterns of proteins controlling each step of

oocyte maturation and formation of CRs could be further carried out for better understanding the reproductive maturation of *P. monodon* in captivity.

In this study, proteins differentially expressed in ovaries of *P. monodon* were identified and characterized by proteomic approaches (1D-gel electrophoresis, molecular imager and nanoESI-LC-MS/MS). The full-length cDNA of the identified proteins (e.g. protein kinase C, Rac GTPase-activating protein 1) were isolated. Recombinant proteins and their polyclonal antibodies were produced. Expression profiles of interesting genes and proteins during ovarian development of *P. monodon* were examined by quantitative real-time PCR and western blot analysis and ELISA, respectively. Localizations of their mRNA and proteins are examined by *in situ* hybridization and immunofluorescence, respectively.

1.2 Objective of this thesis

The objectives of this thesis were determination of protein profiles in domesticated and wild female *P. monodon* broodstock by GeL-LC-MS/MS (SDS-PAGE followed by nanoESI-LC-MS/MS. In addition, the full-length cDNA, expression and localization of interesting genes and proteins involved in oogenesis were examined.

1.3 General introduction

1.3.1 Taxonomy of *P. monodon*

The giant tiger shrimp is taxonomically classified as a member of Phylum Arthropoda; Subphylum Crustacea; Class Malacostraca; Subclass Eumalacostraca; Order Decapoda; Suborder Natantia; Infraorder Penaeidea; Superfamily Penaeoidea; Family Penaeidae, Rafinesque, 1985; Genus Penaeus, Fabricius, 1798 and Subgenus *Penaeus*. The scientific name of shrimp is *Penaeus monodon* (Fabricius, 1798) where

the English common name is giant tiger shrimp or black tiger prawn (Bailey-Brock and Moss, 1992).

The external morphology of *P. monodon* and sex characteristics of male (petasma) and female (thelycum) are illustrated in Figure 1.1.

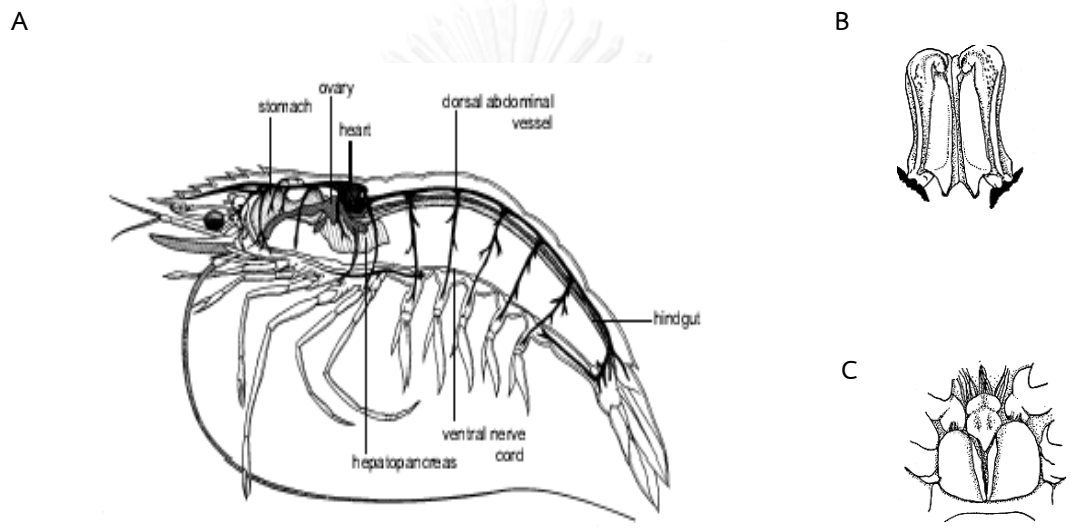


Figure 1.1 External morphology of *P. monodon* (A). Sexes of juveniles and broodstok of penaeid shrimp can be externally differentiated by petasma of male (B) and thelycum of female (C).

1.3.2 Oogenesis and formation of cortical rods in shrimp

In penaeid shrimp, ovarian development is characterized by the accumulation of a major yolk protein and cortical rods formation in the oocytes (Okumura *et al*., 2006). The oocytes of shrimp develop from mitotically dividing oogonia. As the daughter oogonia develop, they increase in size and enter the first stage of meiotic division and migrate away from the zone of proliferation toward the periphery of the ovarian follicles.

During this time, the follicular cell surrounding the growing oocytes produced the vitellogenin protein which constitutes the yolk. The yolk vitellogenin is also produced at extraovarian sites, especially the hepatopancreas. The process of synthesis and accumulation of vitellogenin in the oocytes is called vitellogenesis (Kruevaisayawan et al., 2010). The knowledge about ovarian development stage and morphological characteristic of the oocytes have reported in *Marsupenaeus jarponicus* and *Metapenaeus ensis*.

Recently, oogenesis and formation of cortical rods in the black tiger shrimp *Penaeus monodon* have studied the ultrastructure of the developing oocytes by transmission electron microscopy (Kruevaisayawan et al., 2010). The unique characteristics of the stage 1 oocyte (Oc_1) were the presence of several prominent nucleoli and abundant ribosomes. These characteristics indicated that there is a highly rate nucleoli into the cytoplasm via the numerous nuclear pores (Figure 1.2A). The above characteristics are similar to those of other shrimps, including *P. aztecus*, *P. setiferus*. The stage 2 oocyte (Oc_2) was characterized by an increase in the number of rough endoplasmic reticulum (RER) which started expansion. Thus, Oc_2 represent the initial stage of active protein synthesis, possibly involved with yolk production which later becomes a major component in developing oocytes (Figure 1.2B). Synthesis of other major proteins, particularly CRs, would also be initiated. In the stage 3 oocyte (Oc_3), the RER was well-developed and appeared highly dilated, and distributed throughout the cytoplasm, indicating that this stage of the oocyte is highly active in the protein synthetic activity. Moreover, the same event is also to appear on vitellogenic oocytes of many crusteceans, including lobters and other shrimps. Additionally, an increasing number of nuclear pores in this stage signify an

increased tramsport between the nucleus and cytoplasm. Furthermore, the cytoplasm of Oc_3 started to be filled with yolk granules reflecting the high rate of vitellogenasis another unique ultrastructeral feature of Oc_3 is the presence of numerous of lipid droplets in the cytoplasm.

It is not known how lipid droplets are formed. In *P. kerathurus*, it was suggested that lipid droplets in oocytes are formed from complex vesicular bodies derived from the nuclear envelope, ER and Golgi complexs. In mature oocyte (Oc_4), the appearance of rod-like structures which marks the main feature of this stage in many penaeid shrimp but lacking of in other crusteceans. In contrast to Oc_3, the dilated RER is not as noticeable in the Oc4 suggesting a reduction of synthetic activity during the final stage of maturation. The synthesis of CR is initiated in Oc_2 and increases significantly in the well-developed RER of Oc_4 as shown by the deposition of numerous gold particles in dilated RER of Oc_4. Some CR granules appear to fuse with the Oc_4 plasma membrane that bordered the CR crypt. Thus, this material may be released by exocytosis of CR granules at the periphery of an oocyte into the crypts where it is assembled into CR structure during oocyte maturation (Figure 1.2C-D).

1.3.3 Ovarian development of *Penaeus monodon* and hormonal control in reproductive maturation in shrimp

In penaeid shrimp, the major part of ovaries is found within the cephalothorax area. Ovaries are paired, but partially fused in the cephalothoracic region, and consist of a number of lateral lobes. The intensity of the ovarian shadow is due to the different density of the ovaries and the pigmentation of the egg mass. The ovarian development of penaeid shrimp are generally classified to four different

stages; underdeveloped stage (Stage I), developing or early vitellogenic stage (Stage II), nearly ripe or late vitellogenic stage (Stage III) and ripe or mature stage (Stage IV; Figure 1.3).

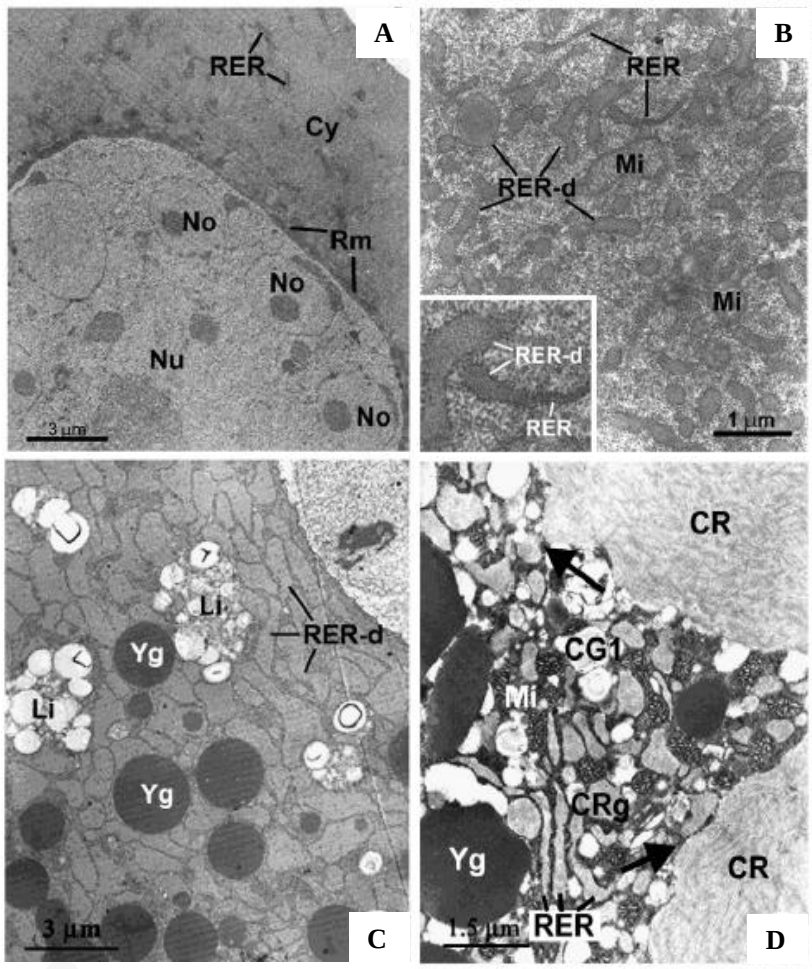


Figure 1.2 TEM micrograph and ultrastructure of oocyte of *P. monodon* stage I-IV. (A) Each nucleolous (No) becomesenlarged and forms a dense peripheral ring surrounding a central dense mass in the late OC1. (B) OC2 showing numerous rough endoplasmic reticulums (RER) that are well-developed throughout the cytoplasm and some of which begin to be dilated (RER-d). (C) The cytoplasm is filled with the electron dense yolk granules (Yg), (RER), ribosome and aggregates lipid droplets (Li). RER are well-developed highly dilated (RER-d) and filling the entire cytoplasm. OC4 showing the cortical rod (CR) at the cells periphery and the cytoplasm is filled with yolk granules (Yg), mitochondria (Mi), RER, and cortical rod granules (CRg). The sites where cortical rod granules join the oolemma at the crypts containing cortical rod are indicated by arrows (Kruevaisayawan et al., 2010).

In an undeveloped stage, the ovaries either do not cast any shadow or a thin opaque line is seen along the length of the tail. At this point the ovaries are composed of a connective tissue capsule surrounding a soft vascular area containing oogonia, and accessory cells (called follicle or nurse cells, Figure 1.3A). The internal wall of the ovary capsule is lined with epithelial cells (called the germinal epithelium). Once the female is sexually mature, the germinal epithelium will produce oogonia by mitosis division throughout the reproductive life of the females.

The eggs develop from oogonia in an area known as the zone of proliferation. As the oogonia develop they increase in size and enter the first stage of meiotic division and henceforth are irreversibly destined to become haploid, with only one set of maternal chromosomes. At this point, although the developing eggs are increasing in size (Figure 1.3B) they are not as yet producing yolk, and are known as previtellogenic oocytes. At this stage the ovaries can be visualized with a light beam as a large centrally located opaque rope-like structure, and classified as the stage 2.

As the oocytes develop, they migrate towards the margins of the ovarian lobes in preparation for ovulation. During this migration, follicle cells are attached to the periphery of each oocyte. It is believed that the follicle cells produce the yolk that is internal in the oocytes in a process called vitellogenesis. As vitellogenesis proceeds, oocytes mature synchronously as yolk accumulates and develop a characteristic dark green colour as a result of deposition of carotenoid pigments. It is the carotenoid pigmentation that mainly causes the dark ovarian shadow during illumination of the female by the torchlight. The female is now in the stage 3 (Figure 1.3C).

By the end of vitellogenesis, the eggs develop cortical granules filled with a jelly-like substance destined to form part of the egg shell membrane after ovulation. At this time the shadow cast by the ovaries is large, resulting in a very distinct dark thick region extending the length of the abdomen, with an enlarged bulbous region directly behind the carapace, called the saddle. The saddle may not be as apparent in some broodstock. The female is now in a pre-spawning state and is regarded as in the stage 4.

Penaeid shrimp are divided into two groups: open-thelycum and closed-thelycum species. In the life history of penaeid shrimps, final maturation, spawning and mating behaviour presage successful reproduction. The final phase of maturation, spawning, mating and their interrelationships differ significantly between the groups. The giant tiger shrimp is one of the closed-thelycum species. Typically, mature males insert their spermatophore into the soft thelycum of newly moulted immature females. Final maturation with germinal vesicle breakdown (GVBD) immediately precedes spawning in a closed thelycum species (Yano, 1988). Two phases are involved: the appearance of ripe ovary and germinal vesicle breakdown (GVBD) in preparation for fertilization after spawning (Figure 1.4).

Ovulation occurs when nuclei, shrunken during the late prematuration phase, have migrated to the peripheral cytoplasm of the oocytes. In the late phase of the maturation cycle, meiotic metaphase is arrested and remains visible just beneath the cytoplasmic membrane of the oocyte, indicating that GVBD is completed after ovulation.

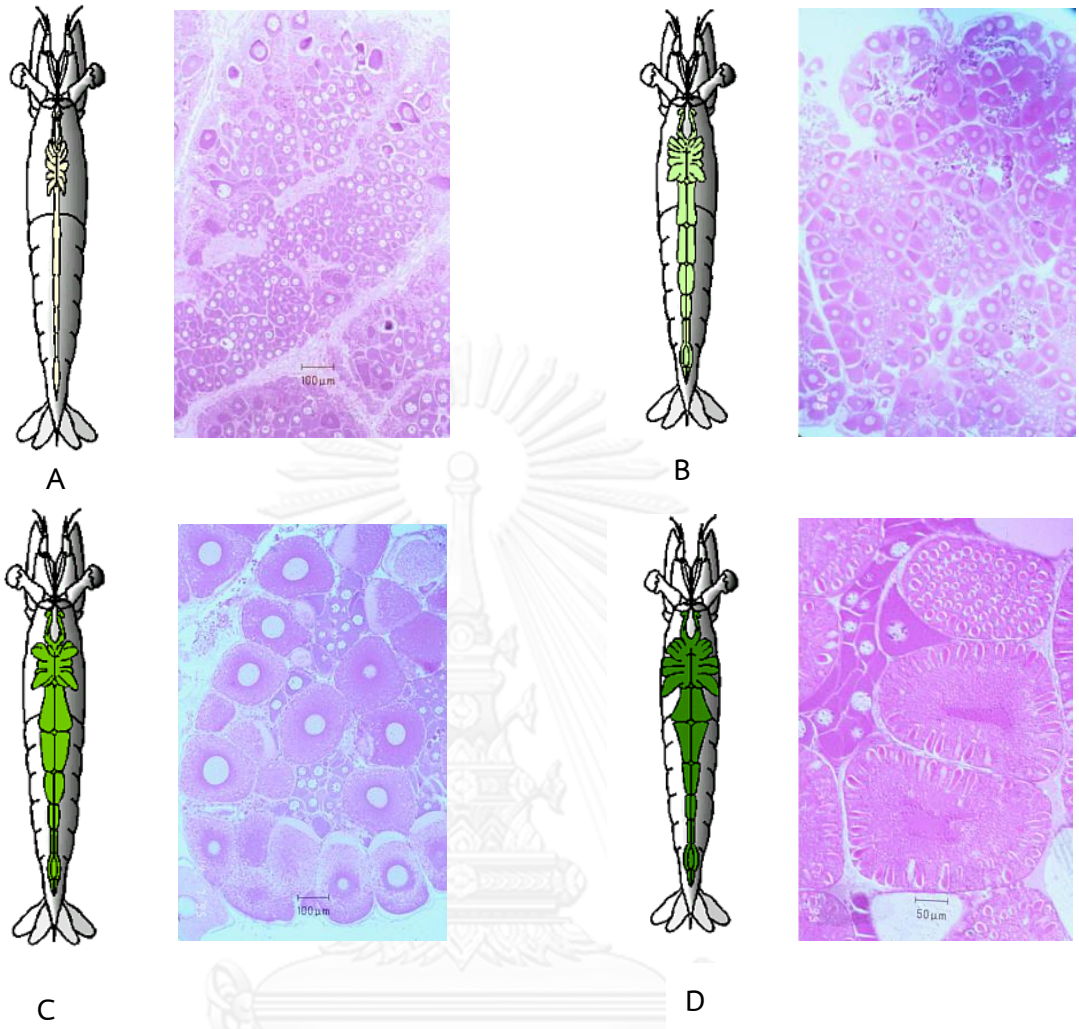


Figure 1.3 Different ovarian development stages of *P. monodon.* Panel A., theunderdeveloped ovaries (Stage I), B., the developing stage (Stage II), C., the nearly ripe stage (Stage III) and D., the ripe stage (Stage IV) (www.aims.gov.au/.../mdef/images/fig01-4a.gif).

Immediately after release from the female gonopore, the mature eggs, still in metaphase (for example in *Marsupenaeus. japonicus*), are fertilized by sperm released into the seawater from the spermatophore held in the thelycum. Once begun, spawning is continuous, females releasing batches of eggs from the ripe ovaries and sperm from the spermatophore into the seawater, where fertilization takes place. Therefore, female shrimp have to repeat the process of molting, mating, and sexual maturation in order to achieve several spawning during their life.

Prematuration accumulation of egg-yolk protein (vitellin) in developing oocytes at the yolk-granule stage, occurs approximately 1 month after spawning without mating in spent shrimps (for example in *M. japonicus*). Female shrimp (closed thelycum), do not mature for several months, even after mating with spermatophore transfer in the season from late autumn to early spring (Yano, 1995). These observations indicate that mating dose not directly accelerate vitellogenesis in closed-thelycum species.

1.3.4 Hormonal studies in shrimp

Biological and physiological processes (growth, reproduction, body color, and metabolism etc.) are hormonal controlled (Figure 1.5). Knowledge from shrimp endocrinology is necessary to develop the hormonal manipulation techniques in shrimp

Eyestalk hormones play the important role for regulating several physiological mechanisms and unilateral eyestalk ablation is practically used for induction of ovarian development and oviposition. The technique gives predictable peaks of maturation and spawning but many associated problems, which leads to an eventual

loss in egg quality and causes high mortality and death (Benzie, 1998). Predictable maturation and spawning in captive shrimp without the use of eyestalk ablation is a long–term goal for the industry (Quackenbush, 1991).

Crustacean hyperglycemic hormone (CHH) is a member of a structurally related peptide family, which also includes molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH) and mandibular organ-inhibiting hormone (MOIH). It is the most abundant peptide in the eyestalk of crustaceans (Chang et al., 1990).

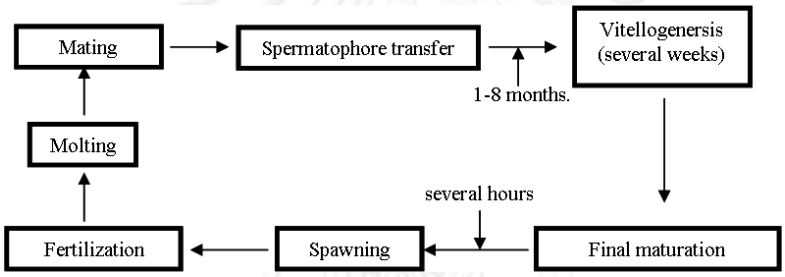


Figure 1.4 Reproductive cycles of the close-thelycum penaeid shrimp (Yano, 1995).

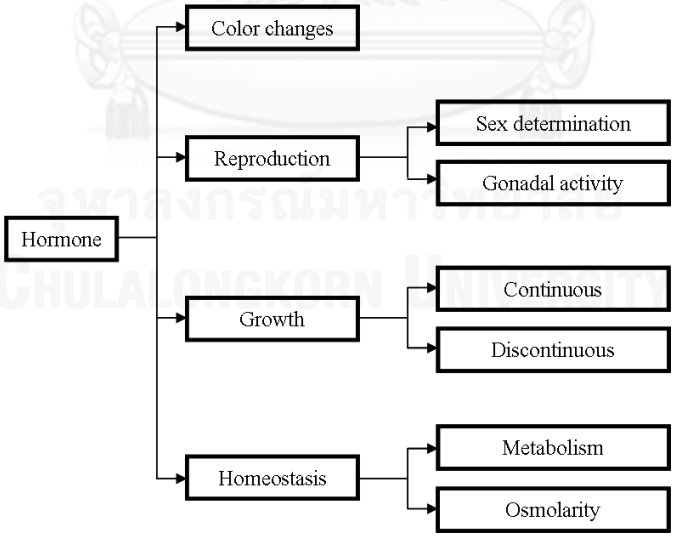


Figure 1.5 Diagram illustrating the hormonal controls of physiological processes of penaeid shrimp (Yano, 1995).

This hormone not only plays its major role in controlling the glucose level in the hemolymph, but is also significantly contributed to other processes such as ecdysteroid synthesis and ovarian maturation. Multiple forms of CHH have been reported. CHH has been isolated from several crustaceans such as crabs, (Kegel et al., 1989) ; (Chung et al., 1998), lobster (Tensen et al., 1991), crayfishes (Kegel et al., 1991) ; (Huberman et al., 1993), shrimp (Sithigorngul et al., 1999) as well as isopod (Martin et al., 1984).

The growth in crustaceans is not continuous because of the rigid exoskeleton. It is often shed to allow periodic growth. Molting is controlled by a complex interplay of hormones (Figure 1.6), in particular, the negative regulation of molt-inhibiting hormones (MIH) from the X-organ/sinus gland (XO/SG) complex which suppresses the synthesis or secretion of molting hormones (ecdysteroids) from the Y-organ (Figure 1.7).

MIH is classified as a member of the crustacean hyperglycemic hormone (CHH) family and has been shown to inhibit the synthesis of the molting hormone, ecdysone, which release from the Y-organ of decapod crustaceans keeps the animal in the intermolt stage that dominates its molting cycle. MIH is thus one of the major keys in mediating growth and reproduction.

Udomkit et al. (2004) cloned and characterized *P. monodon CHH* transcripts and produced recombinant Pem-CHH2 and Pem-CHH3 peptides, a member of a structurally related CHH/MIH/GIM/MO-IH peptide family. Both cDNAs contained 381-bp open reading frame encoding 127 amino acids.

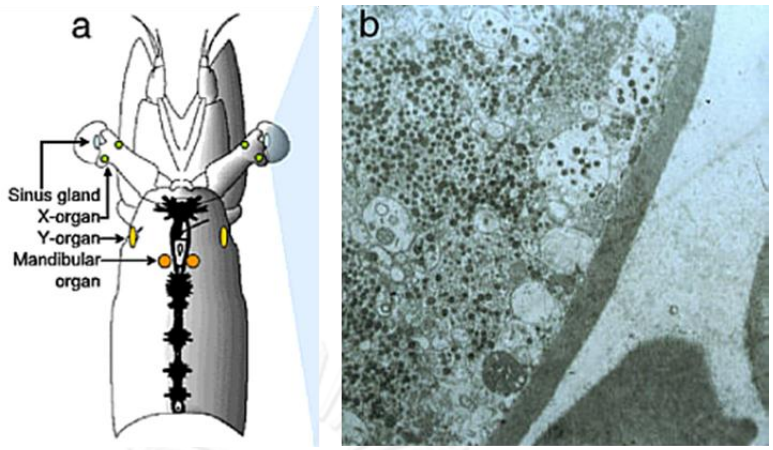


Figure 1.6 (a) A schematic diagram illustrating the major endocrine organs in shrimp. The sinus gland is composed of the terminals from neurons which have their cell bodies in the X-organ and brain. (b) Electron microscopy section (8500X) of the sinus gland demonstrating hormone filled vesicles (dark circles) which are fused and released their contents into the blood. (www.aims.gov.au/.../mdef/images/fig01-4a.gif)

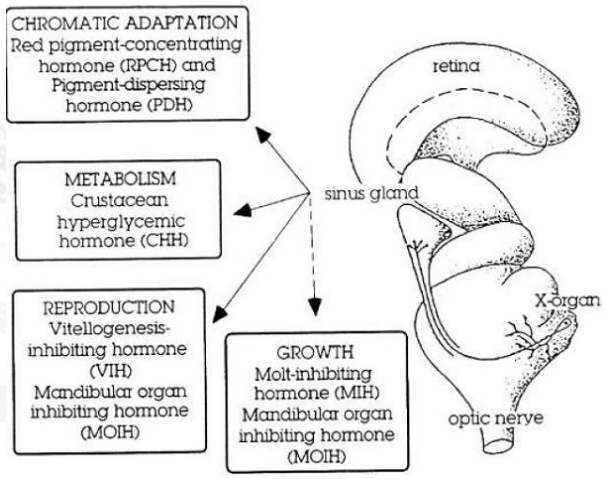


Figure 1.7 Localization of hormones that control several physiological systems from the sinus gland/X-organ complex of *P. monodon* (Okumura, 2004).

Amino acid sequence analysis revealed that Pem-CHH2 and Pem-CHH3 shared 95% identity in their amino acid sequence to that of Pem-CHH1 (Udomkit et

al., 2000). Both recombinant Pem-CHH2 and Pem-CHH3 expressed as secreted proteins in *Pichia pastoris* exhibited the hyperglycemic activity at the comparable level to that of Pem-CHH1. The *Pem-CHH* transcript in several tissues of *P. monodon* was examined by RT-PCR. Expression of Pem-CHH1, Pem-CHH2, Pem-CHH3 was not restricted only to the eyestalk but also detectable in heart. In addition, the transcript of Pem-CHH1 was also present in gills. CHHs form various origins may play different physiological roles.

Yodmuang et al. (2004) isolated cDNA encoding two types of *MIH*, *Pem-MIH1* and *Pem-MIH2* of *P. monodon* by direct PCR amplification and PCR-based genome walking strategies. *Pem-MIH1* cDNA contained a 318 bp ORF encoded for a translated product containing 28 amino acids of the signal peptide and a putative mature *Pem-MIH* of 77 amino acids. *Pem-MIH1* and *Pem-MIH2* genes have the same structures. The interruption of the three exons by the two introns occurs at the same positions in both genes. RT-PCR was used to detect the expression of *Pem-MIH1* and *Pem-MIH2* in several tissues of *P. monodon* and found that *Pem-MIH1* was abundantly detected in eyestalk and thoracic ganglia, whereas no transcript was present in heart. A lower expression level was detected in gill and muscle, *Pem-MIH2* was detected only in eyestalk but not in other tissues. The recombinant *Pem-MIH1* was expressed in *Pichia pastoris* as a secreted protein. Pem-MIH1 exhibited the ability to extend molting duration of *P. monodon* from 11.8 days to 16.3 days suggesting that Pem-MIH1 played the molt-inhibiting function in this shrimp.

In crustacean females, the late phase of gonadal maturation to form mature oocytes is named vitellogenesis. This process comprises the synthesis or deposition,

or both, of yolk or vitellus. The major component of this nutritive material is the lipoprotein vitellin, derived from a precursor called vitellogenin that can be synthesized in extraovarian tissues or in the ovaries.

Penhouse (1943 and 1944 cited in (Huberman, 2000)) described that unilateral eyestalk ablation has been used to accelerate ovarian maturation and spawning in different shrimp species used as broodstock in aquaculture. The effect has been attributed to the presence of gonad inhibiting hormone, GIH (or vitellogenin inhibiting hormone, VIH) in the X- organ-sinus gland complex.

Gonad inhibiting hormone (GIH or vitellogenin inhibiting hormone, VIH) is a member of the CHH/MIH/GIH family. It plays an important role on inhibition of ovarian development. The removal of GIH by unilateral eyestalk ablation is practically used for stimulation of ovarian development in shrimp but identification of GIH was only reported in lobsters. Two isoforms of the GIH were isolated and sequenced by Soyez et al. (1991) from the sinus gland of the lobster *H. americanus*. Both consisted of 77 residues and MWs of 9.135 Kda.

Treerattrakool et al. (2008) characterized a cDNA encoding a GIH (Pem-GIH) from the eyestalk of *Penaeus monodon*. *Pem-GIH* cDNA is 861 bp in size with an ORF of 288 bp. The deduced Pem-GIH consists of a 17-residue signal peptide and a mature peptide region of 79 amino acids with features typical of type II peptide hormones from the CHH family. Pem-GIH transcript was detected in eyestalk, brain, thoracic and abdominal nerve cords of *P. monodon* adults. The gonad-inhibiting activity of Pem-GIH was investigated using the RNA interference technique. Double-stranded RNA, corresponding to the mature Pem-GIH sequence, triggered a decrease

in *Pem-GIH* transcript levels both *in vitro* (eyestalk ganglia and abdominal nerve cord culture) and *in vivo* (female *P. monodon* broodstock). The conspicuous increase in *Vg* transcript level in the ovary of GIH-knockdown shrimp suggests a negative influence for Pem-GIH on *Vg* gene expression, and thus implies its role as the gonad-inhibiting hormone.

Recombinant peptides related to vitellogenesis-inhibiting hormone (VIH) of the American lobster (*Homarus americanus*) were expressed in bacterial cells, and then purified after being allowed to refold. Biological activities of the recombinant VIHs having an amidated C-terminus (rHoa-VIH-amide) and a free carboxyl-terminus (rHoa-VIH-OH) were examined using an ovarian fragment incubation system derived from the kuruma prawn, *Marsupenaeus japonicus*. The rHoa-VIH-amide significantly reduced vitellogenin mRNA levels, while rHoa-VIH-OH had no effect on vitellogenin mRNA levels in ovaries (Ohira et al., 2006).

Gonad stimulating hormones, GSH (or vitellogenin stimulating hormone, VSH), believed to be secreted by the supraesophageal and thoracic ganglia has been proposed to have the opposite effects of GIH (stimulates the gonadal maturation) of shrimp. However, this hormone has not been identified and characterized in any shrimps. Ecdysteroids are known as the molting hormones in crustacean and insects. In crustacean, the inactive forms are secreted and converted to 20-hydroxyecdysone by the Y-organ. Ecdysteriods stimulate vitellogenesis in some insects. However, the levels of ecdysteriods in hemolymph of the giant freshwater shrimp (*Macrobrachium rosenbergii*) were not related to vitellogenesis and showed no distinct relation to the

molt cycle suggesting that ecdysteroids are not involved in vitellogenesis in *M. rosenbergii* (Okumura and Aida, 2000).

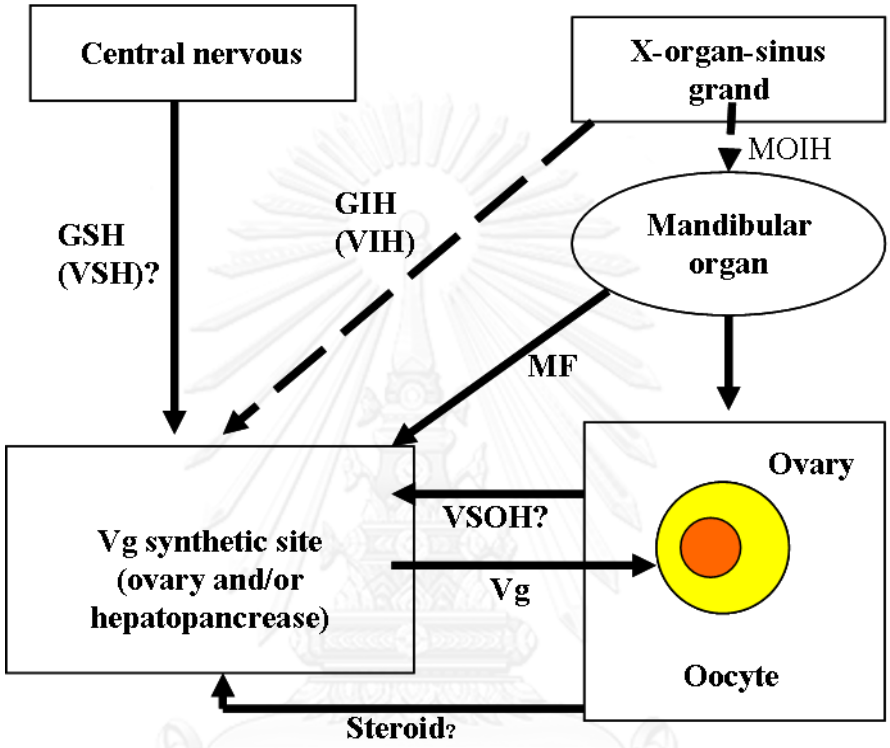


Figure 1.8 Schematic diagram of the endocrine control of vitellogenesis in shrimp (Okumura, 2004) GIH: gonad inhibiting hormone, GSH: gonad stimulating hormone, MF: methyl farnesoate, MOIH: mandibular organ inhibiting hormone, Vg: vitellogenin, VIH: vitellogenesis-inhibiting hormone, VSH: vitellogenesis stimulating hormone.

MF is structurally related to the juvenile hormone and synthesized by mandibular organ (MO). MF has been implicated in the regulation of crustacean development and reproduction in conjunction with eyestalk molt inhibiting hormone and ecdysteroids. The presence of MF in the medium for culturing of ovarian tissue *in vitro* of *L. vannamei* resulted in a significant increase in sizes of oocytes. In

contrast, MF inhibits ovarian development in the tadpole shrimp, *Troops longicaudatus* (Tsukimura et al., 2006).

Farnesoic acid O-methyltransferase (*FAMeT*) catalyzes the methylation of farnesoic acid (FA) in the terminal step of MF synthesis. A schematic diagram of regulatory mechanism of vitellogenersis in shrimp is shown in (Figure 1.8).

Laufer et al. (1998) reported stimulating effects of MF on ovarian maturation in the red swamp crayfish *Procambarus clarkii* in three different trials of MF administration. After 30 days of treatment, ovaries of pre-reproductive females were 2- to 10-fold larger and were in the later stages of vitellogenesis than those of the controls. Similar and statistically significant results were observed in a second 30-day trial, which was begun during the middle of the vitellogenic cycle. The control ovaries were white or yellowish colored but ovaries from the MF-fed groups were larger and showed a dose response for which the 1-µg MF group had tan colored ovaries. A group receiving the higher dose of 2 µg MF had dark brown to black colored ovaries.

Hui et al. (2008) studies functions of *L. vannamei FAmeT* (*LvFAMeT*) in molting using the RNA interference (RNAi) technique. Injection of double stranded RNA (dsRNA) of *LvFAMeT* successfully knocked down the expression of LvFAMeT in juvenile shrimp for at least 3 days and shrimp did not proceed to the final stage of molt cycle. Furthermore, the expression of molt-related genes encoding *cathepsin-L* and *hemocyanin* gene was disturbed. Subsequently, 100% mortality of shrimp was observed in LvFAMeT dsRNA-injected shrimp. In contrast, control shrimp completed their molt and proceeded to the next molt cycle. Therefore, RNAi injection knocked

down the expression of *LvFAMeT* which could potentially result in a decrease in the production of MF and subsequently, could affect the molting process of *P. monodon* were also analyzed.

1.4 Control of cortical rods formation and germinal vesicle breakdown

As the eggs of penaeid shrimp reached maturation, cortical rods start forming around the oocytes. Its function during the egg activation process includes establishment of protective jelly layer (Clark et al., 1990), and contribution to the induction of the sperm acrosome reaction (Kruevaisayawan et al., 2007).

Medina et al. (1996) reported the inability of pond-reared shrimp *Penaeus kerathurus* to synthesie cortical rods leading to the lack of fully mature oocyes (stage IV). It was suspected that hormones play a more significant role since the sizes of oocytes are not different between then wild and pond-raised shrimp. Eyestalk ablated shrimp was not used in Medina et al experiment.

Palacios et al. (2003) reported no significant differences in eyestalk-ablated wild or pond-reared *L. vannamei* oocyte's ability of form cortical rods while Peixoto *et al* (2008) reported significantly higher frequency of oocytes with cortical rods in domesticated shrimp than that of the wild shrimp.

There are several potentials biomarkers for cortical rods formation. Thrombospondin (TSP) major protein component of cortical rods were cloned and characterized in kuruma prawn *M. japonicus* (Yamano et al., 2004b) and Chinese shrimp *F. chinensis* (Sun et al., 2006) High expression of cathepsin C gene in the ovaries of kuruma prawn coincides with the onset of cortical rod formation suggesting that the gene might play a role during CR synthesis and final oocyte maturation (Qiu

et al., 2005). The level of cortical rod protein (CRP) and MjTSP expression in the ovary of kuruma prawn did not change after eyestalk ablation, but the protein levels in the ovary did increase (Okumura et al., 2006). The results suggested that the regulatory mechanism of the CRP and MjTSP control is occurred during translation.

Kim et al. (2005) reported high CRP expression during the previtellogenic and early vitellogenic stage. They suggested that CRP and VG synthesis are regulated by closely-related mechanisms. Cortical rod protein has been cloned and characterized in a species whose oocytes does not form a cortical rod structure such as giant freshwater prawn *Macrobrachium rosenbergii* (Kim et al., 2007).

One important step toward oocyte maturation that has yet to be extensively studied in crustacean is that of germ-vesicle breakdown (GVBD), the breakdown of the nuclear membrane surrounding the chromosome prior to meiosis resumption of the oocyte.

In vertebrate, GVBD and oocyte maturation are triggered by gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). In fish and amphibians, a steroid hormone intermediate, maturation inducing hormone, is also involved in oocyte maturation (Jalabert, 2005; Patio et al., 2001). Examples of maturation inducing hormone in fish include 17,20β-dihydroxy-4pregnen-3one (Jalabert, 2005) and 17β-2-hydroxy-estradiol (Mishra and Joy, 2006a, b, c, d; Senthilkumaran and Joy, 2001).

Yano (1995) hypothesized that final maturation in penaeid shrimp can be induced by prostaglandin whose concentration is related to fatty acid precursors (such as arachidonic acid and eicosapentaenoic acid) in the diet of shrimp. Other

stimulating factor can include mating (for some species), UV-irradiated water, temperature shock or filtration of seawater.

Biogenic amines (e.g., serotonin or 5-HT, epinephrine, and dopamine) and peptide neuroregulators are known to modulate the release of neuropeptide hormones from the sinus gland (Fingerman, 1997) ; (Sarojini et al., 1995) ; (Okumura, 2004). Serotonin (5-HT) in oocyte maturation, particularly GVBD, has been observed in several animal phyla. Simultaneous injections of 5-HT (25 µg/g body weight) and the dopamine antagonist spiperone (1.5 or 5 µg/g body weight) induced ovarian maturation and spawning in wild *L. stylirostris* and pond-reared *L. vannamei* (Alfaro et al., 2004).

Wongprasert et al. (2006) reported the effect on serotonin induction of ovarian maturation and spawning in *P. monodon.* They noted that spawning occurred while the ovaries were in Stage III and the quality of spawns (numbers of eggs, hatching rate, and numbers of nauplii) was better in serotonin-induced group. The stage of ovarian maturation was determined by visual observation in their study, but the correlation to oocyte development was not made. The study provides the promising evidence for serotonin beneficial role on crustacean reproduction.

1.5 Proteomic studies in shrimp reproduction

Changes in protein expression profiles in hemocytes of *P. monodon* upon white spot syndrome virus (WSSV), yellow head virus (YHV) and Vibrio harveyi infection using proteomic studies have been reported (Rattanarojpong et al., 2007) ; (Bourchookarn et al., 2008) ; (Somboonwiwat et al., 2010).

Spermatogenesis and oogenesis are complex cell differentiation processes which require a coordinated series of both mitosis and meiosis cycle events. Accordingly, identification and characterization of genes and/or proteins functionally involved in gonad development are the initial step toward understanding molecular mechanisms of testicular development and sperm quality in *P. monodon* (Leelatanawit et al., 2004) ; (Leelatanawit et al., 2008).

Cellular proteomic analysis was carried out to identify reproduction-related proteins in testes of wild and domesticated broodstock of *Penaeus monodon*. In total, 642 protein spots were characterized and 287 spots (44.70%) significantly matched protein sequences in the databases ($P < 0.05$). To examine a role of the proteasome system in testicular development of *P. monodon*, the expression profiles of *proteasome alpha 3 subunit* (*PmPsma3*) and *proteasome beta 6* (*PmPsmb6*) mRNA in different groups of domesticated shrimp and in wild broodstock were examined. The expression levels of these transcripts in testes of 18-month-old domesticated shrimp were significantly lower than those of wild broodstock ($P < 0.05$). Interestingly, the expression levels of testicular *PmPsma3* and *PmPsmb6* in 18-month-old shrimp were significantly increased at 24 hr following serotonin injection (50 µg/g body weight). Results suggested that reduced degrees of maturation in captive *P. monodon* males may be partially resolved by exogenous 5-HT administration.

Recently, cellular protein profiles in different ovarian stages of *P. monodon* have not been reported. Proteomic analysis was carried out for identification of proteins functionally involved in ovarian development of the giant tiger shrimp (*Penaeus monodon*). A total of 335 protein spots including 183 spots from

vitellogenic (stage II) and 152 spots from mature (stage IV) ovaries of intact *P. monodon* broodstock were examined. Of these, 75 (40.98%) and 59 (38.82%) spots significantly matched known proteins in the databases, respectively. In addition, 270 protein spots including 167 and 103 spots from respective ovarian stages of eyestalk-ablated broodstock were also characterized. A total of 95 (56.89%) and 62 (60.19%) spots matched known proteins, respectively. Among differentially expressed reproduction-related proteins, the full-length cDNA of *protein disulfide isomerase A6* (*PmPDIA6*) was further characterized by RACE-PCR. *PmPDIA6* was 1946 bp in length containing an open reading frame (ORF) of 1293 bp corresponding to a polypeptide of 430 amino acids. PmPDIA6 was up-regulated at stage III ovaries in intact shrimp ($P < 0.05$). Interestingly, eyestalk ablation resulted in a lower expression level of PmPDIA6 in each stage of ovarian development compared to that of intact broodstock ($P < 0.05$). Results in this study clearly indicated the potential of cellular proteomic studies and gene expression analysis for identification of proteins/genes differentially expressed during ovarian development of *P. monodon* (Talakhun et al., 2012).

In addition, 2-DE patterns of total proteins from previtellogenic (stage III) and vitellogenic (stage IV) ovaries of the crayfish (*Procambarus clarkii*) were compared for the first time. A total of 52 significantly differentially expressed protein spots (up/down-regulated ≥ 2 fold) were excised for further analysis using matrix-assisted laser desorption/ionization-tandem time of flight mass spectrometry. There were 29 spots successfully identified, including 22 unique proteins. Compared with stage III ovaries, 13 proteins in the stage IV ovaries were down-regulated (e.g., tropomysin, 70 kD heat shock protein, protein disulfide-isomerase A6, glutathione S transferase D1,

arginine kinase, and Na+/K+-ATPase) and 9 were up-regulated (e.g., peritrophin, cyclin B, elongation factor 2, transketolase, checkpoint kinase 1, and I-connectin). The results indicated the potential of cellular proteomic studies for the identification of proteins functionally involved in the ovarian development of *P. clarkia* (Shui et al., 2012).

Conventional proteomic analysis (2-DE and mass spectrometry were used to identify proteins that are differentially expressed during ovarian maturation in *Metapenaeus ensis*. 87 spots with consistently significant quantitative differences (1.5-fold for vol%) among stage I, III and V ovaries were chosen for MS/MS analysis. 45 spots were significantly matched to known proteins in the database (Mascot score > 40). Half of them were down-regulated, in contrast to 9 out of 45 proteins that were up-regulated as ovarian maturation proceeded. Functionally, these identified proteins could be classified into five major groups, including cytoskeleton (11 %), metabolism (18 %), signal transduction (32 %), gene expression (14 %) and immune response (7 %). Among the differentially expressed reproduction-related proteins, the mRNA expression level of cellular retinoic acid/retinol binding protein in *M. ensis* (MeCRABP) during ovarian maturation was further characterized by quantitative real-time PCR. It was down-regulated during ovarianmaturation. *In situ* hybridization further revealed that MeCRABP transcript was localized in ooplasm of previtellogenic oocytes but not in vitellogenic oocytes (Cui et al., 2014).

Although 2-DE is the traditional method for proteomic analysis, proteins with extremely low or high p*I* and/or molecular mass cannot be fractionated efficiently. In addition, Talakhun et al. (2012) characterized ovarian proteins having p*I* 4 to 7. Accordingly, the basic proteins playing the important role in ovarian development of

this species have not been reported. In this thesis, proteins in ovaries of domesticated and intact wild broodstock of *P. monodon* were size-fractionated by one dimensional gel electrophoresis (SDS-PAGE) and further identified by nanoESI-LC-MS/MS (collectively called GeLC-MS/MS). Several different protein families were characterized. Moreover, the full-length cDNAs of several reproduction-related proteins were further characterized. The expression profile of reproduction-related transcripts and proteins during ovarian development and maturation of *P. monodon* were examined.

CHAPTER II
MATERIALS AND METHODS

2.1 Experimental samples

For cellular proteomics analysis, domesticated 10-month-old (Group A, average body weight = 44.04 ± 4.38 g and GSI = 0.40 ± 0.07%; *N* = 3) and 14-month-old females were collected from Broodstock management Center (BMC), Burapha University, Chanthaburi Campus. The latter was further divided to 2 groups according to the gonadosomatic index (GSI, ovarian weight/body weight x 100; Group B, average body weight = 81.94 ± 2.85 g and GSI = 0.36 ± 0.04% and Group C, average body weight = 71.68 ± 7.08 g and GSI = 1.11 ± 0.07%; *N* = 3 for each group). In addition, 2 groups of wild broodstock were live-caught from the Andaman Sea (west of peninsular Thailand; Group D, average body weight = 209.81 ± 4.45 g and GSI = 2.32 ± 0.13% and Group E, average body weight = 213.93 ± 31.92 g and GSI = 9.70 ± 1.22%; *N* = 3 for each group).

For identification and characterization of nuclear proteins and nuclear membrane proteins, wild broodstock were live-caught from the Andaman Sea. Wild shrimp possessing ovaries stage I (body weight = 212.98 g, GSI = 1.45%) and III (body weight = 217.62 g, GSI = 4.29%).

For real-time analysis, juvenile shrimp (4-month-old, average body weight approximately 20 g; *N* = 6). Female broodstock were collected (average body weight of 142.98 ± 28.37 g) and acclimated under the farm conditions for 2-3 days. The post-spawning group was immediately collected after shrimp were ovulated (*N* = 6). Ovaries were dissected out from each shrimp and weighed. For the eyestalk ablation

group, wild broodstock were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2-7 days after ablation. The ovarian developmental stages of wild shrimp were classified according to the GSI values: <1.5, >2–4, >4–6 and >6% for stages I (*N* = 4 and 4 for intact and eyestalk-ablated broodstock, respectively), II (*N* = 10 and 4), III (*N* = 10 and 5) and IV (*N* = 5 and 9) ovaries, respectively. The ovarian developmental stages of wild shrimp were confirmed by conventional histology. For RT and tissue distribution analysis, various tissues of female and testes of male of juveniles and broodstock (*N* = 6 for each group) were collected, immediately placed in liquid N_2 and kept at -80°C until needed.

Five groups of domesticated 14 month-old are single injected intramuscularly with progesterone (0.1 µg/g of body weight) into the first abdominal segment of each shrimp. Ovaries of progesterone treated were collected for vehicle control (*N* = 5) (at 0 and 12 hour after ethanol injection), negative control (intact shrimp) (*N* = 5), at 12, 24, 48 and 72 hour after PG injected (*N* = 4). For female of domesticated 18 month-old were injected intramuscularly into the first abdominal segment with 5-HT (50 µg/g body weight, *N*=5 for each group). In addition, ovaries of serotonin treated were collected for vehicle control (*N* = 4) (normal saline injection), at 0, 1, 3, 6, 12, 24, 48 and 72 hour after 5HT injection (*N* = 4). Ovarian developmental stages were classified by conventional histology (Qiu and Yamano, 2005) and divided to previtellogenic (I, *N* = 10 and 4 for normal and eyestalk-ablated broodstock, respectively), vitellogenic (II, *N* = 7 and 7), early cortical rod (III, *N* = 7 and 10) and mature (IV, *N* = 10 and 11) stages, respectively. The average body weight of *P. monodon* broodstock was 142.98 ± 28.37 g.

2.2 Protein extraction

2.2.1 Total protein extraction

Approximately 0.5 gram of frozen ovaries of *P. monodon* were ground in the extraction buffer: 10% TCA in acetone (V/V), 0.1% DTT (W/V) and protease inhibitor cocktail. After centrifugation at 10000 g for 10 minutes at 4°C, the supernatant were collected. The extraction buffer is added and left at -20°C 2 hours. The mixtures were centrifuged at 10000 g for 30 minutes at 4°C. The supernatant is discarded. The pellet is air-dried and dissolved in 0.5% SDS (for proteomic) or lysis buffer (for western blot). The amount of extracted proteins was measured by a dye binding assay (Bradford, 1976).

2.2.2 Nuclear membrane and nuclear proteins extraction

Twenty micrograms of frozen ovarian tissues was washed with 1 ml of ice-cold PBS buffer (50 mM NaH_2PO_4, 150 mM NaCl, pH7.2). Different components of proteins from cytoplasm, nuclear membranes, nucleus and cytoskeleton of ovarian cells were extracted using a Qproteome Cell Compartment kit (QIAGEN) following the protocol recommended by the manufacturer. The fractions containing nuclear membrane proteins and nuclear proteins were collected and desalted using Bio-Spin®6 (BioRad). The concentration of extracted were measured (Lowry et al., 1951).

2.2.3 Membrane proteins extraction

Membrane proteins from ovaries of wild broodstocks *P. monodon* undergoing various developmental stages (stages I to IV ovaries) of ovarian maturation were prepared by homogenization of the ovarian tissue in buffer M (100 mM NaCl, 20 mM Tris–HCl, 2 mM MgCl2, 1 mM EDTA and 1 mM PMSF, pH 7.4). The tissue debris was removed by centrifugation at 600×g for 10 min and then at 6000×g for 10 min. After

centrifugation at 20,000×g for 30 min at 4 °C, the membrane pellet was dissolved in 0.2% Triton X-100 in buffer M (Ongvarrasopone et al., 2006). Protein concentration was determined by using Bradford assay (Bradford, 1976).

2.3 Nucleic acid extraction

2.3.1 RNA extraction

Total RNA was extracted from ovaries and testes of each the shrimp using TRI-PURE (Roach). A piece of tissue was immediately placed in mortar containing liquid nitrogen and ground to the fine powder. The tissue powder was transferred to a microcentrifuge tube containing 500 µl of TRI REAGENT (1 ml/50-100 mg tissue) and homogenized. Additional 500 µl of TRI REAGENT were added. The homogenate was left at room temperature for 5 minutes before 0.2 ml of chloroform was added. The homogenate was vortexed for at least 15 seconds, left at room temperature for 2 - 15 minutes and centrifuged at 12000 *g* for 15 minutes at 4°C. The mixture was separated into the lower phenol-chloroform phase (red), the interphase, and the upper aqueous phase (colorless).

The aqueous phase (inclusively containing RNA) was carefully transferred to a new 1.5 ml microcentrifuge tube. RNA was precipitated by an addition of 0.5 ml of isopropanol and mixed thoroughly. The mixture were left at room temperature for 10 - 15 minutes and centrifuged at 12000 *g* for 10 minutes at 4 °C. The supernatant was removed. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7500g for 10 minutes at 4°C. The ethanol was removed. The RNA pellet was air-dried for 5-10 minutes. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80 °C freezer for long storage.

Total RNA was also extracted from other tissues including eyestalks, gills, heart, hemocytes, hepatopancreases, lymphoid organs, intestine, stomach, pleopods and throracic ganglion of *P. monodon* using the same extraction procedure. The quality of extracted total RNA was examined by electrophoresed through 1.0% agarose gels.

2.3.2 Preparation of DNase I-free total RNA

Fifteen micrograms of total RNA were treated with DNase I (0.5 U/1 µg of RNA, Promega) at 37°C for 30 minutes. After the incubation, the sample was gently mixed with a sample volume of phenol : chloroform : isoamylalcohol (25:24:1) for 10 minutes. The mixture was centrifuged at 12,000 g for 10 minutes at 4°C, and the upper aqueous phase was collected. The extraction process was then repeated once with chloroform : isoamylalcohol (24:1) and one with chloroform. The final aqueous phase was mixed with one-tenth final sample volume of 3 M sodium acetate (pH 5.2). After that, RNA was precipitated by adding two point five volume of -20°C-cold absolute ethanol. The mixture was incubated at -80°C for 30 minutes, and the precipitated RNA was recovered by centrifugation at 12,000 g for 10 minutes at room temperature. The RNA pellet was then washed twice with 1 ml of -20°C cold 75% ethanol. Alternatively, the RNA pellet was kept in absolute ethanol at -80°C until required.

2.3.3 Genomic DNA extraction

Genomic DNA was extracted from the pleopod of *P. monodon* using a phenol-chloroform-proteinase K method. The pleopod was placed in a centrifuge tube containing 2 ml of the extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl; pH 8.0) and briefly homogenized with a micropestle and aliquoted into

microcentrifuge tubes (500 µl). SDS (10%) and RNase A (10 mg/ml) solution were added to a final concentration of 0.1% (w/v) and 100 µg/ml, respectively. The resulting mixture was then incubated at 37 °C for 1 hour. At the end of the incubation period, a proteinase K solution (10 mg/ml) was added to the final concentration of 200 µg/ml and further incubated at 55 °C for 3 hours. An equal volume of buffer-equilibrated phenol was added and gently mixed for 15 minutes. The upper aqueous phase was transferred to a new sterile microcentrifuge tube. This extraction process was then repeated once with phenol and twice with chloroform : isoamylalcohol (24:1). The aqueous phase was transferred into a sterile microcentrifuge. One-tenth volume of 3 M sodium citrate, pH 5.2 was added. DNA was precipitated by an addition of two volume of pre-chilled absolute ethanol and mixed thoroughly. The mixture was incubated at -80 °C for 30 minutes and centrifuged at 12000 g for 10 minutes at 4 °C. The precipitated DNA was washed twice with 1 ml of 70% ethanol (10 and 5 minutes, respectively). After centrifugation, the supernatant was removed. The DNA pellet was air-dried and resuspended in 30-50 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0). The DNA solution was incubated at 37 °C for 1-2 hours for compete solubilization and kept at 4 °C until further used.

2.4 Estimation of extracted total protein, total RNA and DNA concentration

The concentration of extracted protein sample is estimated by a dye binding assay. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm. This assay is rapid, reproducible and virtually complete in approximately 2 minutes with the color stability for 1 hour. The concentration of extracted protein sample is estimated corresponding to the BSA standard.

The concentration of extracted RNA and DNA samples is estimated by measuring the optical density at 260 nm (OD_{260}). An OD_{260} of 1.0 corresponds to a concentration of 40 µg/ml single stranded RNA, 50 µg/ml double stranded DNA and 33 µg/ml single stranded DNA (Sambrook and Russell, 2001). Therefore, the concentration of RNA and DNA sample was estimated in µg/ml by using the following equation;

[RNA of DNA] = OD_{260} x dilution factor x (40, 50 or 33 for RNA, double strand DNA and single stranded DNA)

The ratio between OD_{260}/OD_{280} provides an estimate on the purity of extracted DNA/RNA. For the extracted DNA, a pure preparation of DNA has OD_{260}/OD_{280} ratio of 1.8-2.0. The ratio of approximately 2.0 indicates the good quality of the extracted RNA. The ratios that much lower than those values indicate contamination of residual proteins or phenol in extracted DNA or RNA (Kirby, 1992).

2.5 One dimensional gel electrophoresis

2.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Ovarian proteins (30 µg) extracted from different stages of ovaries of captive and intact wild broodstocks and nuclear and nuclear membrane proteins (15 µg) of stages I and III ovaries were size-fractionated in 12% SDS-PAGE (Laemmli, 1970). A low molecular weight protein standard marker (BioRad) was used to estimate sizes of electrophoresed proteins. At the end of each run, the protein gels were silver-stained. The protein bands were excised according to marker proteins range (> 225 kDa, 176-225 kDa, 150-176 kDa, 102-150 kDa, 76-102 kDa, 52-76 kDa, 38-52 kDa and <38 kDa), then 4-5 pieces of approximately 1 square millimeter of gel pieces for trypsination.

2.5.2 Silver staining

At the end of each run, the gel protein was fixed in the fixing solution (50% methanol, 12% acetic acid and 50 µl of 37% formaldehyde to 100 ml fixing solution) for 2 h. The gel was removed in the washing solution (35% ethanol) 3 times for 5 min each and sensitizing in 0.02% sodium thiosulfate for 2 min. After washing in water 3 times for 5 min each, the gel was stained with silver nitrate (2%) for 20 min. The gel was shaken in the developing solution (60% $NaCO_3$ w/v, 0.04% $Na_2S_2O_3$ v/v, 37% formaldehyde CH_2O) until regarded protein spots were visualized and stopped quickly in the stopping solution (14.6% w/v sodium EDTA $C_{10}H_{12}N_2Na_4O_8$) for 20 min and the gel was kept in 0.1% acetic acid at 4°C.

2.6 Mass spectrometry analysis

2.6.1 In-gel digestion for protein identification

Pieces of electrophoresed proteins were excised manually from silver-stained 1DE gels. Gel pieces were placed in a 96 well plate and subjected to in-gel trypsin digestion. Acetonitrile (ACN) were added to the proteins for 5 min following by additional reduction and alkylation step using 10 mM DTT at room temperature for 1 h and 100 mM iodoacetamide in the dark at room temperature for 1 h. The gel pieces were hydrated with acetonitrile for 5 min. The dried gels were rehydrated by addition of appropriate volume of the digestion buffer 20 µl (10 ng/µl of trypsin in 50% acetonitrile and 10 mM ammonium bicarbonate) and incubated for 20 min at RT and 50 µl of 30% acetonitrile was added to cover the gel pieces. Digestion was performed overnight at RT. Tryptic peptide were extracted by an addition of 30 µl of 30% ACN and shaken for 10 min and transferred to a new 96 well plate followed by the addition of 50 µl of 50% ACN/0.1% FA then shaken for 10 min, and 50 µl of 70%

ACN/0.1% FA and shaken for 10 min, respectively. The digested proteins were dried at 40°C for 3-4 hours and stored in -80°C.

2.6.2 nanoLC-MS/MS

Nano-electrospay liquid chromatography ionization tandem mass spectrometry (nanoLC-MS/MS) was performed as followed. Selected protein spots were submitted to an integrated the HCTultra ETD II system™ operated under HyStar™ (Bruker Daltonics). This system was controlled by the Chromeleon Chromatography Management system and comprised a two-pump Micromass/Loading Iontrap system with an autosampler. Injected samples were first trapped and desalted on an AccLaim PepMap C18 µ Precolumn Cartridge (5 µm, 300-µm inside diameter by 5 mm) for 3 min with 0.1% formic acid delivered by a loading pump at 20 µl/min, after which the peptides were eluted from the pre-column and separated on a nano column, AccLaim PepMap 100 C18 (15 cm x 3 µm) connected inline to the mass spectrometer, at 300 nl/min using a 30 min fast gradient of 4 to 96% solvent B (80% acetronitrile in 0.1% formic acid).

2.6.3 Database searches

After data acquisition, MS/MS ion from nanoLC-MS/MS were analyzed using DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) for quantitative protein analysis (Johansson et al., 2006) ; (Thorsell et al., 2007). Acquired LC-MS/MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analyzed MS/MS data from DeCyderMS were submitted to database search using the Mascot software (Matrix Science, London, UK, (Cottrell and London, 1999). The data searched against of the

local shrimp database. In addition, data from nanoLC-MS/MS were searched against data of the National Central for Biotechnology Information (NCBI, nr) and SWISSPROT. For MS/MS ion search, the peptide charge was 1+, 2+ and 3+, MS/MS ion mass tolerance was ±1.2 Da, fragment mass tolerance ± 0.6 Da, and allowance for 1 miss cleavage. Variable modification was methionine oxidation and cysteine carbamidomethylation. Proteins with having scores were selected.

2.7 First strand cDNA synthesis

One and half micrograms of total RNA from various tissues of *P. monodon* were reverse transcribed to the first strand cDNA using an ImProm-II™ Reverse Transcription System Kit (Promega). Total RNA was combined with 0.5 µg of oligo dT_{12-18} and appropriate amount of DEPC-treated H_2O in a final volume of 5 µl. The reaction was incubated at 70 °C for 5 minutes and immediately placed on ice for 5 minutes. The 5x reaction buffer, $MgCl_2$, dNTP mix, RNasin were added to final concentration of 1x, 2.25 mM, 0.5 mM and 20 units, respectively. Finally, 1 µl of ImProm-II™ Reverse transcriptase was added and gently mixed by pipetting. The reaction mixture was incubated at 25 °C for 15 minutes and 42 °C for 90 minutes. The reaction was terminated by incubated at 70 °C for 15 minutes to terminate reverse transcriptase activity. Concentration and rough quality of newly synthesized first strand cDNA was spectrophotometrically examined (OD_{260}/OD_{280}) and electrophoretically analyzed by 1% agarose gel.

2.8 Reverse transcription (RT)-PCR of gene homologues in *P. monodon*

2.8.1 Primer design

Eight primer pairs were designed from EST sequences of gene homologues from hemocyte, ovary and testis cDNA libraries of *P. monodon* (Table 2.1).

2.8.2 RT-PCR

The first strand cDNA (100 ng) of ovaries of female *P. monodon* broodstock were used as the template in a 25 µl RT-PCR reaction composing of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 0.1 mM of each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer and 1 unit of Dynazyme ^{TM} DNA polymerase (FINNZYMES). RT-PCR was carried out with the temperature profile of predenaturation at 94 °C for 3 minutes followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 53 °C for 45 second and extension at 72 °C for 30 seconds. The final extension was carried out at the same temperature for 7 minutes.

Fives microliters of the amplification products are electrophoresed though 1.2- 2.0% agarose gel dependent on size of the amplification products. The electrophoresed band was visualized under a UV transilluminator after ethidium bromide staining (sambrook and Russell, 2001).

2.8.3 Agarose gel electrophoresis

An appropriate amount of agarose was weighed out and mixed with the desired volume of 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.3). The gel slurry was boiled in a microwave oven to complete solubilization and allowed to lower than 60 °C before poured into the gel mold. A comb was inserted. The agarose gel was left to solidify. When needed, enough amount of 1x TBE buffer covering the gel for approximately 0.5 cm. The comb was removed. The PCR product was mixed with the one-fourth volume of the 10x loading dye (0.25% bromophenol blue and 25% ficoll in water) and loaded into the well. A 100 bp DNA ladder was used as the standard DNA marker. Electrophoresis was carried out at 5-6 volt/cm until bromophenol blue moved to approximately one-haft of gel. The

electrophoresed gel was stained with an ethidium bromide solution (25 µg/ml) for 5 minutes and destained in running tap water to remove unbound ethidium bromide from the gel. DNA fragments were visualized under a UV transluminator and photographed through a Gel Doc using a Quality One software (BioRad).

2.9 Tissue distribution analysis of interesting genes or differential expression pattern

2.9.1 Total RNA extraction and the first strand cDNA synthesis

Total RNA was extracted from eyestalk, gills, heart, hemocytes, hepatopancrease, lymphoid organ, intestine, ovaries, pleopods, stomach, thoracic ganglion, antennal gland and subcuticular epithelium of wild females and testes of male *P. monodon*. The first strand cDNA was synthesized as described previously.

2.9.2 Tissue expression analysis

For the target genes, 150 ng of the first strand cDNA from various tissues was used as the template in 25 µl reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.1% Triton X-100, 2 mM $MgCl_2$, 100 µM each of dATP, dGTP, dTTP and dCTP, 0.2 µM of each primer and 1 unit of Dynazyme ™ DNA polymerase (FINNZYMES). *Elongation factor-1α* (F: 5′-ATGGTTGTCAACTTTGCCCC-3′ and R: 5′-TTGACCTCCTTGATCACACC-3′) were also amplified from the same template and considered as the positive control. The reactions were predenaturation at 94 ºC for 3 min followed by 30 cycles composing of a 94 ºC denaturation step for 30 s, a 53 ºC annealing step for 45 s and 72 ºC extension step for 30 s. The final extension was carried out at 72 ºC for 7 min. Fives microliters of the amplification product was electrophoretrically analyzed though a 1.5-1.7% agarose gel base on size of product.

Table 2.1 Gene homologue, primer sequences and expected sizes of the PCR product designed from EST of *P. monodon*

Gene	Primer sequence	size
Beta Thymosin (*PmTmsb*)	F: 5′AGGTTTGCCAGGAACACATC3′	176
	R: 5'ATCGCC TTGCTATTGGTGAG3′	
Valosin containing protein	F: 5'GCAGTTGAACGAGGTGGGCTAC3'	276
(*PmVCP*)	R: 5'TTGCGAAGGTTGCTCTCGCATT3'	
Rac GTPase-activating protein 1	F: 5'CATGTGGCAAAAGAATTAAGTT3'	172
(*PmRacgap1*)	R: 5'GGGCATACGAGATGTGTAGTCA3'	
Protein kinase C	F: 5′ACCACCATT TACACCTAAGATA3′	139
(*PmPKC*)	R: 5'AAGAAAATCCTCTAAACTCGTC3′	
Cyclic AMP regulated protein	F: 5′TGTTCAAGTATGAGGGCAGCCAGGT3′	169
like protein (*PmcAMP-RPL*)		
	R: 5'GTGCCCACCCAAGTTAGCATCAGGA3′	
	F: 5'AAGCGAAAATGTCCACCGATAAG3'	234
	R: 5'AAGAGAACACAGAGCCAGATTGA3'	
Nuclear pore complex protein	F: 5′TGCTCCTCTCACAACGCTTCCTT 3′	139
NUP133 (*PmNUP133*)		
	R: 5'ACTATCCCCTGCTCCAGAGAACT3′	
Semaphorin-2a (*PmSema*)	F: 5'GCTTGGTGGTGATGAATGTAAAT3'	242
	R: 5'CTGGCTGATGTTATTCCTATTGG3'	

2.10 Isolation and characterization of the full-length cDNA of functionally important gene homologues of *P. monodon* using Rapid Amplification of cDNA Ends-Polymerase Chain Reaction (RACE-PCR)

2.10.1 Preparation of the 5' and 3' RACE template

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent. The quality of extracted of total RNA was determined by agarose gel electrophoresis. Messenger (m) RNA was purified using a QuickPrep micro mRNA Purification Kit (Amercham Phamacia Biotech) according to the protocol recommended from the manufacturer. RACE cDNA template was prepared by combining 1 µg of ovarian mRNA with 1 µl of 5'-CDS primer and 1 µl of 10 µM SMART II oligonucleotide for 5' RACE-PCR or 1 µg of ovarian mRNA with 1 µl of 3' CDS primer A for 3' RACE-PCR (Table 2.2). The component were mixed and centrifuged briefly. The reaction was incubated at 70°C for 2 min and snap-cooled on ice for 2 minutes. The reaction tube was centrifuged briefly. After that, 2 µl of 5x First-Strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP Mix (10 mM each) and 1 µl of PowerScript Reverse Transcriptase were added. The reaction were mixed by gently pipetting and centrifuged briefly to collect the contents at the bottom of the tube. The reaction tube was incubated at 42 °C for 1.5 h in a thermocycler. The first strand reaction products were diluted with 125 µl of TE buffer and heated at 72 °C for 7 min. The first strand cDNA templates were kept at -20 °C until needed.

2.10.2 Primer designed for RACE-PCR and primer walking

Gene-specific primers (GSPs) were designed from ovary and hemocyte cDNA libraries. The antisense primer (and nested primer) for 5′ and/or 3′ RACE-PCR of each gene was designed (Table 2.3).

For sequencing of genes that showed the full-length from the 5′ direction, the product from colony PCR was considered. If the insert of a particular gene was larger than that of its homologues, the 3′ direction was further sequenced. Internal primers were designed for primer walking of the inserted cDNA.

2.10.3 RACE-PCR

The master mix sufficient for 5′ and/or 3′ RACE-PCR and the control reactions was prepared (Tables 2.4 and 2.5). For each 25 µl amplification reaction, 14.0 µl sterile deionized H_2O, 2.5 µl of 10x Advantage® 2 PCR buffer, 0.5 µl of 10 µM dNTP mix and 0.5 µl of 50x Advantage® 2 polymerase mix were combined.

The primary 5′ and 3′ RACE-PCR product were electrophoretically analyzed through 1.2-1.5% agarose gels. If the discrete expected bands were not obtained from the primary amplification, nested PCR was performed using the recipes illustrated in Tables 2.4 and 2.5. The primary PCR product was 50-fold diluted. The secondary PCR was performed using 1 - 5 µl of the diluted first PCR product as a template using the conditions described in Table 2.6.

Table 2.2 Primer sequence for the first strand cDNA synthesis and RACE-PCR

Primers	Sequence
SMART II A Oligonucleotide	5′-AAGCAG TGG TATCAACGCAGAGTACGC GGG-3′
3′ RACE CDS Primer A	5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
5′ RACE CDS Primer	5′-$(T)_{25}$ N_{-1} N-3′ (N=A, C, G orT; N_{-1}= A,G or C)
10X Universal PrimerA Mix (UPM)	Long : 5′–CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAG AGT-3′ Short : 5′–CTAATACGACTCACTATAGGG C – 3′
Nested Universal Primer A (NUP)	5 – AAG CAG TGG TAT CAA CGC AGA GT -3′

Table 2.3 Gene-specific primers (GSPs) and nested GSP used for isolation of the full-length cDNA of functionally important genes in *P. monodon*

Gene specific primer	Sequence	Tm (°C)
Thymosin-β		
5′ RACE	R: 5′GGCAGGTGAATCTTCTCCTCCGTC3′	65.38
3′ RACE	F: 5′AGGTTTGCCAGGAACACATC3′	
3'Nested	F: 5' CAATAGCAAGGCGATGGCAAGAAGTG3'	63.57
Valosin containing protein		
3′ RACE	F: 5' TGCCACAAACCGCCCCAACTCCATCG3'	68.03
Protein kinase C		
5′ RACE-I	R: 5′GTTTCCTGGGGAGACTTT AGCACTTT 3′	61.99
5′ RACE-II	R: 5'CACCAATCAACACTG GCACCATAA TC3'	61.99
5′ RACE-III	R: 5' TCCCTAGACCCCATATGAATTCTCGAC3'	63.53
3′ RACE	F: 5' TGTTCGTGCTATCAACCAAGAC3'	
Cyclic AMP-regulated protein like protein		
3′ RACE	F: 5' CGTCTCGTCTCGGCTTCGTTCGGATA 3'	66.72
Nuclear pore complex protein NUP133		
5′ RACE	R: 5'ACTATCCCCTGCTCCAGAGAACT3′	60.4
3′ RACE	F: 5' TGCTCCTCTCACAACGCTTCCTT 3'	65.2
5′ Nested	R: 5'CAGTATCCATTGTGGCAAGGGCGTA 3'	69.1
Semaphorin-2a		
5′ RACE	R: 5'CTGGCTGATGTTATTCCTATTGG3'	58.6
3′ RACE	F: 5'GCTTGGTGGTGATGAATGTAAAT3'	58.6

Table 2.4 Compositions for amplification of the 5′ end of gene homologues using 5′ RACE-PCR

Component	5′ RACE-PCR	UPM only (Control)	GSP1 only (Control)
5’ RACE–Ready cDNA template	1.5 µl	1.5 µl	1.5 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10 uM)	1.0 µl	-	1.0 µl
GSP2 (10 uM)	-	-	-
H_2O	-	1.0 µl	5.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25µ	25 µl

Table 2.5 Compositions for amplification of the 3′ end of gene homologues using 3′ RACE-PCR

Component	3′ RACE-PCR	UPM only (Control)	GSP1 only (Control)
5’ RACE–Ready cDNA template	1.5 µl	1.5 µl	1.5 µl
UPM (10x)	5.0 µl	5.0 µl	-
GSP1 (10 uM)	1.0 µl	-	1.0 µl
GSP2 (10 uM)	-	-	-
H_2O	-	1.0 µl	5.0 µl
Master Mix	17.5 µl	17.5 µl	17.5 µl
Final volume	25 µl	25 µl	25 µl

Table 2.6 The amplification conditions for RACE-PCR of various gene homologues of *P. monodon*

Gene homologue	Amplification condition
PmTmsb	
5′ RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
3′ RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
3′ Nested RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
PmVCP	
3′ RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
PmPKC	
5′ RACE-PCR	20 cycles of 94 °C for 30 s, 65 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
3′ RACE-PCR	20 cycles of 94 °C for 30 s, 65 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
PmcAMP-RPL	
3′ RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
PmNUP133	
5′ RACE-PCR	5 cycles of 94 °C for 30 s and 70 °C for 2 min 5 cycles of 94 °C for 30 s, 68°C for 30 s and 72 °C for 2 min 20 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min
3′ RACE-PCR	5 cycles of 94 °C for 30 s and 70 °C for 2 min 5 cycles of 94 °C for 30 s, 68°C for 30 s and 72 °C for 2 min 20 cycles of 94 °C for 30 s, 66 °C for 30 s and 72 °C for 2 min and the final extension at 72 °C for 7 min
5′ Nested RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
PmSema	
5′ RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min
3′ RACE-PCR	20 cycles of 94 °C for 30 s, 66 °C for 45 s, 72 °C for 2 min and the final extension at 72 °C for 7 min

2.10.4 Elution DNA fragments from agarose gels

After electrophoresis, the desired DNA fragment was excised from the agarose gel using a sterile scalpel and placed in a pre-weighed microcentrifuge tube. DNA was eluted out from the gel using a illustra GFX PCR DNA and Gel Band Purification Kit (GE). Three hundred microlitters of the capture buffer type 3 was added to the sample and mixed by vortexing. The mixture was incubated at 60 °C for 10 - 15 min until the gel slice was completely dissolved. During the incubation period, the tube was inverted every 2-3 min. A column was placed in a collection tube and 600 µl of the sample mixture was applied into the column and centrifuged at 6,000 *g* (8,000 rpm) for 1 min. The flow-through was discarded. The column was placed back in the collection tube. The column was washed by the addition of 500 µl of the ethanol-added wash buffer type 1 and centrifuged at 6,000 *g* for 1 min. After discarding the flow-through, the column was centrifuged for 2 min at the full speed (14,000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 15 µl of the elution buffer type 4 was added to the center of the column matrix. The column was left at room temperature for 2 min before centrifuged for 2 minutes at the full speed to recover the gel-eluted DNA.

2.11 Cloning of the PCR product

2.11.1 Ligation of the PCR product to the pGEM®-T Easy vector

DNA fragments was ligated to the pGEM®-T Easy vector in a 10 µl reaction volume containing 5 µl of 2x Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DDT, 2 mM ATP and 10% PEG8000), 3 unit of T4 DNA ligase, 25 ng of the pGEM®-T Easy vector and approximately 50 ng of the DNA insert. The reaction

mixture was incubated overnight at 4 - 8 °C before transformed to *E.coli* JM 109 (or DH5α).

2.11.2 Transformation of the ligation product to *E.coli* host cells

2.11.2.1 Preparation of competent cell

A single colony of *E. coli* JM109 (or DH5α) was inoculated in 10 ml of LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 0.5% NaCl, pH 7.0) with vigorous shaking at 37 °C overnight. The starting culture was then inoculated into 50 ml of LB broth and continued culture at 37 °C with vigorous shaking to OD_{600} of 0.5 to 0.8. The cells was briefly chilled on ice for 10 minutes and recovered by centrifugation at 2700 *g* for 10 minutes at 4 °C. The pellets were resuspended in 30 ml of ice-cold $MgCl_2$/$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged as above. The cell pellet was resuspended with 2 ml of ice-cold 0.1 M $CaCl_2$ and the cell suspension was divided into 100 or 200 µl aliquots. These competent cells was used immediately or stored at -80°C for subsequently used.

2.11.2.2 Transformation

The competent cells were thawed on ice for 5 min. Fives microlitres of the ligation mixture were added and gently mixed by pipetting. The mixture was left on ice for 30 min. During the incubation period, the ice box was gently moved forward and backward a few times every 5 min. The transformation reaction was heat-shocked in a 42 °C water bath (without shaking) for exactly 45 seconds. The reaction tube was immediately placed on ice for 5 min. The mixture were removed from the tubes and added to a new tube containing 1 ml of pre-warmed SOC (2% Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). The cell suspension was incubated with shaking at 37 °C

for 90 min. The mixture were centrifuged for 1 minutes at room temperature, and resuspended in 100 µl of the SOC medium and spread onto a selective LB agar plates (containing 50 µg/ ml of amplicillin and spread with 20 µl of 25 µg/ml of X-gal and 25 µl of 25 µg/ml of IPTG for approximately 1 hr before using) and further incubated at 37 °C overnight. The recombinant clones containing inserted DNA are white whereas those without inserted DNA are blue (Sambrook and Russell, 2001).

2.12 Colony PCR and digestion of the amplified inserts by restriction endonucleases

Colony PCR was performed in a 25 µl reaction mixture containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.1% Triton X-100, 0.1 mM of each dNTP, 4 mM $MgCl_2$, 0.1 µM each of pUC1 (5′-CCGGCTCGTATGTTGTGTGGA-3′) and pUC2 (5′-GTGGTGCAAGGCGATTAAGTTGG-3′), 0.5 unit of *Taq* DNA polymerase (FINZYME). A colony was picked by a pipette tip, placed in the culture tube and served as the template in the reaction. PCR was carried out in a thermocycler consisting of predenaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 50°C for 1 min and extension at 72°C for 1.5 min. The final extension was carried out at the same temperature for 7 min. The colony PCR products were electrophoresed through a 1.5 % agarose gel and visualized after ethidium bromide staining.

The colony PCR products containing the insert were separately digested with *EcoR* I (Promega) in a 12 µl reaction volume containing 1x buffer (6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl and 1 mM DDT, pH 7.5 for *EcoR* I 0.1 mg/ml BSA, 2 units of enzyme and 5 µl of the colony PCR product. The reaction mixture was incubated at 37°C overnight. The reaction was analyzed by 1.5% agarose gel electrophoresis.

2.13 Extraction of recombinant plasmid DNA

Plasmid DNA was isolated using a illustraTM plasmidPrep Mini Spin Kit (GE). A discrete white colony was inoculated into a sterile culture tube containing 3 ml of LB broth supplemented with 50 µg/ml of amplicillin and incubated with shaking (250 rpm) at 37 ℃ overnight. The culture was transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm for 1 min. The supernatant was discarded. The bacterial pellet was resuspended in 175 µl of the lysis buffer type 9 containing RNase A and thoroughly mixed by vortexed. The resuspended cells were lysed by the addition of 175 µl of the lysis buffer type 7 and mixed gently by inverting the tube for 6 times. The mixture was left for 2 minutes at room temperature. After that, 350 µl of the lysis buffer type 8 was added to neutralize the alkaline lysis step and mixed immediately by inverting the tube for 6 times. The mixture was then centrifuged at 14,000 rpm for 15 min. A mini spin column was placed in a collection tube and the clear lysate was applied into the mini spin column and centrifuged at 12,000 rpm for 30s. The flow-through was discarded. The mini spin column was placed back in the collection tube. The column was washed by the addition of 400 µl of the lysis buffer type 8 and centrifuged at 12,000 rpm for 30s. After discarding the flow-through, 500 µl of the ethanol-added Wash buffer type 1 was added and centrifuged as above. The mini spin column was further centrifuged for 2 min at the full speed (14,000 rpm) to dry the column matrix. The dried column was placed in a new microcentrifuge tube and 30 µl of the elution buffer type 4 was added at the center of the column matrix. The column was left at room temperature for 2 min before centrifuged for 2 min at the full speed to recover the

purified plasmid DNA. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.14. Examination of expression levels of interesting genes in ovaries of *P. monodon* by quantitative real-time PCR

Expression levels of several transcripts including *valosin containing protein* (*PmVCP*), *thymosin-β* (*PmTmsb*), *rac GTPase-activating protein 1* (*PmRacgap1*), *protein kinase C* (*PmPKC*) and *cyclic AMP-regulated protein-like protein* (*PmcAMP-RPL*) were examined using quantitative real-time PCR analysis.

2.14.1 Experimental animals

Intact and eyestalk-ablated wild broodstock of *P. monodon* (ABW = 142.98 ± 28.37) possessing different stages of ovarian development (GSI values: <2, >2–4, >4–6 and >6% for stages I (*N* = 4 and 4 for intact and eyestalk-ablated broodstock, respectively), II (*N* = 10 and 4), III (*N* = 10 and 5) and IV (*N* = 5 and 9) ovaries, respectively.

For progesterone administration, 14 month-old of domesticated shrimp were single injected with progesterone (0.1 µg/g of body weight and average body weight = 100.79-±-17.59 g). Ovaries of progesterone treated were collected for vehicle control (*N* = 5) (at 0 and 12 hour after ethanol injection), negative control (intact shrimp) (*N* = 5), at 12, 24, 48 and 72 hour after PG injected (*N* = 4).

For 5-hydroxytryptamine (5-HT) administration, 18-month-old of domesticated shrimp were injected with 5-HT (50 µg/g body weight, average body weight = 107±16.24g). Ovaries of serotonin treated were collected for vehicle control (*N* = 4) (normal saline injection), at 0, 1, 3, 6, 12, 24, 48 and 72 hour after 5HT injection (*N* = 4).

2.14.2 Primers and construction of the standard curve

Primers for RT-PCR of *PmVCP, PmTmsb, PmPKC* and *PmcAMP-RPL* (Table 2.1) were applied for real-time PCR analysis. For construction of the standard curve of each gene, the PCR product of the target gene and *EF-1α* was amplified, electrophoresed through agarose gel and eluted out from the gel. The gel-eluted product was cloned into pGEM-Teasy vector and transformed into *E. coli* JM109. Plasmid DNA were extracted and used as the template for construction of the standard curve. Templates of each gene homologues and *EF-1α* were ten-fold diluted covering 10^3 – 10^8 copy numbers. Real-time RT-PCR was carried out (see below) and each standard point was run in duplicate.

2.14.3 Quantitative real-time PCR

The first strand cDNA was reverse-transcribed. The target transcript (*PmVCP, PmTmsb, PmPKC* and *PmcAMP-RPL*) and internal control (*EF-1α*) of each shrimp were amplified in reaction volume 10 µl containing 5 µl of 2x SYBR Green Master Mix (Roche). The specific primer pairs were used at a final concentration of 0.1, 0.15, 0.2 and 0.3 µM, respectively. The thermal profile for quantitative real-time RT-PCR was 95°C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 53 °C, 55 °C or 56 °C for 30 s and extension at 72 °C for 20 s. Continually, cycles for the melting curve analysis was carried out at 95 °C for 15 s, 65 °C for 1 min and at 98°C for continue and cooling 40 °C for 30 s. Real-time RT-PCR assay was carried out in 96 well plate and each sample was run in duplicate Relative expression levels of different group of samples were statistically test by one way ANOVA followed by Duncan's new multiple rang test ($P < 0.05$). While serotonin and progesterone administration were statistically test by multiple pairwise kruskal wallis test ($P < 0.05$).

2.15 *In situ* hybridization (ISH)

2.15.1 Sample preparation

Ovaries of intact and eyestalk-ablated *P. monodon* broodstock were fixed in 4% paraformaldehyde prepared in 0.1% phosphate-buffered saline (PBS, pH 7.2) overnight at 4°C. The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at -20°C until used. Tissue was histogically prepared, embedded in paraffin and Conventional paraffin sections (5 µm) were carried out onto poly-L-lysine-coated slides.

2.15.2 Preparation of cRNA probes

For *PmVCP* the template used for synthesis of the cRNA probes were PCR-amplified. The T7 (TAATACGACTCACTATAGGG) and SP6 sequence (ATTTAGGTGACACTATAGAA) (Table 2.7) promoter sequences were added to the 5′ of forward and reverse primers, respectively. PCR was carried out in a 25 µl reaction volume containing 10 ng of recombinant plasmid partial cDNA of *PmVCP* of the target transcripts were used as the template. The PCR condition was initially performed by predenaturation at 94˚C for 3 minutes followed by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 65˚C for 30 seconds and at 72˚C for 1 minute. The PCR product was purified using a illustra GFX PCR DNA and Gel Band Purification Kit (GE). The concentration of purified PCR product was estimated by comparing with the DNA marker after electrophoresis and also spectrometrically estimated.

Table 2.7 Nucleotide sequences and Tm of primers for synthesis of the cRNA probes of *PmVCP*

Gene	Sequence	Tm
PmVCP-T7F	F: TAATACGACTCACTATAGGGAGGGTGACCTGTTCCTGGTGCGA	**66**
PmVCP-SP6R	R: ATTTAGGTGACACTATAGAACGATGGAGTTGGGGCGGTTTGTG	**60**

For *PmVCP*, the sense and anti-sense cRNA probes were synthesized from a recombinant plasmid containing partial cDNA of *PmVCP* using primers (PmVCP-T7F (5′-TAATACGACTCACTATAGGGAGGGTGACCTGTTCCTGGTGCGA-3′) and PmVCP-SP6R (5′ATTTAGGTGACACTATAGAACGATGGAGTTGGGGCGGTTTGTG-3′). Briefly, 100 ng of recombinant plasmid *PmVCP* was reacted with the condition containing 1X transcription buffer, 0.2 mM dNTP, 0.75 unit of *pfu* Taq polymerase (Promega). The PCR condition was initially performed by predenaturation at 95˚C for 3 minutes followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 65˚C for 30 seconds and at 72˚C for 1 minute. The PCR product was purified using a illustra GFX PCR DNA and Gel Band Purification Kit (GE). The concentration of purified PCR product were estimated by comparing with the DNA marker after electrophoresis and also spectrophotometrically estimated.

2.15.3 Synthesis of the cRNA probes

For synthesis of the cRNA probe, 1 µg of the gel-eluted PCR product was used as the template using the protocol recommended by the manufacturer (Roche). The mixture was incubated at 40˚C for 2 hours for the antisense probe and 37˚C for 2 hours for the sense probe. The template DNA was eliminated by treating with DNase I at 37˚C for 20 minutes. The reaction was terminated by adding 2 µl of 0.2 M EDTA

(pH 8.0). The synthesized probe (1 µl) was determined by electrophoresis and the remaining reaction mixture was purified using an RNeasy® MinElute® Cleanup kit (Qiagen). The cRNA probe concentration was spectrophotometrically measured and stored at -80˚C until needed.

2.15.4 Dot blot analysis

The quality of cRNA probes was determined before used for *in situ* hybridization using dot blot analysis. Serial dilutions of the pre-diluted probe and control cRNA were made. The diluted probe (1 µl) was spotted on a piece of the Hybond N^+membrane. The spotted probe was fixed to the membrane by cross-linking with UV-light for 1 minute. The membrane was washed with the washing buffer for 1 minute and incubated in the blocking solution for 1 minute. After that, the membrane was incubated in Anti-DIG-alkaline phosphatase (1:5,000 in the blocking solution) for 3 minute, washed with the washing buffer for 1 minute and incubates in the detection buffer. The positive hybridization signals was developed using NBT/BCIP solution. The intensities of the control and the dilution of probe were compared to estimate the concentration of the cRNA probe.

2.15.5 Hybridization and detection

Tissue sections were dewaxed with xylene and dehydrated in absolute ethanol. The sections were prehybridized with 2x SSC containing 50% deionized formamide, 1 µg/µl yeast tRNA, 1 µg/µl salmon sperm DNA, 1 µg/µl BSA and 10% (w/v) dextran sulfate at 50ºC for 30 min and hybridized with either the sense or antisense probe in the prehybridization solution overnight at 50ºC. After hybridization, the tissue sections were washed twice with 4x SSC for 5 min each and once with 2x SSC containing 50% (v/v) formamide for 20 min at 50ºC. The sections

were immersed in prewarmed RNase A buffer (0.5 M NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) at 37 ºC for 30 min and treated with RNase A (10 µg/ml) at 37 ºC for 30 min. Tissue sections were washed four times with the RNase A buffer (37ºC, 10 min each) and 2x SSC (50ºC, 15 min each). Then tissue sections was wash twice with 2x SSC for 15 min at 50ºC. And high stringent washing was carried out twice in 0.2x SSC at 50ºC for 20 min each. Detection of the positive hybridization signals was carried out with a DIG Wash and Block Buffer kit (Roche) (Qiu and Yamano, 2005).

2.16 *In vitro* expression of recombinant proteins using the bacterial expression system

2.16.1 Primer design

A pair of primer was designed to amplify the complete ORF or functional domain sequence of each gene. The forward and reverse primers contained a restriction enzyme site and six His encoded nucleotides, respectively were designed (Table 2.8).

2.16.2 Construction of recombinant plasmid in cloning and expression vectors

The recombinant plasmid of each genes were amplified by PCR, ligated to pGEM®-T Easy vector and transformed in to *E. coli* JM109. Plasmid was extracted from a positive clone and used as the template for PCR amplification using 0.2 µM of each primer, 0.75 unit *Pfu* DNA polymerase (Promega) and 0.2 mM of each dNTP.

The thermal profiles were predenaturation at 95˚C for 3 minutes followed by 30 cycles of denaturation at 95˚C for 30 seconds, annealing at 58˚C for 30/45 seconds and at 72˚C for 2 minutes and the final extension at 72˚C for 7 minutes. The amplification product and expression vector were digested with restriction enzymes

and analyzed by agarose gel electrophoresis. The gel-eluted product was ligated into vector digested and transformed into *E. coli* JM109. The recombinant plasmid was subsequently transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL.

Table 2.8 Gene specific overhang primers, their sequences and melting temperature ™ of *PmVCP*, *PmRacgap1*, *PmTmsb*, *PmNUP133* and *Pmsema*

Gene	Sequence	Tm
PmCdc48-VCP-F/ *Bam H* I	F: 5'-TTT CATATG GCCGAACAGGACGACTTAGC-3'	59
PmCdc48-VCP-R/ *Eco R*I	R: 5'-GCC GAATTC *TCA* **ATG ATG ATG ATG A TG ATG** TCGCTTAACTGGCTCTCCTT-3'	57
PmRacgap1-ORF-F/ *Nde* I	F: 5'-GCC CATATG GAGTCCCTTTCAGCA-3'	62
PmRacgap1-ORF-R/ *Bam H* I	R: 5'-GCC GGATCC *TCA* **ATG ATG ATG ATG ATG ATG** CTTCCGGAAGACATA -3'	65
PmTmsb-ORF-F / *Nde* I	F: 5'-GCC CATATG AGCGCTGAAACT CCCCTCAAG-3'	63
PmTmsb-ORF-R/ *Bam H* I	R: 5'-GCC GGATCC *TCA* **ATG ATG ATG ATG ATG ATG** GGCCTTCTTCTCTTCCTC AAT-3'	57
PmNPC-NUP133-Nucleoporin_N-F/ *Nde* I	F: 5'-GCC CATATG GAAGGCAGTGGC CAG-3'	64
PmNPC-NUP133-Nucleoporin_N-R/ *Eco R*I	R: 5'-GCC GAATTC *CTA* **ATG ATG ATG ATG ATG ATG** CACCCGATCCCCACT GTC-3'	58
PmSema-F/ *Nde* I	F: 5'-GCC CATATG CTT CCAGACAACGTG CTG -3'	52
PmSema-R/ *Bam H* I	R: 5'-GCC GGATCC *TCA* **ATGATGATG ATGATGATG** GAC GAA GGG ATT GCT CCT -3'	54

*Restriction site (underlined) and reverse stop codon (italicized) and six His encoded nucleotides (boldfaced)

2.16.3 Expression of recombinant proteins

A bacterial colony carrying recombinant plasmid of each gene was inoculated into 3 ml of LB medium containing 50 µg/ml ampicillin or 30 µg/ml kanamycin and 50 µg/ml chloramphenicol at 37°C and 250 µl of the overnight culture was transferred to 50 ml of LB medium containing ampicillin or kanamycin and chloramphenicol and further incubated until an OD_{600} was obtained at 0.4-0.6, one

OD_{600} was taken at 0 hour after induced by 1mM IPTG. After IPTG induction, one OD_{600} was time-interval taken at 1, 2, 3, 6, 12 and 24 hr. The cultures were centrifuged at 12000 g for 1 min, resuspended with 2X loading buffer and 1XPBS (pH 7.4) and analyzed by SDS-PAGE (Laemmli, 1970). In addition, for soluble or inclusion bodies protein was investigated subsequently by cultured 20 ml of the IPTG induced-culture (3 /6 hr). The cultured was centrifuged and resuspended in lysis buffer (0.05 M Tris-HCl; pH 7.5, 0.05 M NaCl; containing 1 mg/ml lysozyme) and sonicated at 15% amplitude, pulsed on for 30 seconds and pulsed off for 45 seconds in a period 3 minutes until the solution is cleared. The protein concentration of both soluble and insoluble fractions was measured (Bradford, 1976). Overexpression of recombinant protein was analyzed by SDS-PAGE. For western blot analysis, the electrophoresed proteins were transferred to a PVDF membrane (Towbin et al., 1979).

Recombinant protein was size-fractionated using SDS-PAGE. For western blpt analysis, the electrophoresed proteins was transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) in 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at 100 V for 90 min. The membrane was treated in 5% BSA blocking solution for overnight, then the membrane was incubated with the Anti-His antibody IgG2a (GE Healthcare; 1:7500 in the blocking solution) for 1 h at room temperature. The membrane was washed 3 times with 1× Tris Buffer Saline-Tween20 (TBST; 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween20) and incubated with goat anti mouse IgG (H+L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:7500 for 1 h and washed 3 times with 1× TBST. Immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate.

2.16.4 Purification of recombinant proteins

Recombinant protein was purified using a His GraviTrap kit (GE Healthcare). Initially, 1 liter of IPTG-induced cultured 3-6 hr at 37°C was harvested by centrifugation at 7000 rpm for 15 min. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4), sonicated and centrifuged at 12000 rpm for 45 min. The insoluble fraction was purified by using a His GraviTrap kit (GE Healthcare) under denaturing .The insoluble fraction was loaded into the column after binding with binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) and washed with 10 ml of the binding buffer containing 20 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, pH 7.4 and 8M urea), 10 ml of the binding buffer containing 40 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, pH 7.4 and 8M urea) and 5 ml of the binding buffer containing 80 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, 80 mM imidazole, pH 7.4 and 8M urea), respectively. The recombinant protein was eluted with 6 ml of the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4 and 8M urea). For soluble fraction, the soluble protein was loaded into column after binding column with binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4 containing 40 mM imidazole). The recombinant protein was obtained subsequently by washing the column with binding buffer containing 40 mM imidazole, 80 M imidazole, 150 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) respectively. The recombinant protein was eluted by binding buffer containing 500 mM imidazole (20 mM sodium phosphate, 500 mM NaCl, pH 7.4 and 500 mM imidazole). Fractions from the washing and eluting steps were analyzed by SDS-PAGE

and western blotting. The purified protein was stored at 4°C or -20°C for long term storage.

2.16.5 Peptide sequencing of recombinant proteins

Peptide sequencing was applied to confirm that the expressed proteins were rPmCdc48-VCP, rPmTmsb, rPmRacgap1, rPmNPC-NUP13 and rPmsemaphorin-2a_sema by using NanoLC-MS/MS.

2.16.6 Polyclonal antibody production and polyclonal antibody purification

Polyclonal antibody against rPmVCP, rPmTmsb, rPmRacgap1, rPmNPC-NUP133 and rPmSema was immunologically produced in rabbit by Faculty of Associated Medical Sciences, Chiangmai University. Western blot analysis was carried out to examine specificity and sensitivity of the antibody.

For polyclonal antibody purification: purification of polyclonal antibody using protein A (Thermo Scientific), three milliliter of polyclonal antibody was centrifuged prior to loading into the column after binding with binding buffer that followed by manufacturer protocol and some modified. For purification of polyclonal antibody using Hitrap NHS-activated HP (GE), recombinant protein was incubated by injection into the column for 15-30 minutes at room temperature or 4 hour at 4°C then followed by recommended the manufacturer protocol.

2.16.7 Western blot analysis

For western blot analysis, ovarian tissues of *P. monodon* were ground in the extraction buffer: 10% TCA in acetone (V/V), 0.1% DTT (W/V) and protease inhibitor cocktail. After centrifugation at 10000 g for 10 minutes at 4°C, the supernatant were collected. Acetone is added and left at -20°C 2 hours. The mixtures were centrifuged

at 10000 g for 30 minutes at 4°C. The supernatant is discarded. The pellet is air-dried and dissolved in lysis buffer. The amount of extracted proteins were measured by a dye binding assay (Bradford, 1976). Twenty micrograms of ovarian proteins were heated at 100°C for 5 min, immediately cooled on ice and size-fractionated by SDS-PAGE (Laemmli, 1970). The percentage of SDS-PAGE was depended on size of the protein. Electrophoretically separated proteins were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) in the 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at a constant current of 100 V for 90 minutes. The membrane was treated with the 5% BSA blocking solution for overnight and incubated with the primary antibody in the blocking solution for 1 hr at room temperature. The membrane was washed 3 times with 1x Tris Buffer Saline-Tween-20 (TBST; 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween-20) and incubated with goat anti-rabbit IgG (H+L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:3000 for 1 h and washed 3 times with 1x TBST. Immunological signals were visualized using NBT/BCIP (Roche) as the substrate.

2.17 Localization of reproduction-related proteins

2.17.1 Immunohistochemistry

The paraffin sections were prepared from pieces of ovaries fixed with 4% paraformaldehyde. Deparafinized sections were autoclaved in 0.01 sodium citrate (pH 6.0) containing 0.1% Tween-20 at 120˚C for 5 minutes. Then incubated in the blocking solution I (3% H_2O_2 in methanol) for 15 minutes. After treatment in the blocking solution II (Roche) for 4 hours, section were incubated with purified anti-VCP-cdc48, anti-RacGap1, anti-NPC133_N, anti-semaphorin-2a_sema, preimmunise and blocking solution (control) for 1 hour in the humid chamber. The sections were

rinsed three times for 5 minutes with 1XPBS (pH 7.2) and incubate with goat anti-rabbit IgG conjugated with horse radish peroxidase for 1 hour. The sections were again rinse three times for 5 minutes with 1XPBS (pH 7.2). Localization of antigen was visualized using diaminobenzidine (Wako) as the substrate. Tissue section were dehydrated and mounted for long term storage.

2.17.2 Immunofluorescence

Ovaries were dissected out from *P. monodon* broodstock and fixed in Davidson's fixative overnight at 4°C. Standard paraffin sections (6 µm) were carried out. The sections are deparaffinized and rehydrated through a grade ethanol series for 5 minutes each then immerse in TBST containing 10% glycine for 10 min. After treatment in a blocking solution (10% normal goat serum) for 2 hours, the sections are incubated with the first antibody (1:20) overnight at room temperature, and rinsed with 1x TBST three times for 20 min each. The tissue sections are incubated with goat anti-rabbit IgG conjugated with Alexa 635 or Alexa 488 (1:200 in blocking solution) for 2 hours and rinsed with 1x TBST. The sections are mounted in Mounting Medium (Thermo scientific). The slides are viewed and image-captured by a confocal laser scaning microscope. Tissue sections were also incubated pre-immune rabbit sera and the blocking solution as the negative controls.

2.18 Pull down assay

Pull-down assay were carried out by a Sulfo-NHS-LC-Biotin kit (Pierce) according to the manufacturer protocol. The biotin-labeled recombinant protein was immobilized with the affinity ligand (Streptavidin) and incubated at 4°C for 30 minutes with gentle rotation and centrifuge at 1250 g for 30-60 seconds. Available streptavidin sites are blocked with free biotin by gently inverted before centrifuged at

1250 g for 30-60 sec. The ovarian lysate was added into immobilized column and incubated at 4°C for 1 hour. The column were washed by the addition of 250 µl of the wash buffer and incubated for 1 minute at room temperature. The reaction is centrifuged and the neutralization buffer (Tris-HCl ; pH 9) was added. The eluted complexes protein elution was obtained by adding 250 µl of the elution buffer and the tube is mixed by gently inverting the column and the column is further incubated for 3-5 minutes at room temperature. After centrifugation, the complex proteins were resolved by SDS-PAGE. Bound proteins are further identification protein using nanoESI-LC/MS/MS (Proteomics Laboratory, BIOTEC).

CHAPTER III
RESULTS

3.1 Cellular proteomic profiles of *P. monodon* ovaries examined by 1-DE and nanoESI-LC-MS/MS

The new approach for proteomic analysis was carried out using the GeLC-MS/MS method. One-dimensional gel electrophoresis (1-DE) was performed to examine protein profiles of both domesticated shrimp and wild intact (non-ablated) *P. monodon* broodstock. Specimens were classified to five groups; 10 month-old (GSI = 0.40 ± 0.07%; A), 14 month-old (GSI = 0.36 ± 0.04%; B and 1.11 ± 0.07%; C) and wild broodstock possessing stages II (GSI = 2.32 ± 0.13; D) and IV (GSI = 9.70 ± 1.22; E) ovaries (*N* = 3 for each group). Total soluble proteins were extracted from ovaries of each shrimp and 30 µg of the extracted proteins was size-fractionated in 12.0% SDS-PAGE (Figure. 3.9).

The protein bands were excised according to marker proteins range (> 225 kDa, 176-225 kDa, 150-176 kDa, 102-150 kDa, 76-102 kDa, 52-76 kDa, 38-52 kDa and <38 kDa) and 4-5 pieces of approximately 1 square millimeter of gel pieces each were subjected to in-gel digestion. Peptide sequencing was carried out using nanoESI-LC-MS/MS.

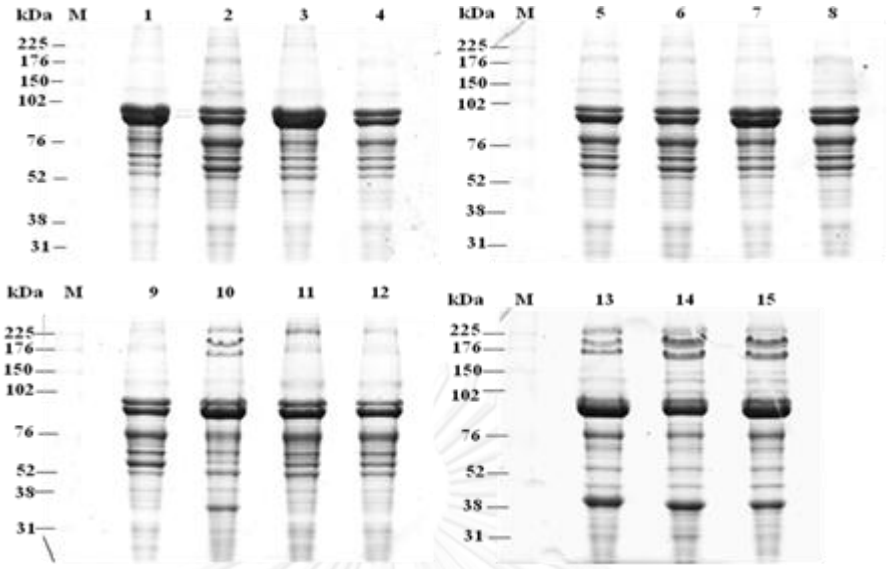


Figure 3.9 Ovarian protein profiles of *P. monodon* analyzed by 12% SDS-PAGE. Lanes 1-3 = 10-month-old (GSI < 0.6%), Lanes 4-6 = 14-month-old (GSI < 0.5%), lanes 7-9; 14-month-old (GSI < 1.5%), lane 10-12 = wild intact broodstock (GSI = 2.0-2.5%) and lanes 13-15 = wild intact broodstock (GSI > 6.0%), respectively.

In total, 1638 proteins matched those previously deposited in the databases and 1253 (76.50%) of which matched known proteins while the remaining 385 proteins (23.50%) significantly matched hypothetical proteins, unnamed proteins or unknown proteins (Appendix A, Table A1). Characterized proteins in this study could be divided to proteins that matched the local shrimp database (800 proteins which matched deduced peptide sequences *P. monodon* ESTs; https://pmonodon.biotec.or.th), those matched the non-redundant database (672 proteins; http://ncbi.nim.nih.gov) and those matched the SWISS-PROT (166 proteins; http://expasy.org/) respectively.

Among proteins identified in this study, 1451 proteins (e.g. thioredoxin domain-containing protein 3, Importin-7, serine/threonine protein phosphatase, C subunit of V-ATPase, karyopherin alpha 2, RAG cohort 1, importin alpha 1,

DEAD/DEAH box helicase, histone acetylase complex subunit, ovarian peritrophin 1 precursor, dual specificity protein kinase CLK2, F-box/ankyrin repeat protein SKIP35, bystin isoform 1, thymosin beta isoform 2, M-phase inducer phosphatase Cdc25-like protein, Heat shock protein 67B2, cyclophilin A, 14-3-3-like protein, WAP four-disulfide core domain 5 precursor, p47 protein isoform a, peritrophin-like protein 2, vesicle transport protein GOT1B, hormone receptor 3C, saposin, ras-related protein Rab-10, vitellogenin, dual 3',5'-cyclic-AMP and -GMP phosphodiesterase 11, Rho and Rac GTPase) were expressed in ovaries of both domesticated and wild broodstock (Figure 3.10).

No protein was specifically found in ovaries of domesticated 10-month-old shrimp (group A) and both groups of wild broodstock (groups D and E). However, two proteins (L-seryl-tRNA selenium transferase and 60S acidic ribosomal protein P0) were found only in ovaries of domesticated 14-month-old shrimp (groups B and C). Twenty-four proteins (e.g. cytochrome P450 49a1, Vacuolar ATP synthase subunit D, long N-terminal adhesion GPCR, TGF beta-activated kinase, Cdc16-prov protein and regulatory protein, TetR) were found only in domesticated shrimp (groups A, B and C). In addition, 76 proteins (e.g. six transmembrane prostate protein 2 variant 2, adenylate kinase-2 CG3140-PA, E3 ubiquitin-protein ligase Bre1, ras suppressor-1 CG9031-PA, periplasmic sensor hybrid histidine kinase, heterogeneous nuclear ribonucleoprotein A1, A2/B1 homolog, Ser/Arg-related nuclear matrix protein, ovarian peritrophin 2 precursor, magnesium transport protein corA, transmembrane transport protein, cyclic AMP-responsive element-binding protein 3, G2/mitotic-specific cyclin B, ankyrin repeat and SOCS box protein 4, calcium-binding protein p22 and

glyceraldehyde-3-phosphate dehydrogenase) were not foud in mature ovaries of wild broodstock (Group E) (Figure 3.10).

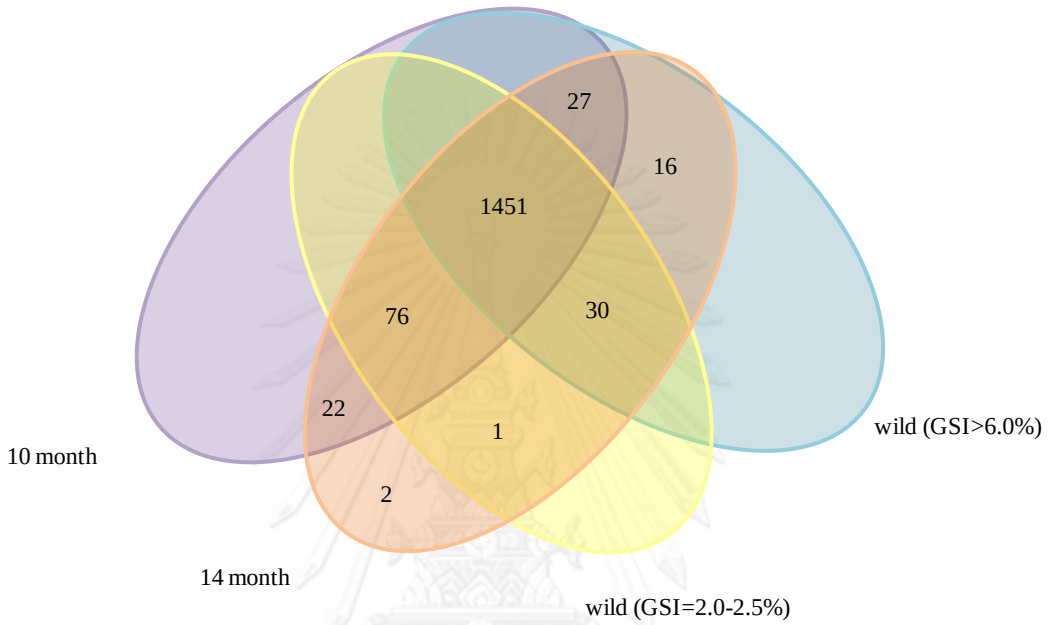


Figure 3.10 Venn diagram representing proteins in each group of *P. monodon* identified by GeLC-MS/MS.

Proteomic spectra of a particular protein in different ovarian stages of domesticated and wild shrimp were compared in order to preliminary identify candidate proteins that play an important role on ovarian development of *P. monodon*.

In total, 514 proteins (354 known proteins, 73 hypothetical proteins and 87 unknown proteins; Appendix A, Table A1) were differentially expressed in different stages of ovarian development. These included, for example, DEAD/DEAH box helicase, ATP-dependent protease ATPase subunit HslU, cytochrome P450, integral membrane sensor signal transduction histidine kinase, membrane protein M1, nuclear

cap-binding protein subunit 1, Cyclic AMP-responsive element-binding protein 3, 2-Cys thioredoxin peroxidase, calmodulin, isoform A, Dual 3',5'-cyclic-AMP and -GMP phosphodiesterase 11, NF-kappa-B inhibitor-interacting Ras-like protein, nuclear pore protein, O-methyltransferase, ubiquitin protein ligase E3A isoform 2, ankyrin repeat domain-containing protein, asparaginyl-tRNA synthetase CG10687-PA, aspartate aminotransferase, ATP-dependent RNA helicase Dbp7, calponin, calreticulin, cytochrome b, GTP-binding nuclear protein RAN1, nuclear transport factor 2, Histone H1, ubiquitin carboxyl-terminal hydrolase 5 and Y-box binding protein isoform 2.

Several reproduction-related proteins exhinbiting differential expression profiles, for example, G2/mitotic-specific cyclin B, receptor for activated protein C kinase (RACK), 14-3-3 like protein, protein disulfide isomerase A6, M-phase inducer phosphatase (Cdc25-like protein), carbonyl reductase 1-like (20β-hydroxysteroiddehydrogenabse) and ubiquitin-activating enzyme E1-domain containing protein, receptor for egg jelly 6, 5-hydroxytryptamine receptor 6 and alpha-2A adrenergic receptor, were identified. In addition, β-thymosin which is abundantly expressed as previously reported by 2-DE analysis (Talakhun et al., 2012) and Rac-GTPase activating protein 1 (PmRacgap1) which was not found in 2-DE analysis were also found. These proteins showed a trend of differential expression level in different groups of samples and were further characterized.

Among identified proteins, those matched unknown function (407 protein, 25.0%). were the most abundant group followed by those functionally categorized into biosynthetic process (118 proteins, 7.20%), transport (89 proteins, 5.43%), metabolic process (69 proteins, 4.21%), translation (42 proteins, 2.56%), regulation (41 proteins, 2.50%), catabolic process (37 proteins, 2.26%) and proteolysis (28

proteins, 1.71%). Proteins in other functional categories (e.g. cell cycle, cell adhesion and protein folding) were found in less than 1% of the characterized proteins (Figure 3.11).

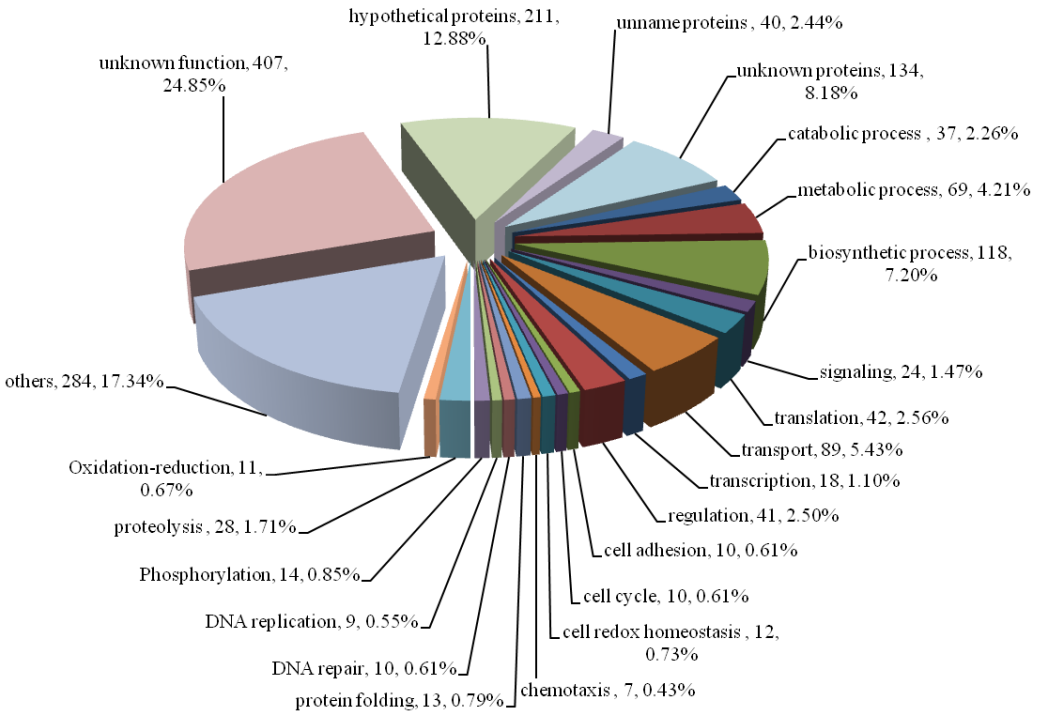


Figure 3.11 A pie chart showing the functional categories of characterized ovarian proteins of *P. monodon* matched those in the databases.

The numbers of characterized proteins in each functional category of different ages of domesticated shrimp and wild intact broodstock possing stage II and IV ovaries were considered. Results indicated no obvious difference of the identified proteins were found among different groups of samples (Figure. 3.12).

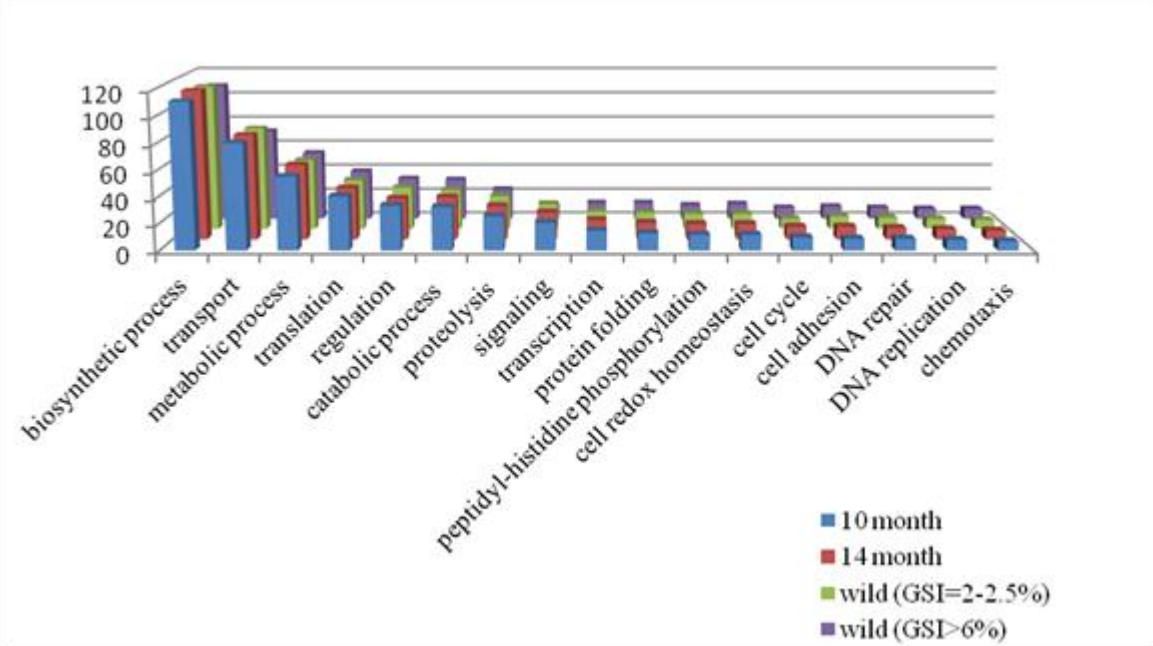


Figure 3.12 Histogram showing numbers of characterized proteins in each functional category that were found in ovaries of different ages of domesticated shrimp and in wild broodstock exhibiting stage II and IV ovaries.

3.2 Characterization of nuclear membrane proteins and nuclear proteins in ovaries of *P. monodon* by GeLC-MS/MS

During oogenesis, oocyte is developed and maturation of oocytes are physically indicated by the germinal vesicle breakdown (GVBD). Therefore, identification of proteins located in the nuclear membrane is the initial step for isolation of biomarkers functional related with oocyte maturation in *P. monodon*.

Different components of proteins from cytoplasm, nuclear membranes, nucleus and cytoskeleton of ovarian cells were extracted from stage I and III ovaries of wild intact *P. monodon* broodstock. The extracted proteins were size-fractionated through 12.0% SDS-PAGE and gels containing different size-range of electrophoresed proteins were excised. Peptide sequencing was carried out using nanoESI-LC-MS/MS.

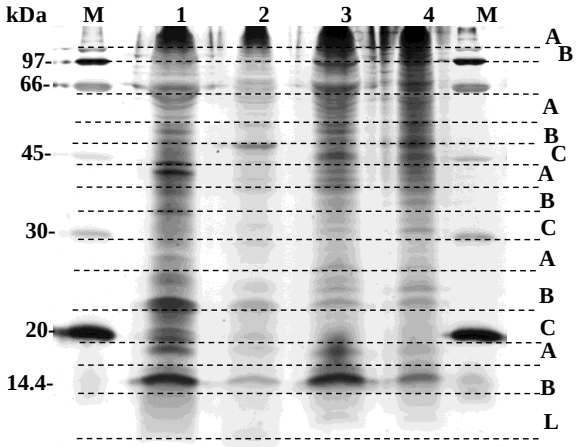


Figure 3.13 Protein profiles of the nuclear proteins and nuclear membrane extracted from stage I and III ovaries of *P. monodon* analyzed by 12% SDS-PAGE. Size-fractionated proteins were excised from the gel by reference to the molecular marker and further analyzed. Lanes 1-2 are nuclear proteins of stage I and III. Lanes 3-4 are nuclear membrane proteins of stage I and III ovaries respectively.

To simplify the proteomic analysis, both nuclear membrane and nuclear protein fractions of stages I and III were pooled and analyzed simultaneously. Bioinformatic analysis was subsequently carried out and divided the discovered proteins to those found in the nuclear membrane (called nuclear membrane proteins), those found in nuclear components (called nuclear proteins) and those with unknown cellular components.

In total, 724 proteins which matched known protein were identified. Analysis of cellular components indicated that 89 proteins were localized at integral to membrane or nuclear membrane (Table A2). Example of these proteins are Lysophospholipid acyltransferase 5-like**,** Ankyrin repeat-containing protein**,** ATP lipid-binding protein like protein**,** Calcium-activated chloride channel family member 1**,**

Glucose-6-phosphate translocase**,** Leucine-rich receptor-like protein kinase-like, ribophorin I, Semaphorin 1a, transmembrane protein 102, voltage-dependent anion-selective channel isoform 1, vacuolar proton ATPase, gap junction Cx32.2 protein, golgi phosphoprotein 3 (coat-protein GPP34), nucleoporin 50kDa, ran gtpase-activating protein, cytochrome c oxidase subunit II, cytochrome P450 and binding-protein-dependent transport systems inner membrane component, aspartyl/asparaginyl beta-hydroxylase, capK capsular polysaccharide biosynthesis protein, Poly [ADP-ribose] polymerase 1, delta12 fatty acid desaturase, cytochrome c oxidase assembly protein COX15 and lysosomal-associated transmembrane protein.

Likewise, 99 proteins were recognized as nuclear proteins as cellular component search revealed that they are localized in nucleus, nucleolus, nucleoplasm or pronucleus (Table A3). Examples of proteins in this group were nuclear autoantigenic sperm protein, DEAD (Asp-Glu-Ala-Asp) box polypeptide 18, DEAD/DEAH box helicase domain-containing protein, vasa, myosin light chain, zinc finger protein, guanine-specific ribonuclease N1 and T1, histone acetyltransferase MYST2, E3 ubiquitin ligase, putative, formamidopyrimidine-DNA glycosylase, TATA box-binding protein, cell division control protein Cdc6, cell division cycle 2 protein, 26S proteasome non-ATPase regulatory subunit 2, cyclin dependent kinase 2, Y-box binding protein isoform 2, importin subunit alpha-2, apoptosis regulator BAX, integrase family protein, mariner transposase, PHP domain-containing protein, DNA topoisomerase I, RNA polymerase factor sigma-54, ATP-dependent RNA helicase, dual specificity tyrosine-phosphorylation-regulated kinase 2 and nuclear transcription factor Y, alpha.

The cellular components of the remaining 536 proteins could not be assigned. Therefore, these characterized proteins were not regarded as nuclear/nuclear memebrane proteins. Examples of proteins in this group were ubiquitin-conjugating enzyme E2 variant 2-like, AMP-binding domain protein, N-acyl-L-amino acid amidohydrolase, ATP-dependent DNA helicase HFM1-like, fast myosin heavy chain, methyltransferase type 11, nickel-dependent hydrogenase large subunit, cytosine deaminase or related metal-dependent hydrolase, O-methyltransferase, PH domain containing protein, calponin homology (CH) domain-containing, chdc/lrch, helix-turn-helix domain-containing protein, regulator of de-novo NAD biosynthesis NadR, malonyl CoA-acyl carrier protein transacylase, ubiquinone binding protein, FAD linked oxidase domain-containing protein, xanthine dehydrogenase, vacuolar protein sorting 8 homolog, ATP-binding ABC transporter protein, methyl coenzyme M reductase alpha subunit, alpha-2-macroglobulin-like, ADP-ribosylation factor GTPase-activating protein, dehydrogenase E1 and transketolase domain-containing protein 1, ubiquitin carboxyl-terminal hydrolase, coatomer subunit delta-like, 14.5 kDa translational inhibitor protein, p14.5 and GTP-binding protein 5.

Notably, several proteins matched those of microorganism (e.g. bacteria and viruses). This suggested that shrimp under study may be infected by infectious agents. Accordingly, results in Tables A2-A4 should be interpreted with cautions

Functional categories of identified nuclear membrane proteins were classified based on biological functions of matched homologues. Disregarding proteins with unknown functions (22 proteins accounting for 22.72%), the most abundant group of nuclear membrane proteins in this study was those in transport (19 protein, 19.10%) followed by biosynthetic process (6 protein, 6.74%), signal transduction (4 protein,

4.49%), ATP catabolic (4 protein, 4.49%), regulation (3 protein, 3.37%), homeostasis (3 protein, 3.37%), regulation (3 protein, 3.37%), apoptotic (2 protein, 2.25%) and cell differentiation (2 protein, 2.25%). Proteins with other functions were collectively accounted for 23.60% (Figure 3.14).

Similarly, functional categories of nuclear proteins identified in ovaries of *P. monodon* were classified. Disregarding proteins with unknown functions (20 protein accounting for 20.20%), the most abundant group of nuclear membrane proteins in this study was those in regulation (18 protein, 18.8%) followed by transcription (5 protein, 5.05%), catabolic process (4 protein, 4.04%), DNA topological change (5 protein, 5.05%), DNA integration (3 protein, 3.03%), DNA repair (3 protein, 3.03%), nucleosome assembly (3 protein, 3.03%), cell division (3 protein, 3.03%), cell differentiation (2 protein, 2.02%), signal transduction (2 protein, 2.02%). Proteins with other functions (21 proteins) were collectively accounted for 27.27% (Figure 3.15).

Functional categories of protein with unknown cellular components found from proteomic analysis of nuclear and nuclear protein fractions extracted from ovaries of *P. monodon* were also classified. Disregarding proteins with unknown functions (368 proteins accounting for 68.66%), the most abundant group of nuclear membrane proteins in this study was those in biosynthetic process (28 proteins, 5.22%) followed by metabolic process and catabolic process (15 proteins each, 2.80%), transcription (10 proteins, 1.87%), signal transduction (8, 1.49%), transport and regulation (7 proteins each, 1.31%), DNA integration (4 proteins, 0.75%), DNA repair, Transposition, Tricaboxylic acid cycle (3 proteins each, 0.56%). Proteins with other functions (62 proteins) were collectively accounted for 27.27% (Figure 3.16).

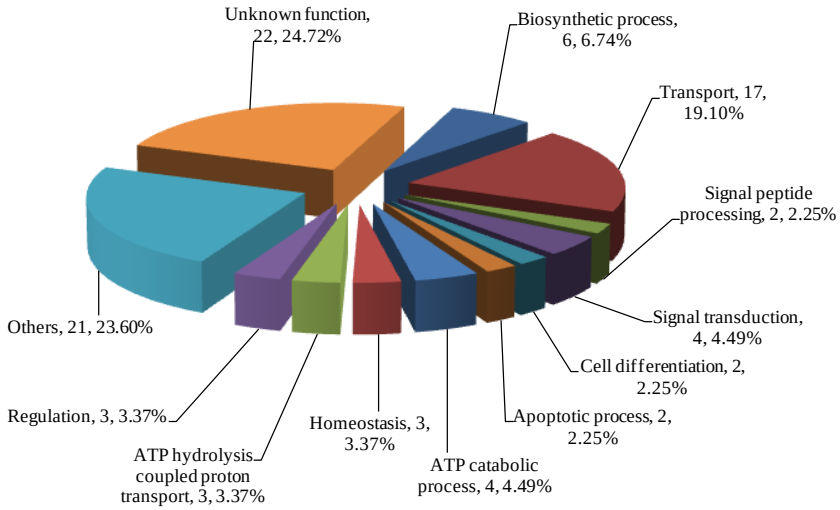


Figure 3.14 Pie chart showing functional categories of nuclear membrane proteins in ovaries of *P. monodon* analyzed by GeLC-MS/MS.

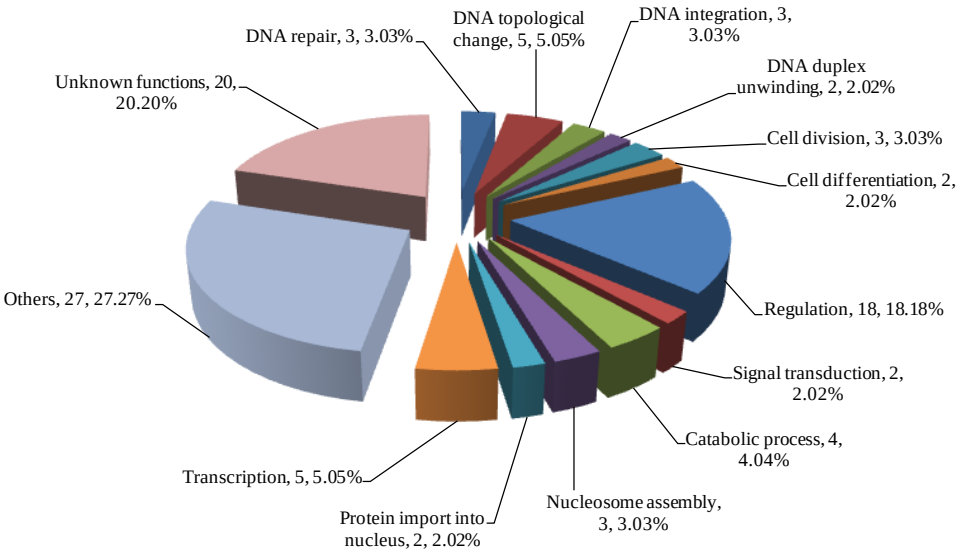


Figure 3.15 Pie chart showing functional categories of nuclear proteins in ovaries of *P. monodon* analyzed by GeLC-MS/MS.

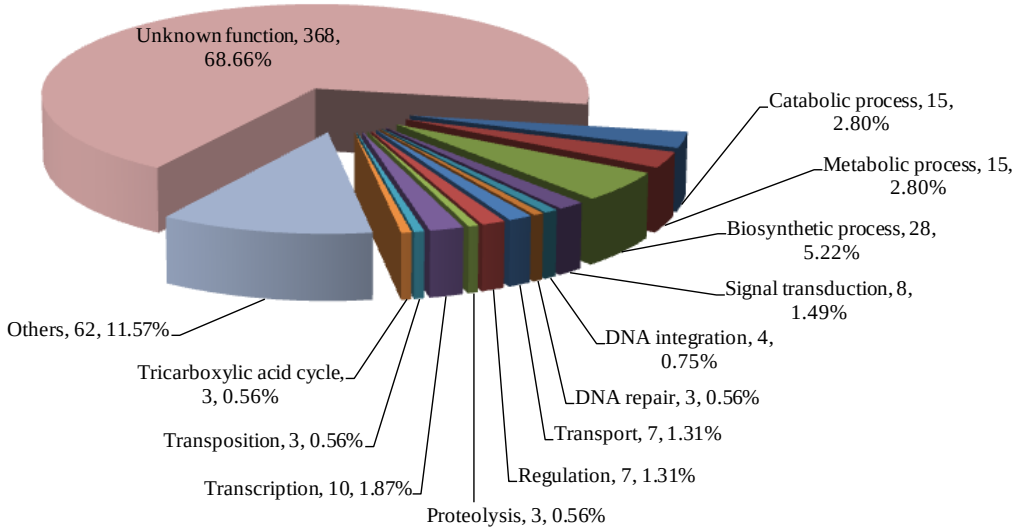


Figure 3.16 Pie chart showing functional categories of protein with unknown cellular components found from proteomic analysis of nuclear and nuclear protein fractions extracted from ovaries of *P. monodon*.

3.3 Isolation and characterization of the full-length cDNA of reproduction-related genes of *P. monodon*

3.3.1. *Valosin containing protein 1*

Two discrete bands were obtained from nested 3'RACE-PCR of *P. monodon valosin-containing protein (PmVCP)*. A 1604 bp fragment was cloned and sequenced (Figure 3.7). Nucleotide sequences of this fragment and the original EST (Figure 3.18) were assembled.

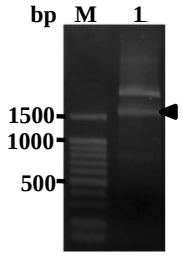


Figure 3.17 Agarose gel eletrophoresis illustrating the nested 3'RACE-PCR product of *PmVCP*. Arrowheads indicate the RACE-PCR product that was cloned and sequenced. Lane M is a 100 bp DNA ladder.

The full-length cDNA of *PmVCP* was 2725 bp in length containing an ORF of 2481 bp deducing to a polypeptide of 826 amino acids and 5' and 3' UTRs of 101 and 113 bp (excluding the poly A tail), respectively (Figure 3.19A). The closest sequence according to the best hit approach of this characterized sequence is *valosin containing protein 1* of *Eisenia fetida* (*E*-value = 0.00).

A

GAGTGTCGAGGAGCCGTCAGAGAGGAAGGAACGCTCGGGGGATTTCTTCGTCTTTTTATTAAAAAAAAC
ACAATAATCCTTCGTTTAATTTATCGAAGCCATGGCCGAACAGGACGACTTAGCTACTGCCATTCTTAA
AGAGAAGAAGAAGCCCAACAGACTCATTGTCGAGGATGCTGTCAACGACGACAATTCCGTGGTGGCACT
CAGCCAGGCCAAGATGGATGAGCTGCAGCTCTTCCGCGGCGACACAGTCCTGCTCAAGGGCAAGAAGCG
CAAACAGACTGTGTGCATTGTGCTCTCAGACGACACCATGCAGGATGACAAGATTCGCATGAACCGTGT
GGTCAGAAACAACCTTCGCATCCGCTTGGGAGACATCGTGTCCATCCAGCCGTGTCCAGACGTCAAGTA
TGGCAAACGTATCCATGTCCTGCCGATTGATGACACTGTTGAAGGTCTCACGGGAAACATCTTTGAGGT
ATATTTGAAGCCCTACTTTCTGGAGGCATACAGGCCCATCCACAAGGGTGACCTGTTCCTGGTGCGAGG
TGGTATGAGGGCTGTGGAGTTCAAGGTGGTGGAGACGGATCCTGCGCCTTATTGCATCGTCTCCCAGGA
CACTGTTATCTACTGTGAAGGAGAGCCAGTTAAGCGAGAGGAGGAGGAAGAGCAGTTGAACGAGGTGGG
CTACGATGACATCGGTGGCTGCCGCAAACAGTTGGCACAGATCAAGGAGATGGTGGAGCTGCCTCTCCG
CCACCCTTCACTCTTCAAGGCCATTGGTGTCAAGCCCCCAAGAGGTATCCTTCTCTATGGTCCTCCTGG
TACTGGTAAGACCCTCATTGCCCGTGCAGTGGCCAACGAGACCGGAGCATTCTTCTTCCTCATCAACGG
GCCTGAGATCATGTCAAAGTTAGCTGGTGAATCCGAGAGCAACCTTCGCAAGGCCTTCGAAGAGGCTGA
GAAGAATGCCCCTGCCATCATCTTCATTGATGAGATTGATGCCATTGCACCCAAGCGTGAAAAGACACA
TGGTGAGGTGGAAAGACGTATAGTGTCACAGCTGCTAACTCTTATGGATGGCCTCAAGCAACGAGCCCA

Valo-F1

TGTTATTGTTATGGCTGCCACAAACCGCCCCAACTCCATCGACCCGGCACTCAGGCGATTTGGACGCTT
TGACAGGGAGGTTGACATCAGTATCCCAGACACTACAGGTCGTCTGGAGATTTTGCGCATCCACACTAA
GAACATGAAGCTGTCAGATGATGTGGACCTGGAACAGATTGCAGCAGAAACCCATGGCCATGTTGGTGC
TGAC

B

Valo-F1

TGCCACAAACCGCCCCAACTCCATCGACCCGGCACTCAGGCGATTTGGACGCTTTGACAGGGAGGTTGA
CATCAGTATCCCAGACACTACAGGTCGTCTGGAGATTTTGCGCATCCACACAAAGAACATGAAGCTGTC
AGATGATGTGGACCTGGAACAGATTGCAGCAGAAACCCATGGCCATGTTGGTGCTGACTTGGCAGCCCT
GTGTTCAGAGGCTGCACTCCAGCAAATCAGAGAAAAGATGGACCTCATAGATCTAGATGATGACCAGAT
TGATGCTGAGGTATTGAATTCCCTGGCTGTCTCTATGGACAACTTTAGGTTTGCTATGGGTAAGAG

3′ RACE-Valosin

CACACCATCAGCTCTGCGTGAGACAGTGGTGGAAGTGCCCAACACCACTTGGAACGATATTGGTGGTCT
CGAAAATGTCAAGAGAGAATTGCAGGAACTTGTGCAGTACCCTGTAGAGCATCCGGGTAAATTCCTCAA
GTTCGGCATGACCCCCAGCAAGGGTGTGCTCTTCTATGGGCCTCCTGGTTGTGGTAAGACCCTCCTGGC
CAAGGCTATTGCCAATGAGTGCCAGGCCAACTTCATTTCCATCAAGGGCCCTGAACTCCTTACTATGTG
GTTTGGTGAATCTGAAGCCAATGTCAGGGATGTGTTTGACAAGGCACGTTCAGCAGCACCTTGTGTTCT
ATTCTTTGATGAATTAGACAGTATTGCCAAGGCACGAGGTGGCTCTGCTGGTGATGCTGGTGGAGCAGC
TGACCGAGTTATCAACCAGGTTCTGACTGAGATGGACGGCATGGGAGCGAAGAAGGACGTGTTCATCAT
TGGTGCCACCAACCGACCTGACATCATTGACCCAGCCATTCTTCGTCCTGGCCGTCTAGATCAGCTCAT
CTACATCCCTCTGCCGGACGAGAAGTCACGCGTACAGATCCTGAAGGCTTGCATGAGGAAGTCTCCAGT
GGCAAAGAGTGTTGACCTGGATTACCTTGCCAAGGTCACTCATGGTTTCTCAGGTGCAGATTTGACTGA
AATTTGCCAGCGTGCATGCAAACTTGCCATCAGACAAGCCATTGAGGCTGATATTCGCCGAGAGAGGGA
AAGAGCAGCAGGAGATACTATGGATATGGAGGAAGAGGATCCCGTTCCCGAAATCACCCGTGACCACTT
TGAGGAGGCCATGAAGTTTGCCCGTCG

3′ Nested- Valosin

GTCAGTGTCAGACAATGACATCAGAAAATACGAGATGTTCTCACAGACGCTACAGCAGAGCCGAGGCCT
GGATCCAATTTCAGATTCCCTGACCAGCAAGGCCAGGGCGGCAGTGGCCAGGGAGGAAACTTTGGTGCC
GATGGAGAGGATGACGACCTCTATTCCTAAAGTGTGTCCCATCCCTTACCCCATCCTGCTCCTCTCCAA
GGGGTTGGCTTGTGGGAAGGGTTTTGACATAGGGCATAAAATACTAGAATCATTC**TGA**GTGGAATGCTT
GGACTATTCCTTAATATAGTGGGGGAGAACCTTTGAGTCTTGAATTTCAGGAACACATTGTAACTTTTG
TTTGTCCGGAAC**AATAAAAA**GGGGACATACAGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.18 Nucleotide sequences of EST (A) and 3' RACE-PCR products (B) of *PmVCP*. A Varo-F1, 3′RACE-Valosin and 3′Nested-Valosin primers used for the primary and secondary 3'RACE-PCR are underlined.

The calculated p*I* and MW of *PmVCP* was 5.48 and 91.67 kDa, respectively. The signal peptide was not found in this putative nonsecretory protein. The *N*-linked-glycosylation domain was found at positions 468- 470 of the deduced PmVCP protein. Two Cdc48 and AAA domains were found at positions 20-103, 120-186 (*E*-value = 3.03e-28 and 1.34e-11) and 232-368, 505-644 (E-value = 3.67e-24 and 3.73e-25; Figure 3.19B) of this deduced protein.

A

```
GAGTGTCGAGGAAGCCGTCAGAGAGGAAGGAACGCTCGGGGGATTTCTTCGTCTTTTTAT   60
TAAAAAAAACACAATAATCCTTCGTTTAATTTATCGAAGCCATGGCCGAACAGGACGACT   120
                                        M  A  E  Q  D  D        6
TAGCTACTGCCATTCTTAAAGAGAAGAAGAAGCCCAACAGACTCATTGTCGAGGATGCTG   180
 L  A  T  A  I  L  K  E  K  K  K  P  N  R  L  I  V  E  D  A   26
TCAACGACGACAATTCCGTGGTGGCACTCAGCCAGGCCAAGATGGATGAGCTGCAGCTCT   240
 V  N  D  D  N  S  V  V  A  L  S  Q  A  K  M  D  E  L  Q  L   46
TCCGCGGCGACACAGTCCTGCTCAAGGGCAAGAAGCGCAAACAGACTGTGTGCATTGTGC   300
 F  R  G  D  T  V  L  L  K  G  K  K  R  K  Q  T  V  C  I  V   66
TCTCAGACGACACCATGCAGGATGGCAAGATTCGCATGAACCGTGTGGTCAGAAACAACC   360
 L  S  D  D  T  M  Q  D  G  K  I  R  M  N  R  V  V  R  N  N   86
TTCGCATCCGCTTGGGAGACATCGTGTCCATCCAGCCGTGTCCAGACGTCAAGTATGGCA   420
 L  R  I  R  L  G  D  I  V  S  I  Q  P  C  P  D  V  K  Y  G   106
AACGTATCCATGTCCTGCCGATTGATGACACTGTTGAAGGTCTCACGGGAAACATCTTTG   480
 K  R  I  H  V  L  P  I  D  D  T  V  E  G  L  T  G  N  I  F   126
AGGTATATTTGAAGCCCTACTTTCTGGAGGCATACAGGCCCATCCACAAGGGTGACCTGT   540
 E  V  Y  L  K  P  Y  F  L  E  A  Y  R  P  I  H  K  G  D  L   146
TCCTGGTGCGAGGTGGTATGAGGGCTGTGGAGTTCAAGGTGGTGGAGACGGATCCTGCGC   600
 F  L  V  R  G  G  M  R  A  V  E  F  K  V  V  E  T  D  P  A   166
CTTATTGCATCGTCTCCCAGGACACTGTTGTCTACTGTGAAGGAGAGCCAGTTAAGCGAG   660
 P  Y  C  I  V  S  Q  D  T  V  V  Y  C  E  G  E  P  V  K  R   186
AGGAGGAGGAAGAGCAGTTGAACGAGGTGGGCTACGATGACATCGGTGGCTGCCGCAAAC   720
 E  E  E  E  E  Q  L  N  E  V  G  Y  D  D  I  G  G  C  R  K   206
AGTTGGCACAGATCAAGGAGATGGTGGAGCTGCCTCTCCGCCACCCTTCACTCTTCAAGG   780
 Q  L  A  Q  I  K  E  M  V  E  L  P  L  R  H  P  S  L  F  K   226
CCATTGGTGTCAAGCCCCCAAGAGGTATCCTTCTCTATGGTCCTCCTGGTACTGGTAAGA   840
 A  I  G  V  K  P  P  R  G  I  L  L  Y  G  P  P  G  T  G  K   246
CCCTCATTGCCCGTGCAGTGGCCAACGAGACCGGAGCATTCTTCTTCCTCATCAACGGGC   900
 T  L  I  A  R  A  V  A  N  E  T  G  A  F  F  F  L  I  N  G   266
CTGAGATCATGTCAAAGTTAGCTGGTGAATCCGAGAGCAACCTTCGCAAGGCCTTCGAAG   960
 P  E  I  M  S  K  L  A  G  E  S  E  S  N  L  R  K  A  F  E   286
AGGCTGAGAAGAATGCCCCTGCCATCATCTTCATTGATGAGATTGATGCCATTGCACCCA   1020
 E  A  E  K  N  A  P  A  I  I  F  I  D  E  I  D  A  I  A  P   306
AGCGTGAAAAGACACATGGTGAGGTGGAAAGACGTATAGTGTCACAGCTGCTAACTCTTA   1080
 K  R  E  K  T  H  G  E  V  E  R  R  I  V  S  Q  L  L  T  L   326
TGGATGGCCTCAAGCAACGAGCCCATGTTATTGTTATGGCTGCCACAAACCGCCCCAACT   1140
 M  D  G  L  K  Q  R  A  H  V  I  V  M  A  A  T  N  R  P  N   346
CCATCGACCCGGCACTCAGGCGATTTGGACGCTTTGACAGGGAGGTTGACATCAGTATCC   1200
 S  I  D  P  A  L  R  R  F  G  R  F  D  R  E  V  D  I  S  I   366
CAGACACTACAGGTCGTCTGGAGATTTTGCGCATCCACACTAAGAACATGAAGCTGTCAG   1260
 P  D  T  T  G  R  L  E  I  L  R  I  H  T  K  N  M  K  L  S   386
ATGATGTGGACCTGGAACAGATTGCAGCAGAAACCCATGGCCATGTTGGTGCTGACTTGG   1320
 D  D  V  D  L  E  Q  I  A  A  E  T  H  G  H  V  G  A  D  L   406
CAGCCCTGTGTTCAGAGGCTGCACTCCAGCAAATCAGAGAAAAGATGGACCTCATAGATC   1380
 A  A  L  C  S  E  A  A  L  Q  Q  I  R  E  K  M  D  L  I  D   426
TAGATGATGACCAGATTGATGCTGAGGTATTGAATTCCCTGGCTGTCTCTATGGACAACT   1440
 L  D  D  D  Q  I  D  A  E  V  L  N  S  L  A  V  S  M  D  N   446
TTAGGTTTGCTATGGGTAAGAGCACACCATCAGCTCTGCGTGAGACAGTGGTGGAAGTGC   1500
 F  R  F  A  M  G  K  S  T  P  S  A  L  R  E  T  V  V  E  V   466
CCAACACCACTTGGAACGATATTGGTGGTCTCGAAAATGTCAAGAGAGAATTGCAGGAAC   1560
 P  N  T  T  W  N  D  I  G  G  L  E  N  V  K  R  E  L  Q  E   486
```

```
TTGTGCAGTACCCTGTAGAGCATCCGGGTAAATTCCTCAAGTTCGGCATGACCCCCAGCA   1620
 L  V  Q  Y  P  V  E  H  P  G  K  F  L  K  F  G  M  T  P  S    506
AGGGTGTGCTCTTCTATGGGCCTCCTGGTTGTGGTAAGACCCTCCTGGCCAAGGCTATTG   1680
 K  G  V  L  F  Y  G  P  P  G  C  G  K  T  L  L  A  K  A  I    526
CCAATGAGTGCCAGGCCAACTTCATTTCCATCAAGGGCCCTGAACTCCTTACTATGTGGT   1740
 A  N  E  C  Q  A  N  F  I  S  I  K  G  P  E  L  L  T  M  W    546
TTGGTGAATCTGAAGCCAATGTCAGGGATGTGTTTGACAAGGCACGTTCAGCAGCACCTT   1800
 F  G  E  S  E  A  N  V  R  D  V  F  D  K  A  R  S  A  A  P    566
GTGTTCTATTCTTTGATGAATTAGACAGTATTGCCAAGGCACGAGGTGGCTCTGCTGGTG   1860
 C  V  L  F  F  D  E  L  D  S  I  A  K  A  R  G  G  S  A  G    586
ATGCTGGTGGAGCAGCTGACCGAGTTATCAACCAGGTTCTGACTGAGATGGACGGCATGG   1920
 D  A  G  G  A  A  D  R  V  I  N  Q  V  L  T  E  M  D  G  M    606
GAGCGAAGAAGGACGTGTTCATCATTGGTGCCACCAACCGACCTGACATCATTGACCCAG   1980
 G  A  K  K  D  V  F  I  I  G  A  T  N  R  P  D  I  I  D  P    626
CCATTCTTCGTCCTGGCCGTCTAGATCAGCTCATCTACATCCCTCTGCCGGACGAGAAGT   2040
 A  I  L  R  P  G  R  L  D  Q  L  I  Y  I  P  L  P  D  E  K    646
CACGCGTACAGATCCTGAAGGCTTGCATGAGGAAGTCTCCAGTGGCAAAGAGTGTTGACC   2100
 S  R  V  Q  I  L  K  A  C  M  R  K  S  P  V  A  K  S  V  D    666
TGGATTACCTTGCCAAGGTCACTCATGGTTTCTCAGGTGCAGATTTGACTGAAATTTGCC   2160
 L  D  Y  L  A  K  V  T  H  G  F  S  G  A  D  L  T  E  I  C    686
AGCGTGCATGCAAACTTGCCATCAGACAAGCCATTGAGGCTGATATTCGCCGAGAGAGGG   2220
 Q  R  A  C  K  L  A  I  R  Q  A  I  E  A  D  I  R  R  E  R    706
AAAGAGCAGCAGGAGATACTATGGATATGGAGGAAGAGGATCCCGTTCCCGAAATCACCC   2280
 E  R  A  A  G  D  T  M  D  M  E  E  E  D  P  V  P  E  I  T    726
GTGACCACTTTGAGGAGGCCATGAAGTTTGCCCGTCGGTCAGTGTCAGACAATGACATCA   2340
 R  D  H  F  E  E  A  M  K  F  A  R  R  S  V  S  D  N  D  I    746
GAAAATACGAGATGTTCTCACAGACGCTACAGCAGAGCCGAGGCCTGGATCCAATTTCAG   2400
 R  K  Y  E  M  F  S  Q  T  L  Q  Q  S  R  G  L  D  P  I  S    766
ATTCCCTGACCAGCAAGGCCAGGGCGGCAGTGGCCAGGGAGGAAACTTTGGTGCCGATGG   2460
 D  S  L  T  S  K  A  R  A  A  V  A  R  E  E  T  L  V  P  M    786
AGAGGATGACGACCTCTATTCCTAAAGTGTGTCCCATCCCTTACCCCATCCTGCTCCTCT   2520
 E  R  M  T  T  S  I  P  K  V  C  P  I  P  Y  P  I  L  L  L    806
CCAAGGGGTTGGCTTGTGGGAAGGGTTTTGACATAGGGCATAAAATACTAGAATCATTCT   2580
 S  K  G  L  A  C  G  K  G  F  D  I  G  H  K  I  L  E  S  F    826
GAGTGGAATGCTTGGACTATTCCTTAATATAGTGGGGGAGAACCTTTGAGTCTTGAATTT   2640
 *
CAGGAACACATTGTAACTTTTGTTTGTCCGGAACAATAAAAAGGGGACATACAGTAAAAA   2700
AAAAAAAAAAAAAAAAAAAAAAAAA                                      2725
```

B

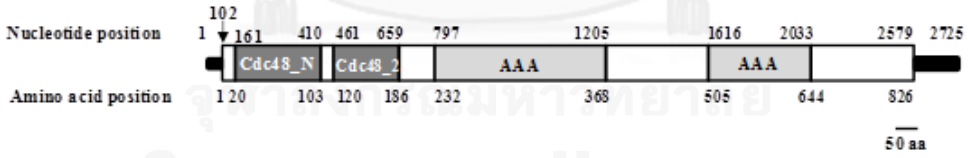


Figure 3.19 (A) The full-length cDNA anddeduced amino sequences of *valosin-containing protein* of *P. monodon* (*PmVCP*). Start and stop codons are illustrated in boldfaced and underlined. A putative *N*-linked-glycosylation site is highlighted and underlined. (B) Diagram illustrating VCP cDNA of *P. monodon*. The CDC48 and AAA domains were found in the deduced protein of this transcript. The scale bar is 50 amino acids in length.

3.3.2 *Thymosin-β*

The primary 5' and 3' RACE-PCR generated several amplification products. PCR was carried out by amplification of the primary RACE-PCR product with the same gene-specific primer and nested universal adaptor primer (nUPM). After electrophoresis, discrete bands approximately 250 bp and 450 bp fragments were obtained from 5' and 3' RACE-PCR of *P. monodn thymosin-β* (*PmTmsb*) (Figure 3.20). These fragments were cloned and sequenced. Their nucleotide sequences (Figure 3.21) were assembled with the original EST sequence.

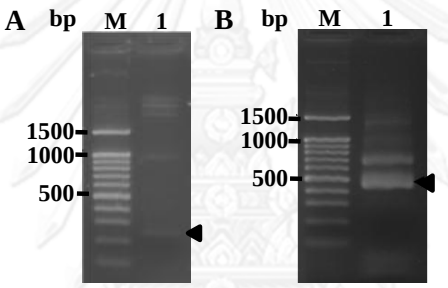


Figure 3.20 Agarose gel electrophoresis showing results from 5' (lane 1, A) and 3' (lane 1, B) RACE-PCR of *PmTmsb*. Arrowheads indicate RACE-PCR products that were cloned and sequenced. Lanes M are a 100 bp DNA ladder.

The full length cDNA of *PmTmsb* was 1084 bp in length containing an ORF of 387 bp corresponding to a polypeptide of 128 amino acids. The 5' and 3' UTRs of *thymosin-β5* were 69 and 602 bp (excluding the poly A tail), respectively. The poly A additional signal (AATAAA) was located between 1043-1048 of this transcript (Figure 3.22A). Its closest similarity according to the best hit approach was *thymosin-β5* of *Pacifastacus leniusculus* (*E*-value = 4e-44).

A

GGGGAGTTGAGCATTGGACTTCAACGAGTTAGCCTTCGAACCAGAATCACCACCACCCACCACCACCAT
C**ATG**AGCGCTGAAACTCCCCTCAAGGACTTGCCCAAGGTTGACCCCACCCTTAAGGGACAGCTCGAGGG
5´RACE
ATTCTCCGCCGTAAACCTTAAGAAGACCGAGACGGAGGAGAAGATTCACCTGCC

B

GAGCGCTGAAACTCCCCTCAAGGACTTGCCCAAGGTTGACCCCACCCTTAAGGGACAGCTCGAGGGATT
5´RACE
CTCCGCCGTAAACCTTAAGAAGACCGAGACGGAGGAGAAGATTCACCTGCCAAACAGGGAGGACGTGGA
AGCAGAGAAGAAAGTACAGGCCCATCTGCAGGCCGTCGAAGGCTTCAATACTGCACAACTCAAGCATGC
CAATACCCAAGAAAAAATTGTTTTACCTGCTCAGGAAGATATTGAGACTGAGAAGGGTCAGCAGGCACT
CCGCCAGGGTATTGAGGGCTTTGACCATGCTGCTTTGAAGAAAGCTCAGACGACAGAGAAGAATACCCT
3´RACE
TCCAACTAAGGAAATGATTGAGGAAGAGAAGAAGGCC**TAA**CAAGGTTTGCCAGGAACACATCTATGTTC
ACTGTGCATCCCAGTGATATCCTCCCTGCTCTCCCTAGAACTTCTAATAGTTCTGCAAGCCAAAATGTT
TTGTACTGTACTTTGATAATCTAAGTGTATAGGTAAACCAACTGATTTTGTAACCGGATATCATATGCT
Nested3´RACE
ATTCTTTGAGTCATTATTATTTGCTTTAGTGATAAGATCAAGGTGCTCACCAATAGCAAGGCGATGGCA
AGAAGTGCAAAAGGGACATTTAGAAACTGCATGATAATTTTCATTGCTTTTGTGCACCCTCCTAGTAAT
TTATCAGCAATGCTAGTGGACCAAAATTTCACCTTGCTTC

C

3´RACE
AGGTTTGCCAGGAACACATCTATGTTCACTGTGCATCCCAGTGATATCCTCCCTGCTCTCCCTAGAACT
TCTAATAGTTCTGCAAGCCAAAATGTTTTGTACTGTACTTTGATAATCTAAGTGTATAGGTAAACCAAC
TGATTTTGTAACCGGATATCATATGCTATTCTTTGAGTCATTATTATTTGCTTTAGTGATAAGATCAAG
Nested3´RACE
GTGCAATAGCAAGGCGATGGCAAGAAGTGCAAAAGGGACATTTAGAAACTGCATGATAATTTTCATTGC
TTTTGTGCACCCTCCTAGTAATTTATCAGCAATGCTAGTGGACCAAAATTTCACCTTGCTTCCGCCTTG
TTCAATAGAAGGTAAAGGTCTCCCATGCAGGTAATGGAAAACTGCCAAAAGGTACAATGAATTTGTAAT
TTGCCAGGATTGTAATGTGTGAGTCTCAATTTTTACCCCATTTAATTTGCATTTAATTGTTAGCATTTA
CATGAATTTTTAAGTGTTTATAAAGAATTTATCATTGCCTTGTTTCAAAATTAATTTGCTTTTTGGGGC
ATCCAAGTAAATGTGATTTTGGGAGCC**AATAAA**GTGCTTTTGCAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.21 Nucleotide sequence of 5' RACE-PCR (A), the original EST (B) and 3' RACE-PCR (C) of *PmTmsb*. Sequences of primers are underlined.

The calculated p*I* and MW of *PmTmsb* was 5.45 and 14.30 kDa, respectively. The predicted signal peptide was not found in the deduced PmTmsb protein. Like thymosin-β5, THY domains were found at positions 18-54 (*E*-value = 9.48e-06), 56-92 (*E*-value = 5.48e-01), 94-128 (*E*-value = 3.68e-03) of the deduced protein, respectively (Figure 3.22B).

A

```
GGGAGTTGAGCATTGGACTTCAACGAGTTAGCCTTCGAACCAGAATCACCACCACCCACC 60
ACCACCATCATGAGCGCTGAAACTCCCCTCAAGGACTTGCCCAAGGTTGACCCCACCCTT 120
         M  S  A  E  T  P  L  K  D  L  P  K  V  D  P  T  L   17
AAGGGACAGCTCGAGGGATTCTCCGCCGTAAACCTTAAGAAGACCGAGACGGAGGAGAAG 180
K  G  Q  L  E  G  F  S  A  V  N  L  K  K  T  E  T  E  E  K   37
ATTCACCTGCCAAACAGGGAGGACGTGGAAGCAGAGAAGAAAGTACAGGCCCATCTGCAG 240
I  H  L  P  N  R  E  D  V  E  A  E  K  K  V  Q  A  H  L  Q   57
GCCGTCGAAGGCTTCAATACTGCACAACTCAAGCATGCCAATACCCAAGAAAAAATTGTT 300
A  V  E  G  F  N  T  A  Q  L  K  H  A  N  T  Q  E  K  I  V   77
TTACCTGCTCAGGAAGATATTGAGACTGAGAAGGGTCAGCAGGCACTCCGCCAGGGTATT 360
L  P  A  Q  E  D  I  E  T  E  K  G  Q  Q  A  L  R  Q  G  I   97
GAGGGCTTTGACCATGCTGCTTTGAAGAAAGCTCAGACGACAGAGAAGAATACCCTTCCA 420
E  G  F  D  H  A  A  L  K  K  A  Q  T  T  E  K  N  T  L  P  117
ACTAAGGAAATGATTGAGGAAGAGAAGAAGGCCTAACAAGGTTTGCCAGGAACACATCTA 480
T  K  E  M  I  E  E  E  K  K  A  *                          128
TGTTCACTGTGCATCCCAGTGATATCCTCCCTGCTCTCCCTAGAACTTCTAATAGTTCTG 540
CAAGCCAAAATGTTTTGTACTGTACTTTGATAATCTAAGTGTATAGGTAAACCAACTGAT 600
TTTGTAACCGGATATCATATGCTATTCTTTGAGTCATTATTATTTGCTTTAGTGATAAGA 660
TCAAGGTGCTCACCAATAGCAAGGCGATGGCAAGAAGTGCAAAAGGGACATTTAGAAACT 720
GCATGATAATTTTCATTGCTTTTGTGCACCCTCCTAGTAATTTATCAGCAATGCTAGTGG 780
ACCAAAATTTCACCTTGCTTCCGCCTTGTTCAATAGAAGGTAAAGGTCTCCCATGCAGGT 840
AATGGAAAACTGCCAAAAGGTACAATGAATTTGTAATTTGCCAGGATTGTAATGTGTGAG 900
TCTCAATTTTTACCCCATTTAATTTGCATTTAATTGTTAGCATTTACATGAATTTTTAAG 960
TGTTTATAAAGAATTTATCATTGCCTTGTTTCAAAATTAATTTGCTTTTTGGGGCATCCA 1020
AGTAAATGTGATTTTGGGAGCCAATAAAGTGCTTTTGCAAAAAAAAAAAAAAAAAAAAA 1080
AAAA                                                          1084
```

B

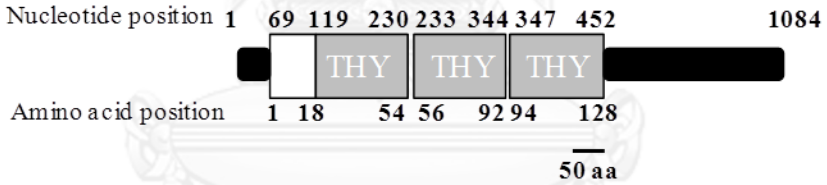


Figure 3.22 (A) The full-length cDNA and deduced protein sequences of *PmTmsb*. Start and stop codon are illustrated in boldfaced and underlined. The poly A additional signal (AATAA) is boldfaced and the THY domain are highlighted. (B) Diagram illustrating *PmTmsb*. Three THY domains were found in the deduced PmTmsb protein. The scale bar is 50 amino acids in length.

3.3.3 *Rac1 GTPase-activating protein*

A fragment of 1881 bp in length was obtained from amplification of cDNA in ovaries using primers designed from ORF of the testes form of *P. monodon Rac GTPase-activating protein 1* (*PmRacgap1*) (Leelatanawit, 2008) (Figure 3.23). The PCR

product was cloned and sequenced. The ORF of *PmRacgap1* was 1881 bp in length corresponding to a polypeptide of 626 amino acids. The closest similarity of this characterized transcript was *Rac GTPase-activating protein 1* of *Capitella teleta* (*E*-value = 2e-120).

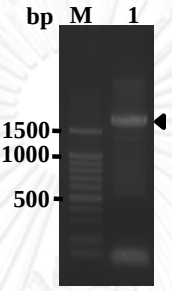


Figure 3.23 Agarose gel electrophoresis showing the amplified ORF of ovarian *PmRacgap1* using primers designed from that previously identified in testes of *P. monodon*.

The calculated p*I* and MW of the deduced PmRacgap1 protein was 8.68 and 71.47 kDa. Two domains (C1 and RhoGAP) were found at the amino acid positions 317-364 (*E*-value = 5.32e-06) and 365-540 (*E*-value = 2.26-47) of the deduced PmRacgap1 protein (Figure 3.24). The putative *N*-linked-glycosylation sites were found at positions 109-111,150-152 and 571-573, respectively.

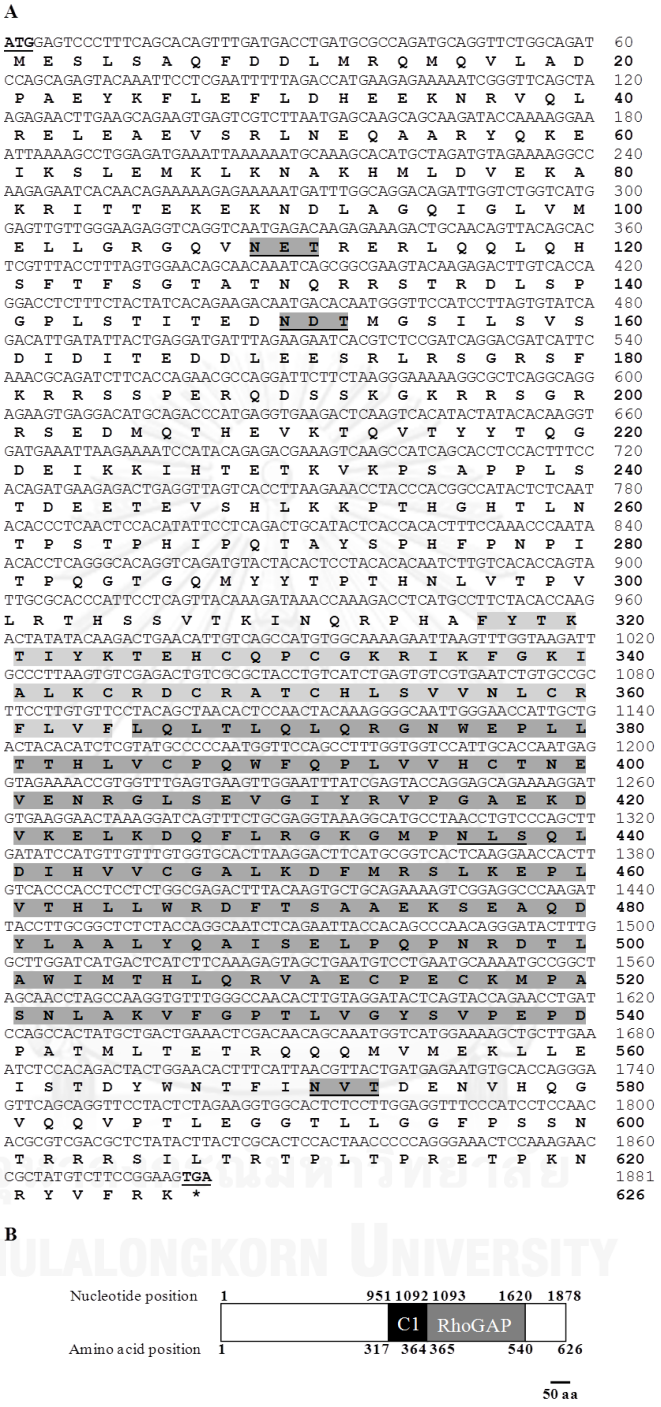


Figure 3.24 The open reading frame and deduced protein sequences of *PmRacgap1*. Start and stop codon are illustrated in boldfaced and underlined. The predicted *N*-link glycosylation sites are highlighted and underline. The C1 and RhoGAP domain are highlighted with light-grey and dark grey, respectively. (B) Diagram illustrating *PmRacgap1*. The C1 and RhoGAP domains were found in the deduced PmRacgap1 protein. The scale bar is 50 amino acids in length.

Alignment nucleotide sequences of *PmRacgap1* in ovaries (this thesis) and testes (Leelatanawit, 2008) of *P. monodon* indicated that differences between these isoforms were resulted from small indels (C and AAACAG; position 1064 and 852 - 857) found in the testes form and a large indel (TAACACTCCAACTACAAAGGGGCAA TTGGGAACCATTGCTGACTACACATCTCGTATGCCCCCAATGGTTCCAGCC; positions 1107-1183) found in the ovary form. In addition, a single substitution from T to C was observed at position 118, 383 and 1375, A to T was observed at position 663 (Figure 3.25).

```
Ovary       ATGGAGTCCCTTTCAGCACAGTTTGATGACCTGATGCGCCAGATGCAGGTTCTGGCAGAT
Testis      ATGGAGTCCCTTTCAGCACAGTTTGATGACCTGATGCGCCAGATGCAGGTTCTGGCAGAT
            ************************************************************

Ovary       CCAGCAGAGTACAAATTCCTCGAATTTTTAGACCATGAAGAGAAAAATCGGGTTCAGCTA
Testis      CCAGCAGAGTACAAATTCCTCGAATTTTTAGACCATGAAGAGAAAAATCGGGTTCAGTTA
            ************************************************************ **

Ovary       AGAGAACTTGAAGCAGAAGTGAGTCGTCTTAATGAGCAAGCAGCAAGATACCAAAAGGAA
Testis      AGAGAACTTGAAGCAGAAGTGAGTCGTCTTAATGAGCAAGCAGCAAGATACCAAAAGGAA
            ************************************************************

Ovary       ATTAAAAGCCTGGAGATGAAATTAAAAAATGCAAAGCACATGCTAGATGTAGAAAAGGCC
Testis      ATTAAAAGCCTGGAGATGAAATTAAAAAATGCAAAGCACATGCTAGATGTAGAAAAGGCC
            ************************************************************

Ovary       AAGAGAATCACAACAGAAAAAGAGAAAAATGATTTGGCAGGACAGATTGGTCTGGTCATG
Testis      AAGAGAATCACAACAGAAAAAGAGAAAAATGATTTGGCAGGACAGATTGGTCTGGTCATG
            ************************************************************

Ovary       GAGTTGTTGGGAAGAGGTCAGGTCAATGAGACAAGAGAAAGACTGCAACAGTTACAGCAC
Testis      GAGTTGTTGGGAAGAGGTCAGGTCAATGAGACAAGAGAAAGACTGCAACAGTTACAGCAC
            ************************************************************

Ovary       TCGTTTACCTTTAGTGGAACAGCAACAAATCAGCGGCGAAGTACAAGAGACTTGTCACCA
Testis      TCGTTTACCTTTAGTGGAACAGTAACAAATCAGCGGCGAAGTACAAGAGACTTGTCACCA
            ********************** ***************************************

Ovary       GGACCTCTTTCTACTATCACAGAAGACAATGACACAATGGGTTCCATCCTTAGTGTATCA
Testis      GGACCTCTTTCTACTATCACAGAAGACAATGACACAATGGGTTCCATCCTTAGTGTATCA
            ************************************************************

Ovary       GACATTGATATTACTGAGGATGATTTAGAAGAATCACGTCTCCGATCAGGACGATCATTC
Testis      GACATTGATATTACTGAGGATGATTTAGAAGAATCACGTCTCCGATCAGGACGATCATTC
            ************************************************************

Ovary       AAACGCAGATCTTCACCAGAACGCCAGGATTCTTCTAAGGGAAAAAGGCGCTCAGGCAGG
Testis      AAACGCAGATCTTCACCAGAACGCCAGGATTCTTCTAAGGGAAAAAGGCGCTCAGGCAGG
            ************************************************************

Ovary       AGAAGTGAGGACATGCAGACCCATGAGGTGAAGACTCAAGTCACATACTATACACAAGGT
Testis      AGAAGTGAGGACATGCAGACCCATGAGGTGAAGACTCAAGTCACATACTATACACAAGGT
            ************************************************************

Ovary       GATGAAATTAAGAAAATCCATACAGAGACGAAAGTCAAGCCATCAGCACCTCCACTTTCC
Testis      GAAGAAATTAAGAAAATCCATACAGAGACGAAAGTCAAGCCATCAGCACCTCCACTTTCC
            ** ************************************************************

Ovary       ACAGATGAAGAGACTGAGGTTAGTCACCTTAAGAAACCTACCCACGGCCATACTCTCAAT
Testis      ACAGATGAAGAGACTGAGGTTAGTCACCTTAAGAAACCTACCCACGGCCATACTCTCAAT
            ************************************************************

Ovary       ACACCCTCAACTCCACATATTCCTCAGACTGCATACTCACCACACTTTCCAAACCCAATA
Testis      ACACCCTCAACTCCACATATTCCTCAGACTGCATACTCACCACACTTTCCAAACCCAATA
```

Figure 3.25 Pairwise nucleotide sequences alignment of *PmRacgap1* found in ovaries and testes.

3.3.4. *Protein kinase C*

The full-length cDNA of *protein kinase C (PKC)* was identified by RACE-PCR. Primers were designed from EST clone no. OV-N-S01-1421-W which significantly matched of protein kinase C 98E-like protein of *Tribolium castaneum*; *E*-value = 5e-26). The amplified fragments of approximately 850 bp, 800 bp, 1250 bp and 800 bp were obtained from the 5' RACE-PCR, nested 5' RACE-PCR and nested-nested 5' RACE-PCR and primary 3' RACE of *PmPKC* respectively (Figure 3.26). These fragments were cloned and sequenced (Figure 3.27). Nucleotide sequences of the original EST, 5' RACE-PCR and 3´ RACE were assembled.

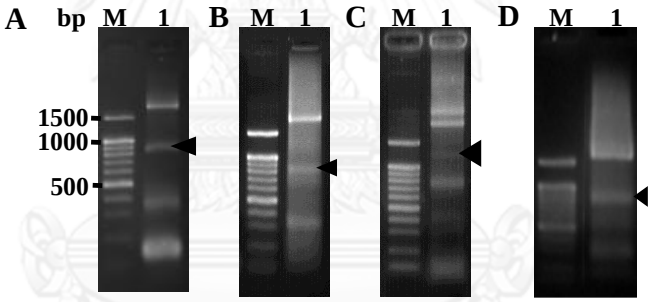


Figure 3.26 Agarose gel electrophoresis showing results from 5' RACE-PCR (A), nested 5' RACE-PCR (B), nested-nested 5' RACE-PCR (C) and 3 'RACE-PCR (D) products of *PmPKC*. Arrowhead indicate the RACE-PCR fragments that were cloned and sequenced. Lanes M are a 100 bp DNA ladder.

The full length cDNA of *PmPKC* was 3404 bp in length containing the ORF of 2235 bp deducing to a polypeptide of 744 amino acids and the 5' and 3' UTRs of 143 and 997 bp (excluding the poly A tail), respectively (Figure 3.28A). The closest match to this transcript was *Protein kinase C* of *Ciona intestinalis* (*E*-value = 1e-110).

A

I

GGGAGTTTTATCGAATGGTGTTGAATGGGTGACAGTACGGTCGGCCTTACAGTCACTTTCGCCCCATTT
CCCTATGCACTACTGGGACCTATTGGTGAGGTGAGACGGGGCTTGTGAGCACCTGAGCAGAGAGAACAC
GGGCG**ATG**TTCACGGGCAGCCTTAAAGTGAAAATATGCGAAGCTACCGATCTCCGCCTTACCGACTGTA
TGACCCGCTATGTCGGAGTAGCAGGGGTAGGAAAAGGTCCTCAAGACCAAACCCTGGATCCTTACGTGA
CGCTGGAGGTGGACGAGGTACACTGGACCAAGACGCAGGCCCGACAGAAGACCTTTACGCCCATATGGA
ACGAGAGCTTTGAGCAAGACGTGATGGGCGCCGTCCAGCTCGGCCTCAAGATCTTCCATGACTCCGCGG
TCGGCAACGATGACTTCGTGGCCGACGCTTCCTTGCTCTTCGAGGAAATATGTGCCGAGAACCAGACCC
ATGCCGACATCTGGGTGGACTTAGAACCTCAGGGGAAGCTACATGTGGTGATTGAACTAAAATGGGCAC
CACCAGAAGATGGAGGCGTCCGCCCACGAGAATTCAGGGAACGGCAGGGCTTCAACCGGCGACGAGGTG
CCATGAGGAGGCGGGTTCATCAGGTCAATGGTCACAAATTCATGGCTACGTTCCTGCGGCAACCTACAT
5′RACE-PKC-III
TCTGCTCGCATTGTCGAGAATTCATATGGGGTCTAGGGA

II

ACGGCGGGTTCATCAGGTCAATGGTCACAAATTCATGGTTACGTTCCTACGGCAACCTACATTCTGCTC
5′RACE-PKC-III
GCATTGTCGAGAATTCATATGGGGTCTAGGGAAACAAGGATATCAATGTCAAGTATGTACTTGCGTAGT
CCACAAACGCTGTCACCAGTCGGTTGTCACACGACGTCCAGGAAGCAAGAGTGAGACCATCAATGAGGA
ACCTTCAGTCCAGGGAAGTTGTCAACCGCGATTCAACGTCAATGTGCCACATCGGTTTTCTGTTCACTC
CTACAAACGGTTCACATTCTGTGACCATTGTGGCTCATTACTTTATGGCCTTATTCGTCAGGGTCTCCA
ATGTGAAGTATGCAACATGAATGTTCACAAGGGATGCCAAAAGAATGTAGCCAACAATTGTGGGATAGA
CGTAAAACAGTTATCAGAAATTCTAGCCACCATGGGTATACGGCCTCAAGATGAAGCAGCCAAACGCAA
GAAGAAGTCTGTTAGTGAGACTAAGTTATCATCGGCCACCGCCCCTGTTAGTGTCCCAGGAATCATCGG
AGAAGTGGAAGTCCCAGGTGAAATGAATGAGGAAGAGCTAAGACTAAGGATTGAAGCTCAGCGTATCAT
GGACAAGAAGATGAAGGAACGCTGTGCAGAAGAAGGACTAGATGCCAAGTCGAAGCCACATGATACTTC
TTTAGATGTTATGCTTGGTGGGAAGAAGGTAGACCTGGATGACTTCTCATTCATTAAAGTACTTGGCAA
AGGAAGTTTTGGAAAAGTAATGCTAGCTGAACTTAAAGGGACAGATGAAGTCTACGCCATCAAGGTGCT
TAAGAAAGACGTGATCCTACAAGATGATGATGTAGAATGTACAATGACTGAGCGGCGGATCTTAGCGAT
GGCTGCACACCACCCCTTCCTCACTGCCCTCCACTCTAGCTTTCAAACCAAGGATCGATTATTCTTTGT
TATGGAGTATGTTAATGGTGGTGACCTAATGTTCCAAATTCAGAAGGCTCGCAAGTTCACAGAGTCACG
TGCAAGATTTTATGCAGCTGAGGTCACGTTGGCCTTACAGTTCCTCCATAAAAATGGTGTTATTTACAG
AGATTTGAAATTAGATAATATTCTACTAGACAGTGAAGGACATTGTAAAATAGCTGATTTTGGTATGTG
TAAGGAAGGCATCAGGGATAATATTACAACAACCACCTTCTGTGGAACACCCGATTACATTGCACCAGA
5′RACE-PKC-II
GATTCTTAAAGAGTTGGATTATGGTGCCAGTGTTGATTGGTG

III

AAGCAGTGGTATCAACGCAGAGTACGCGGGCAAGATTTTATGCAGCTGAGGTCACCTTGGCCTTACAGT
TCCTCCATAAAAATGGTGTTATTTACAGAGATTTGAAATTAGATAATATTCTACTAGACAGTGAAGGAC
ATTGTAAAATAGCTGATTTTGGTATGTGTAAGGAAGGCATCAGGGATAATATTACAACAACCACCTTCC
5′RACE-PKC-II
GTGGAACACCCGATTACATTGCACCAGAGATTCTTAAAGAGTTGGATTATGGTGCCAGTGTTGATTGGT
GGGCACTAGGAGTTCTCATGTATGAAATGATGGCAGGACAGCCACCATTTGAGGCTGATAATGAAGATG
ATCTCTTTGAGTCCATCCTGCATGAAGAAGTTTTGTATCCAGTGTGGCTTTCAAAGGAAGCAGTTTCTA
TACTGAAAGGGTTTATGACAAAGGAACCATCCAAGCGGTTGGGCTGCGTTGCAGAGCGAGGAGGAGAAT
TGGCAATTCGTAACCACAAATTCTTCCAAGAGATAGACTGGGAGGCCCTTGAGCAGCGCAAAGTTAAAC
CACCATTTACACCTAAGATAAAAGGACGTAAGGATACAGTCAACTTTGATGCAGAATTTACCAAGGAAG
AGCCAACTCTCACCTTTATTAATGAAGAAGTTGTTCGTGCTATCAACCAAGACGAGTTTAGAGGATTTT
CTTTCGTTAATCGTGAATTTAAGTCCATGGCGGCTGCACCATGTCAAACCTAAGTGCAAATGCAGCACA
5′RACE-PKC-I
GTACAAAGTGCTAAAGTCTCCCCAGGAAAC

B

GTAACCACAAATTCTTCCGAGAGATAGACTGGGAGGCCCTTGAGCAGCGCAAAGTTAAACCACCATTTA
CACCTAAGATAAAAGGACGTAAGGATACAGTCAACTTTGATGCAGAATTTACCAAGGAAGAGCCAACTC
PKC-F1
TCACCTTTATTAATGAAGAAGTTGTTCGTGCTATCAACCAAGACGAGTTTAGAGGATTTTCTTTCGTTA
ATCGTGAATTTAAGTCCATGGCGGCTGCACCATGTCAAACCTAAGTGCAAATGCAGCACAGTACAAAGT
5´RACE-PKC-I
GCTAAAGTCTCCCCAGGAAACTTGATGTGCAGACTATATGAGAGGATTCTTTAGGGAAGAAGGCAGATG
AAGGAAGGAAAAAAATAATGAGTAATAGTAGGAAAGATTTCAATTTTGACTGCACAAAGAGTTGAATGT
CTCAACAAGCACAACTGAGAATGGCTGTATTGTGATTAACATTGTATATCTAATAATACTCTTTATGAT
GGATTTTTTCATTATTATTATAGTTTTTTTTTGCAAAAGATTCTAATGTGGAAGATCTCTGAATTTATA
AGTTACAATATTTTTAAATATAGATATATTTCATGATGGAAAGATATTACATGGGAGAGTTGTAGAGAG
ATTTTTTTTAAATTGAAGATAGTATTTATATAGTGTTGTTCTGTAAATGCATGATGCATTTGGAATATG
ATAGGAAGATACAAAAGTTCGTAAAGAATTTTACAAAGAA

C.

PKC-F1
TGTTCGTGCTATCAACCAAGACGAGTTTAGAGGATTTTCTTTCGTTAATCGTGAATTTAAGTCCATGGC
5´RACE-PKC-I
GGCTGCACCATGTCAAACC**TAA**GTGCAAATGCAGCACAGTACAAAGTGCTAAAGTCTCCCCAGGAAACT
TGATGTGCAGACTATATGAGAGGATTCTTTAGGGAAGAAGGCAGATGAAGGAAGGAAAAAAATAATGAG
TAATAGTAGGAAAGATTTCAATTTTGACTGCACAAAGAGTTGAATGTCTCAACAAGCACAACTGAGAAT
GGCTGTATTGTGATTAACATTGTATATCTAATAATACTCTTTATGATGGATTTTTTCATTATTATTATA
GTTTTTTTTTGCAAAAGATTCTAATGTGGAAGATCTCTGAATTTATAAGTTACAATATTTTTAAATATA
GATATATTTCATGATGGAAAGATATTACATGGGAGAGTTGTAGAGAGATTTTTTTTAAATTGAAGATAG
TATTTATATAGTGTTGTTCTGTAAATGCATGATGCATTTGGAATATGATAGGAAGATACAAAAGTTCGT
AAAGAATTTTACAAAGAATGATTCTAATGTTTATTTATTTTTTACTGACTTCTTTCAATGTATTGTTAG
GGAAAATTGCCAACTTTTGAACTTCCAGTTCTGGAGGTTTAAAGGATATATATTGCATTTTAATGCAGT
AAGCACTTACTATAGATAATCAACCTATGAGGCTCATGATTTAGCACATTTAAAGAAAGAGAAAAGGAA
GTGAAATTGAAAGTTATAACCTTTGACTAAAATTGCTTGTCCAGTTATGGTACCAGATGATTCCAAAGG
CTAGTAAGAAGTGCACAAAGATAACTAGCATCAGATATATTTAATGTCTGGCAGCGGAAAGAAACTTGG
ATGCAGGTATGGGGGAGACCAGATGTAGGACCATACAACTCATATTAACTTTTGGCCCTCTTTTGTATT
GTTTTTAGATCACTCAGTCACATAAACATACACTCATGTACACTCACATGCTATAACACACTCACAATC
AAACAAGCGCATAAAAATATTTTATATTCGTGAAGTGGTGGAAGCCCTCCTCGTATACTTATGGTGCAA
TATGACAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.27 Nucleotide sequences of 5' RACE (AI-AIII), the original EST (B) and 3' RACE-PCR (C) of *PmPKC*. A forward primer for RT-PCR (PKC-F1 underlined) was used for 3' RACE-PCR.

The calculated p*I* and MW of the deduced *PKC* protein was 6.35 and 84.47 kDa, respectively. The C2 domain was found at the amino acid position 5-116 (1.59e-9). The C1 domains were found at the amino acid positions 171 - 218 (7.57e-11), 248 – 297(2.65e-15) and S_TKc domain 407-667(1.84e-104) and S_TKx 668-731 (3.15e-25) of the deduced protein, respectively (Figure 3.28B).

A

```
GGGAGTTTTATCGAATGGTGTTGAATGGGTGACAGTACGGTCGGCCTTACAGTCACTTTC  60
GCCCCATTTCCCTATGCACTACTGGGACCTATTGGTGAGGTGAGACGGGGCTTGTGAGCA  120
CCTGAGCAGAGAGAACACGGGCGATGTTCACGGGCAGCCTTAAAGTGAAAATATGCGAAG  180
                        M  F  T  G  S  L  K  V  K  I  C  E     12
CTACCGATCTCCGCCTTACCGACTGTATGACCCGCTATGTCGGAGTAGCAGGGGTAGGAA  240
A  T  D  L  R  L  T  D  C  M  T  R  Y  V  G  V  A  G  V  G     32
AAGGTCCTCAAGACCAAACCCTGGATCCTTACGTGACGCTGGAGGTGGACGAGGTACACT  300
K  G  P  Q  D  Q  T  L  D  P  Y  V  T  L  E  V  D  E  V  H     52
GGACCAAGACGCAGGCCCGACAGAAGACCTTTACGCCCATATGGAACGAGAGCTTTGAGC  360
W  T  K  T  Q  A  R  Q  K  T  F  T  P  I  W  N  E  S  F  E     72
AAGACGTGATGGGCGCCGTCCAGCTCGGCCTCAAGATCTTCCATGACTCCGCGGTCGGCA  420
Q  D  V  M  G  A  V  Q  L  G  L  K  I  F  H  D  S  A  V  G     92
ACGATGACTTCGTGGCCGACGCTTCCTTGCTCTTCGAGGAAATATGTGCCGAGAACCAGA  480
N  D  D  F  V  A  D  A  S  L  L  F  E  E  I  C  A  E  N  Q    112
CCCATGCCGACATCTGGGTGGACTTAGAACCTCAGGGGAAGCTACATGTGGTGATTGAAC  540
T  H  A  D  I  W  V  D  L  E  P  Q  G  K  L  H  V  V  I  E    132
TAAAATGGGCACCACCAGAAGATGGAGGCGTCCGCCCACGAGAATTCAGGGAACGGCAGG  600
L  K  W  A  P  P  E  D  G  G  V  R  P  R  E  F  R  E  R  Q    152
GCTTCAACCGGCGACGAGGTGCCATGAGGAGGCGGGTTCATCAGGTCAATGGTCACAAAT  660
G  F  N  R  R  R  G  A  M  R  R  R  V  H  Q  V  N  G  H  K    172
TCATGGCTACGTTCCTGCGGCAACCTACATTCTGCTCGCATTGTCGAGAATTCATATGGG  720
F  M  A  T  F  L  R  Q  P  T  F  C  S  H  C  R  E  F  I  W    192
GTCTAGGGAAACAAGGATATCAATGTCAAGTATGTACTTGCGTAGTCCACAAACGCTGTC  780
G  L  G  K  Q  G  Y  Q  C  Q  V  C  T  C  V  V  H  K  R  C    212
ACCAGTCGGTTGTCACACGACGTCCAGGAAGCAAGAGTGAGACCATCAATGAGGAACCTT  840
H  Q  S  V  V  T  R  R  P  G  S  K  S  E  T  I  N  E  E  P    232
CAGTCCAGGGAAGTTGTCAACCGCGATTCAACGTCAATGTGCCACATCGGTTTTCTGTTC  900
S  V  Q  G  S  C  Q  P  R  F  N  V  N  V  P  H  R  F  S  V    252
ACTCCTACAAACGGTTCACATTCTGTGACCATTGTGGCTCATTACTTTATGGCCTTATTC  960
H  S  Y  K  R  F  T  F  C  D  H  C  G  S  L  L  Y  G  L  I    272
GTCAGGGTCTCCAATGTGAAGTATGCAACATGAATGTTCACAAGGGATGCCAAAAGAATG  1020
R  Q  G  L  Q  C  E  V  C  N  M  N  V  H  K  G  C  Q  K  N    292
TAGCCAACAATTGTGGGATAGACGTAAAACAGTTATCAGAAATTCTAGCCACCATGGGTA  1080
V  A  N  N  C  G  I  D  V  K  Q  L  S  E  I  L  A  T  M  G    312
TACGGCCTCAAGATGAAGCAGCCAAACGCAAGAAGAAGTCTGTTAGTGAGACTAAGTTAT  1140
I  R  P  Q  D  E  A  A  K  R  K  K  K  S  V  S  E  T  K  L    332
CATCGGCCACCGCCCCTGTTAGTGTCCCAGGAATCATCGGAGAAGTGGAAGTCCCAGGTG  1200
S  S  A  T  A  P  V  S  V  P  G  I  I  G  E  V  E  V  P  G    352
AAATGAATGAGGAAGAGCTAAGACTAAGGATTGAAGCTCAGCGTATCATGGACAAGAAGA  1260
E  M  N  E  E  E  L  R  L  R  I  E  A  Q  R  I  M  D  K  K    372
TGAAGGAACGCTGTGCAGAAGAAGGACTAGATGCCAAGTCGAAGCCACATGATACTTCTT  1300
M  K  E  R  C  A  E  E  G  L  D  A  K  S  K  P  H  D  T  S    392
TAGATGTTATGCTTGGTGGGAAGAAGGTAGACCTGGATGACTTCTCATTCATTAAAGTAC  1360
L  D  V  M  L  G  G  K  K  V  D  L  D  D  F  S  F  I  K  V    412
TTGGCAAAGGAAGTTTTGGAAAAGTAATGCTAGCTGAACTTAAAGGGACAGATGAAGTCT  1420
L  G  K  G  S  F  G  K  V  M  L  A  E  L  K  G  T  D  E  V    432
ACGCCATCAAGGTGCTTAAGAAAGACGTGATCCTACAAGATGATGATGTAGAATGTACAA  1480
Y  A  I  K  V  L  K  K  D  V  I  L  Q  D  D  D  V  E  C  T    452
TGACTGAGCGGCGGATCTTAGCGATGGCTGCACACCACCCCTTCCTCACTGCCCTCCACT  1540
M  T  E  R  R  I  L  A  M  A  A  H  H  P  F  L  T  A  L  H    472
```

```
CTAGCTTTCAAACCAAGGATCGATTATTCTTTGTTATGGAGTATGTTAATGGTGGTGACC  1600
S  S  F  Q  T  K  D  R  L  F  F  V  M  E  Y  V  N  G  G  D     492
TAATGTTCCAAATTCAGAAGGCTCGCAAGTTCACAGAGTCACGTGCAAGATTTTATGCAG  1660
L  M  F  Q  I  Q  K  A  R  K  F  T  E  S  R  A  R  F  Y  A     512
CTGAGGTCACGTTGGCCTTACAGTTCCTCCATAAAAATGGTGTTATTTACAGAGATTTGA  1720
A  E  V  T  L  A  L  Q  F  L  H  K  N  G  V  I  Y  R  D  L     532
AATTAGATAATATTCTACTAGACAGTGAAGGACATTGTAAAATAGCTGATTTTGGTATGT  1780
K  L  D  N  I  L  L  D  S  E  G  H  C  K  I  A  D  F  G  M     552
GTAAGGAAGGCATCAGGGATAATATTACAACAACCACCTTCTGTGGAACACCCGATTACA  1840
C  K  E  G  I  R  D  N  I  T  T  T  T  F  C  G  T  P  D  Y     572
TTGCACCAGAGATTCTTAAAGAGTTGGATTATGGTGCCAGTGTTGATTGGTGGGCACTAG  1900
I  A  P  E  I  L  K  E  L  D  Y  G  A  S  V  D  W  W  A  L     592
GAGTTCTCATGTATGAAATGATGGCAGGACAGCCACCATTTGAGGCTGATAATGAAGATG  1960
G  V  L  M  Y  E  M  M  A  G  Q  P  P  F  E  A  D  N  E  D     612
ATCTCTTTGAGTCCATCCTGCATGAAGAAGTTTTGTATCCAGTGTGGCTTTCAAAGGAAG  2020
D  L  F  E  S  I  L  H  E  E  V  L  Y  P  V  W  L  S  K  E     632
CAGTTTCTATACTGAAAGGGTTTATGACAAAGGAACCATCCAAGCGGTTGGGCTGCGTTG  2080
A  V  S  I  L  K  G  F  M  T  K  E  P  S  K  R  L  G  C  V     652
CAGAGCGAGGAGGAGAATTGGCAATTCGTAACCACAAATTCTTCCGAGAGATAGACTGGG  2140
A  E  R  G  G  E  L  A  I  R  N  H  K  F  F  R  E  I  D  W     672
AGGCCCTTGAGCAGCGCAAAGTTAAACCACCATTTACACCTAAGATAAAAGGACGTAAGG  2200
E  A  L  E  Q  R  K  V  K  P  P  F  T  P  K  I  K  G  R  K     692
ATACAGTCAACTTTGATGCAGAATTTACCAAGGAAGAGCCAACTCTCACCTTTATTAATG  2260
D  T  V  N  F  D  A  E  F  T  K  E  E  P  T  L  T  F  I  N     712
AAGAAGTTGTTCGTGCTATCAACCAAGACGAGTTTAGAGGATTTTCTTTCGTTAATCGTG  2320
E  E  V  V  R  A  I  N  Q  D  E  F  R  G  F  S  F  V  N  R     732
AATTTAAGTCCATGGCGGCTGCACCATGTCAAACCTAAGTGCAAATGCAGCACAGTACAA  2380
E  F  K  S  M  A  A  A  P  C  Q  T  *                          744
AGTGCTAAAGTCTCCCCAGGAAACTTGATGTGCAGACTATATGAGAGGATTCTTTAGGGA  2440
AGAAGGCAGATGAAGGAAGGAAAAAAATAATGAGTAATAGTAGGAAAGATTTCAATTTTG  2500
ACTGCACAAAGAGTTGAATGTCTCAACAAGCACAACTGAGAATGGCTGTATTGTGATTAA  2560
CATTGTATATCTAATAATACTCTTTATGATGGATTTTTCATTATTATTATAGTTTTTTT  2620
TTGCAAAAGATTCTAATGTGGAAGATCTCTGAATTTATAAGTTACAATATTTTTAAATAT  2680
AGATATATTTCATGATGGAAAGATATTACATGGGAGAGTTGTAGAGAGATTTTTTTTAAA  2740
TTGAAGATAGTATTTATATAGTGTTGTTCTGTAAATGCATGATGCATTTGGAATATGATA  2800
GGAAGATACAAAAGTTCGTAAAGAATTTTACAAAGAATGATTCTAATGTTTATTTATTTT  2860
TTACTGACTTCTTTCAATGTATTGTTAGGGAAAATTGCCAACTTTTGAACTTCCAGTTCT  2920
GGAGGTTTAAAGGATATATATTGCATTTTAATGCAGTAAGCACTTACTATAGATAATCAA  2980
CCTATGAGGCTCATGATTTAGCACATTTAAAGAAAGAGAAAAGGAAGTGAAATTGAAAGT  3040
TATAACCTTTGACTAAAATTGCTTGTCCAGTTATGGTACCAGATGATTCCAAAGGCTAGT  3100
AAGAAGTGCACAAAGATAACTAGCATCAGATATATTTAATGTCTGGCAGCGGAAAGAAAC  3160
TTGGATGCAGGTATGGGGGAGACCAGATGTAGGACCATACAACTCATATTAACTTTTGGC  3220
CCTCTTTTGTATTGTTTTTAGATCACTCAGTCACATAAACATACACTCATGTACACTCAC  3280
ATGCTATAACACACTCACAATCAAACAAGCGCATAAAAATATTTTATATTCGTGAAGTGG  3340
TGGAAGCCCTCCTCGTATACTTATGGTGCAATATGACAAAAAAAAAAAAAAAAAAAAAA  3400
AAAA                                                           3404
```

B

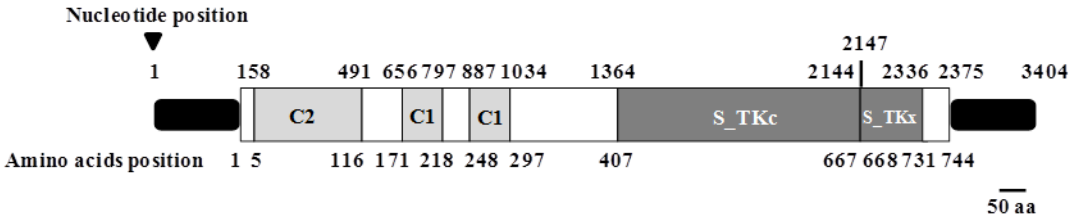


Figure 3.28 (A) The full-length cDNA and deduced protein sequences of *PmPKC*. Start and stop codons are illustrated in boldfaced and underlined. The *N*-linked-glycosylation sites and domain proteins are highlighted and underlined. The poly A additional signal (AATTAA) is boldfaced. (B) Diagram illustrating the full length cDNA of *protein kinase C* of *P. monodon*. The C1, C2, S_TKc domain and S_TKx domain were found in this transcript. The scale bar is 50 amino acids in length.

3.3.5. *Cyclic AMP-regulated protein like protein*

A primer was designed from an EST clone no. HPa-N-N01-0641-LF which significantly matched *cyclic AMP regulated protein-like protein* of *Marsupenaeus japonicus* (*E*-value = 1e-54). RACE-PCR was carried out. An amplified fragment of approximately 1100 bp was obtained from the primary 3' RACE-PCR (Figure 3.29). This fragment was cloned and sequenced. Nucleotide sequences of the original EST and 3' RACE-PCR were assembled (Figure 3.30).

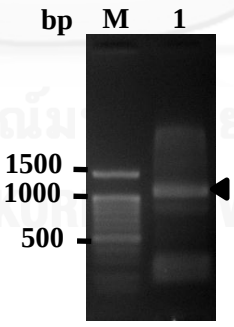


Figure 3.29 The primary 3' RACE-PCR product of *cyclic AMP-regulated protein like protein.* An arrowhead indicates a RACE-PCR product that was cloned and sequenced. Lanes M is a 100 bp DNA ladder.

A

```
CCTCTCTCGCTCGCTCTCCCTCTCTCGCTCGCTCTCCCTCTCTCGCTCTCCCTCTCTCTCTGTGCCTCT
CGTTGGCTCGAAGCTCTCCCTCGGTCTCTCCTCGCTGATCCGTCTCGTCTCGGCTTCGTTCGGATAAAA
TCACGGTGATGGCTAGTACCCAGATCGACCGGGAAGGCCTACACAGCGCTTATAGTGATGTCAGGGACG
                                        3´RACE
CAAAAGCGACCGTCAATTGGGCCGTGTTCAAGTATGAGGGCAGCCAGGTGACTGTGGCGGACAAGGGAC
AGACCTTTGATGACTTCAAGGCGCAGTTTGGGGATGATGAGCGTGCGTTCGCTTACCTGCGCATCCAGA
CTGGGGATGAGATGAGCAAGAGGTCGAAGTTCCTGATGCTAACTTGGGTGGGCACCGAAGTCTCGACCA
TCAAGACAGCGAAAATGTACACC
```

B

```
          3´RACE
TGTTCAAGTATGAGGGCAGCCAGGTGACTGTGGCGGCCAAGGGACAGACCTTTGATGACTTCAAGGTGC
AGTTTGGGGATGATGAGCGTGCGTTCGCTTACCTGCGCATCCAGACTGGGGATGAGATGAGCAAGAGAT
CGAAGTTCCTGATGCTAACTTGGGTGGGCACCGAGGTCTCGCCCATCAAGAAAGCGAAAATGTCCACCG
ATAAGGCCCTCGTCAAAGAGGTTCTTGCAAACTTTGCAGTGGAGTTGACAATAGAAAGCGCTCACGAAC
TGGACCACGAAGCCTTTTTACAAGAGCTCGTGAAAGCTGGTGGAGCCAACTATGGCACAGGATTCCGCG
ATTAGGCTTGTGGGTGCTCGCGTTTGGCATTTAGATATGATAGGACACTGAAATGTAATCAATCTGGCT
CTGTGTTCTCTTCAAAAATTGCTAATGTCAGGGAATGGAACAGAATCGTTTTAATAAATGAAATACAAT
CAAATTATATAAAGTGCTTTGTGGGTCCATGATCAAAATATTGCAGGTACTTTTGTTAATATTCCCATA
TTTTTATTTATTTTTTGATTATTATCTTTTGGGCAGGAGAGAATTATCATTATTTGGAACTACACTGAA
TTTTCATAGCATTTATGATGAGTTTCATTATGCATGCAACATTTTTCATTGAGGAGTTTAACAGACAGG
CATTTTTATATATACATAGCTTTCAAAAGTTGACTTGAAATACATTCATTAAAAAAACATTACTGTAAA
CTAGTATTAACCTAGAATTAAGCAGTGCCATCAGTTATTCAATTTTTTTCTGTAAATCCTCTATTTTTA
GCATTACAATTGATAGACTGGTCATATTTAATTCTACAAAAAATACAATATATAATCAGATATAGATTT
TAAATAGAAGTATCATTCTTTTCTGGTTTTAAAATTTTTGTATTGTTTTTAGGCCATACACAATCTTGG
GGCTGGTTCTGTATTGTCATATGTAATAAAAATATAAAATAAATAGTAAAAAAAAAAAAAAAAAAAAAA
AAAAAA
```

Figure 3.30 Nucleotide sequences of an original EST (A) and 3' RACE-PCR (B). Start and stop codons are illustrated in boldfaced and underlined. 3' RACE-PCR primer for isolation of the full-length cDNA of this gene is underlined.

The full-length cDNA of *P. monodon cyclic AMP-regulated protein-like protein* (*PmcAMP-RPL*) was 1272 bp in length consisting an ORF of 435 bp corresponding to a polypeptide of 144 amino acids and 5' and 3' UTRs of 146 and 663 bp (excluding the poly A tail) (Figure 3.31A). The closest similarity of this transcript was *cyclic AMP-regulated protein like protein* of *Marsupenaeus japonicus* (*E*-value = 3e-95).

The calculated p*I* and MW of the deduced cyclic AMP regulated protein like protein was 5.39 and 16.02 kDa, respectively. The ADF domain was found at the amino acid position 9-131 (1.63e-15) of the deduced protein (Figure 3.31B).

A

```
CCTCTCTCGCTCGCTCTCCCTCTCTCGCTCGCTCTCCCTCTCTCGCTCTCCCTCTCTCTC   60
TGTGCCTCTCGTTGGCTCGAAGCTCTCCCTCGGTCTCTCCTCGCTGATCCGTCTCGTCTC  120
GGCTTCGTTCGGATAAAATCACGGTGATGGCTAGTACCCAGATCGACCGGGAAGGCCTAC  180
                          M  A  S  T  Q  I  D  R  E  G  L      11
ACAGCGCTTATAGTGATGTCAGGGACGCAAAAGCGACCGTCAATTGGGCCGTGTTCAAGT  240
H  S  A  Y  S  D  V  R  D  D  K  S  D  V  N  W  A  V  F  K    31
ATGAGGGCAGCCAGGTGACTGTGGCGGCCAAGGGACAGACCTTTGATGACTTCAAGGTGC  300
Y  E  G  S  Q  V  T  V  A  A  K  G  Q  T  F  D  D  F  K  A    51
AGTTTGGGGATGATGAGCGTGCGTTCGCTTACCTGCGCATCCAGACTGGGGATGAGATGA  360
Q  F  G  D  D  E  R  A  F  A  Y  L  R  I  Q  T  G  D  E  M    71
GCAAGAGATCGAAGTTCCTGATGCTAACTTGGGTGGGCACCGAGGTCTCGCCCATCAAGA  420
S  K  R  S  K  F  L  M  L  T  W  V  G  T  E  V  S  P  I  K    91
AAGCGAAAATGTCCACCGATAAGGCCCTCGTCAAAGAGGTTCTTGCAAACTTTGCAGTGG  480
K  A  K  M  S  T  D  K  A  L  V  K  E  V  L  A  N  F  A  V   111
AGTTGACAATAGAAAGCGCTCACGAACTGGACCACGAAGCCTTTTTACAAGAGCTCGTGA  540
E  L  T  I  E  S  A  H  E  L  D  H  E  A  F  L  Q  E  L  V   131
AAGCTGGTGGAGCCAACTATGGCACAGGATTCCGCGATTAGGCTTGTGGGTGCTCGCGTT  600
K  A  G  G  A  N  Y  G  T  G  F  R  D  *                     144
TGGCATTTAGATATGATAGGACACTGAAATGTAATCAATCTGGCTCTGTGTTCTCTTCAA  660
AAATTGCTAATGTCAGGGAATGGAACAGAATCGTTTTAATAAATGAAATACAATCAAATT  720
ATATAAAGTGCTTTGTGGGTCCATGATCAAAATATTGCAGGTACTTTTGTTAATATTCCC  780
ATATTTTTATTTATTTTTTGATTATTATCTTTTGGGCAGGAGAGAATTATCATTATTTGG  840
AACTACACTGAATTTTCATAGCATTTATGATGAGTTTCATTATGCATGCAACATTTTTCA  900
TTGAGGAGTTTAACAGACAGGCATTTTTATATATACATAGCTTTCAAAAGTTGACTTGAA  960
ATACATTCATTAAAAAAACATTACTGTAAACTAGTATTAACCTAGAATTAAGCAGTGCCA 1020
TCAGTTATTCAATTTTTTTCTGTAAATCCTCTATTTTTAGCATTACAATTGATAGACTGG 1080
TCATATTTAATTCTACAAAAAATACAATATATAATCAGATATAGATTTTAAATAGAAGTA 1140
TCATTCTTTTCTGGTTTTAAAATTTTTGTATTGTTTTTAGGCCATACACAATCTTGGGGC 1200
TGGTTCTGTATTGTCATATGTAATAAAAATATAAAATAAATAGTAAAAAAAAAAAAAAAA 1260
AAAAAAAAAAAA
```

B

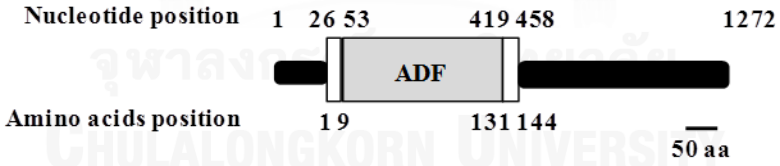


Figure 3.31 (A) The full-length cDNA and deduced protein sequences of *cyclic AMP-regulated protein like protein* of *P. monodon*. Start and stop codon are illustrated in boldfaced and underlined. The poly A additional signal (AATAAA) is boldfaced. (B) Diagram illustrating the full-length cDNA of *cyclic AMP-regulated protein like protein* of *P. monodon*. The ADF domain was found in this deduced protein. The scale bar is 50 amino acids in length.

3.3.6. *Nuclear pore complex protein NUP133*

Primers were designed from an EST clone no. HC-N-S01-0025-LF significantly matched *nuclear pore complex protein nup133* (*Pediculus humanus corporis*; *E*-value = 8e-34). The amplified fragments of approximately 900 bp and 2.3 kb were obtained from nested 5′ and primary 3' RACE-PCR (Figure 3.32). The amplification fragments were cloned and sequenced. Nucleotide sequences of the original EST, 5′ and 3' RACE-PCR were assembled (Figure 3.33).

The full-length cDNA of *PmNUP133* was 4130 bp in length consisting an ORF of 3228 bp corresponding to a polypeptide of 1085 amino acids and 5' and 3' UTRs of 438 and 464 bp (excluding the poly A tail) (Fig. 3.34A). The closest similarity of this newly characterized transcript was *nuclear pore complex protein nup133* of *Pediculus humanus corporis* (*E*-value = 0.00).

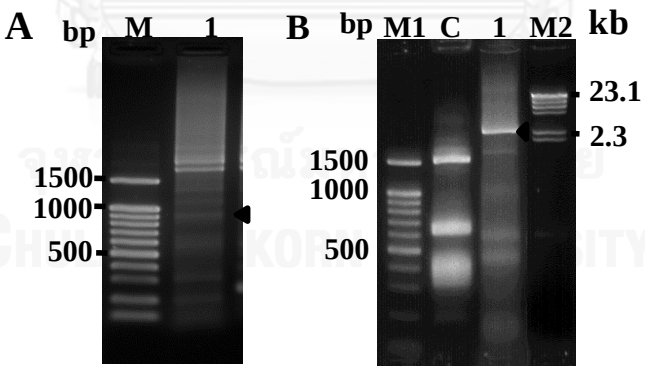


Figure 3.32 Agarose gel electrophoresis showing results from nested 5'RACE-PCR product (A) and primary 3′RACE-PCR product of *PmNPC133.* An arrowhead indicates a RACE-PCR product that was cloned and sequenced. Lanes M is a 100 bp DNA ladder.

A

I

GGTGCCACTTTAAAGGGTTTTTTTATTGTGTGGAAAGAGATGCCAGTTACTTAATGCACAGTAAGGACA
GGTAATTGAGGTGCCACATAGATTGTTTGTGAAGAAAAGATAACTTTGTAATGAAAATTGTGATCATGG
CCGACATATTTATTGAATTTAAAGTCACTGATTTGAGTTGAAGACATCGGCGGGAATACGACGTACGCT
TATATCCGGTGGATTTTTACTTTGCTCTGTCATTTGATATCTACAGCCCCATTTGCAAGATGTACACTC
CAGTCTCAGACCGAGGGGGAGGAGGGGCAGCCAATACTTCCTTCACATCTGCGAGTGCAAGAGCAGCAG
CAGCAGCTGCTGCACGCAGACGCAGTGCAGTTGGCTTCAATACCAGTGCAAGACGATCACCATTTACAA
CACCAGGTCGCTCCACACCTCTCAACCGATCTCTGCAAGCATCACAGCTC**ATG**GAAGGCAGTGGCCAGT
ATACCCTTGAGAGCTATGGGGCCTCCCTGCCAGCCTTGGTAATGGAAGCACTGACGTTTGCTGACCGAA
ATGTGGAGATGTCAGCAGTTTTATCACAGTGTGGCTGGGCGTGGCTTGTGTGTGGCCGACGTCTCTTAG
TGTGGAGGTATAAAGTAGATGACTCACGACGCCTTGTCAACCACCAGTTCCGGGAACTTACTTTACCTC
CATCAGACTTGGCACACAGAGCCCAGCTGGTGGTTGTATATGCCGCAAATGAAGGCCAGGTACCAGCGT
GTGTAGCAGCATCACCCGAGGGGTTCGTCAGATTCTGGCCCAGTATTGCGCATGAAGGCTCATATTTTG

Nested 5′RACE

AAGTCAGCACTGAACTACAGGGCCAAGAGTGTGACAGCCTGGTGTTCCTGGGGTCATCCTTAGGGTGTC
TGCTGGCCACAACCACATCCACCACCCTGCTCATCCAACCAGGCCAGGGCTCTGGTGGGAACCAGACCA
TTAATGTGAGGCAGTTGAAGGTTCCTGCTGGACTCTTAGGGGGTATATCACGCAGAGTTTCCTCACTAG
TGTTTGGATCCACGCCCACGCAAGCCCCAGAAGCGCGCTTGGTCCGTGCAGTAGCAGTTCCTGGTGCAG
AGTCAGATGAACGTTCAGTGCTGGTGCTGTCTGCCAACTCCTTGCAGAAGTGGTACTTGATCCCTAATG
AACCTGACAAGCTTGTTTATGAGTGTAATGTTGAAAAATACATCAGAGAAGGTTTTGTTGATCATGTTT
GGGGCCGTGAGAGAGCAGGAGCGACACAGTTGAGGGTGTGGTTGGTTGATATGCAGCCGACCTCAGGGG
TGTCATCAGTCATGGTCCTGACAGCAGCAGTTAACCCTCAGGTCTCACAGCAGTTGGTTTACGCCCTTG
CCACAATGGATACTGGTGGCTCAGCTCCACCCCTTGCAGTTGGCAATTTCTCGGTATTAAAGCATTCCG

NPC133-F1

AATATTATCAAGACTCTGCGGAGAATGTGCTCCTCTCACAACGCTTCCTTCTTTGTGCAAACACAGCTT
ATATTTACAACAAGAACACAGTCCTTTGTGTCTCAGCTGTGGGCGAAGGGGACAGTGGGGATCGGGTGG

NPC133-R1

AGTTCTCTGGAGCAGGGGATAGT

B

CAGAGTCAGATGAACGTTCAGTGCTGGTGCTGTCTGCCAACTCCTTGCAGAAGTGGTACTTGATCCCTA
ATGAACCTGACAAGCTTGTTTATGAGTGTAATGTTGAAAAATACATCAGAGAAGGATTTGTTGATCATG
TTTGGGGCCGTGAGAGAGCAGGAGCGACACAGTTGAGGGTGTGGTTGGTTGACATGCAGCCGACCTCAG
GGGTGTCATCAGTCATGGTCCTGACAGCAGCAGTTAACCCTCAGGTCTCACAGCAGTTGGTTTACGCCC
TTGCCACAATGGATACTGGTGGCTCAGCTCCACCCCTTGCAGTTGGCAATTTCTCGGTATTAAAGCATT

NPC133-F1

CCGAATATTATCAAGACTCTGCGGAGAATGTGCTCCTCTCACAACGCTTCCTTCTTTGTGCAAACACAG
CTTATATTTACAACAAGAACACAGTCCTTTGTGTCTCAGCTGTGGGCGAAGGGGACAGTGGGGATCGGG

NPC133-R1

TGGAGTTCTCTGGAGCAGGGGATAGTATCCTGGGTGTTGGCAGATCGCAGGCTTGCCCCTTTCTTCTCT
GCAAACCATGGCATTGTGTCTATCACGCCCAGTCAGCACCTGAACCAGTCAAGCATGTTGTTGAATGAG
AGTGTGGCTGAAGATACGTCTCGGCTTTGTGAGGCTCTCAACATCAGCAAG

C

NPC133-F1

TGCTCCTCTCACAACGCTTCCTTCTTTGTGCAAACACAGCTTATATTTACAACAAGAACACAGTCCTTT

NPC133-R1

GTGTCTCAGCTGTGGGCGAAGGGGACAGTGGGGATCGGGTGGAGTTCTCTGGAGCAGGGGATAGTATCC
TGGGTGTTGGCAGATCGCAGGCTTGCCCCTTTCTTCTCTGCAAACCATGGCATTGTGTCTATCACGCCC
AGTCAGCACCTGAACCAGTCAAGCATGTTGTTGAATGAGAGTGTGGCTGAAGATACGTCTCGGCTTTGT
GAGGCTCTCAACATCAGCAAGATCCTGGGTGTTGGCAGATCGCAGGGCTTGCCCCTCTTCTTCTCTGCA
AACCATGGCATTGTGTCTATCACGCCCAGTCAGCACCTGAACCAGTCAAGCATGTTGTTGAATGAGAGT
GTGGCTGAAGATACGTCTCGGCTTTGTGAGGCTCTCAACATCAGCAGTGTGGGCCTGGAAGCTATCACA
TCCAGCCAAGACCATACGGCCCGACTCCAGGCAGCCTTCCTGCATTTCAATAAGAACAACATTCCACAG
GCTCAGGCTCTTCTGGATGAGCTCTTCCCAGGGGCAGACAACTCCACTCTAGATGCTACTGTCATCAGC
CTTTCAACCAATCTGCTGGATGACTCTCCAGCAACAGATCCTCGCTGGGCTGAGAGCAATGAGGCAGGT
GGTACAGGTGCTCCGATGTCCCTCATTTTACAGAATCAGCTGAGAGACAAGACAACAGCTCATCAGTAC
TACATTAACTTTTTGCACCAGACTGGACTATGGCAGCGCTTGAGTGTTGGTCAGGTAGAAGAAACCCGC
GTCCTGACTCGAGTATTGCTTGCAGAACATGCAGAACAGTTAGCATTTGCAACATCTCTACGCACAAGA
CATAATGACCATCAGCATCTCATTGATGCTGCCATTAGGCATGTGCTAAATGAACGAGGAGAGACTCCA
AAAGGGAAACTGACCCATGCAGACCTCTTCTATCGCCGTGTTAGCCAGATTGAAGGCATCATCTGGGGA
TTGTTACGTGCACAGAGGAGGTCTTGGCTGCTGATGTCGCACCTCGCGATGCTCCGGGCCACTATTCAC
TCGGTAAACAGCCTTGTCTTAGCACTGTTACAAAGTGCCAGGGTAGGCAGTGCATCCATTTCTGGGATC
ACAGGGACTGTTAACGATCCTCCTCTTGAACACATACCTTGGACTGCCACACAGGGGTCCCGTGGAGTG
CGAACCCTTCTGCTGGAGCAGCATAACACCACAGTGGAGGTTGGCCTAGCAAGAGCTGAGGATGGAGCA
ACCCGAGCTAAACTCTACACACAGATGGTTGACCTTGCTGATTGCATTCTGACTGGATACCAGCCACAG
CTTCGTTCTCTTGTATCTGTTAGCCACGACTTGCACCAGTCCCTTCTGCGCTCATATGAGAGGGACCGC
TACAATTTGATTCAACCTCTTGTACAAGGCGAGCAGTACGATCAAGCAGTTGGACTTGCTGAGAAGTAC
TGTGACTTCCGCACGCTGGTAGAAGTCTGCGATCGTACCGACAACCAAGAAAGACTGAGCCAGTACATG
ACACAGTTTGGCTCTGAGGGTTTCTCGGACTTTGTGTTCCGTTGGTACTTGGAGACAGGTAAACGAGGT
CGCTTGCTGAGCCATGGTGACCAAGGGGGCCTTTCTCGCTTCTTGCAAGACTACACTTCCCTCGCTTGG
TTGCACCAAATCCAAACCAGAGATTTTTCATCAGCCTCTACCACTTTACGTCAGCTTGGCTTGGATGAG
ATGACTTACCTTAGCAGAAAAAAGACATTGTTAAGCCTTAGTAAATTATGCAACCTGGCTGCTAGTGTG
CCAGGCAGCGCAGATACAAGTGTGATGGAGGATGACACTATGACCTTAGAAGAGGACTTATTCTGTACC
AGGAGCAGTTGCCTGAACCGGTCTCATGCGCACTCCCTTGAGCTGATACCATGAAGTCTTTCACTACTG
AGCTGTCATCTCTAATACTGGAGATGAAACACTATGCATGATCGACTTCAGGCGCTAGATTGCTGCTTG
TTACCCTGGAGAGAAGTTCTTGGAGTGAGAAAGACTTCTGGGTGCGTGCTGTGGCTTCACTCTGCATCA
TGGAACTGCAATTCCATTGAAGCCAGTATGGACACGTGCCCTCCGGAGCCTCTCGGTTCGGGCAGAGAG
ATTACACTCCCTTGGTGGTGGACCCATCAACCAGGATTTCATTACGCCTTAACCATTACGTCAGCTTGG
CTGGGATGAGATGACTAACTAGCAGAAAAAGACATTGTTAGGCTAGTAATTATGCAACCTGCTGCTAGT
GTGCAGCAGCGCAGAATACAAGTGTGATGGAGGATGACACTATGACTAGAGAGACTTATTCTGTACCAG
AGCAGTTGCTGAACCGGTCCTCAATGCGCACTCCTTGAGGCTGATACCATGAAGGTCCTTTCACTACTG
AGCTTGTTCATCTCTATACTGGAGATGAAAACACCTATGCCCAATGAATTCGACTTCAAGAAGGCTTTA
GATTTGCTTGCCTTTGTACCTGGAGAAGAAGTTCCTGAGTTGAAGAAAAGACATCTGGGTGCGTGCTGT
GCTTCGCAACTCCTGGCATCACATGGACACAGACAATCCACTAGAAGCAATTAGTGACACGTTGCTTTT
CAAGACTATAGAGCTGGCATTCACACAAGGGACTGACATAAAGGAACTGCTACTTCCCGCTGAAGAATT
GCTGGAGTGTGAAGAACTAGGGGACCTCAAGGAAGATGCCACATTCAAGTTCCTGGTGAATGCTGGCTA
TGAGAAGATCACTCAGCTGGTTGGG**TAG**ATTTATTTGCAAGAAAAGGGAACAACAGGAAGGAACCTCAA
GTAATTGAAGTGGTCATGAACTTTGGAACTGTGATAAGTTTAGTAAAGGATAAGTGATAGAGCTTGTTT
TAGTGGCATTTTTTTTCTTGTGTCGGCCTGGTTAGAGAGAACATGTTTACCTAGTGTTATTTAAGATGT
GATTGTTGGCTTTATATACAAAGAAAAGATTAAAGTCAGTGTTCAAGAATTATGTGAGTTCAGGTACTA
GAAGCATTTTGAAGTTTGTGCATGAAATATAAGGTACTCCCTTTTTTATAGTTTTATGTACTGTTAGCT
TGTTTTATTTAAGATACATGGAAGTACTGATGCATTTTATGGTTTGCTGTACCTGAGATTTGAAATATT
ACTGCCACATGTAGTGTATTATTTTTTTTTACAAT**AATAA**TTTTTAAAATGTAAAAAAAAAAAAAGAAA
AAAAAAAAAAAAAAAAAAAAAAA

Figure 3.33 Nucleotide sequence of 5' RACE-PCR (A), the original EST (B) and 3′RACE (C) of *PmNPC133.* Forward and reverse RT-PCR primer (NPC133-F1 and NPC133-R1, underlined) was used for 5' and 3′ RACE-PCR of this gene.

The calculated p*I* and MW of the deduced PmNUP133 protein was 5.18 and 118.78 kDa, respectively. The nucleoporin_N and nucleoporin_ C domains were found at the amino acid position 5-374 (*E* value = 1.1e-16), 518-1033 (*E* value = 5.7e-59) of the deduced PmNUP133 protein (Figure 3.34B).

A

```
GGTGCCACTTTAAAGGGTTTTTTTATTGTGTGGAAAGAGATGCCAGTTACTTAATGCACA  60
GTAAGGACAGGTAATTGAGGTGCCACATAGATTGTTTGTGAAGAAAAGATAACTTTGTAA  120
TGAAAATTGTGATCATGGCCGACATATTTATTGAATTTAAAGTCACTGATTTGAGTTGAA  180
GACATCGGCGGGAATACGACGTACGCTTATATCCGGTGGATTTTTACTTTGCTCTGTCAT  240
TTGATATCTACAGCCCCATTTGCAAGATGTACACTCCAGTCTCAGACCGAGGGGGAGGAG  300
GGGCAGCCAATACTTCCTTCACATCTGCGAGTGCAAGAGCAGCAGCAGCAGCTGCTGCAC  360
GCAGACGCAGTGCAGTTGGCTTCAATACCAGTGCAAGACGATCACCATTTACAACACCAG  420
GTCGCTCCACACCTCTCAACCGATCTCTGCAAGCATCACAGCTCATGGAAGGCAGTGGCC  480
                                            M  E  G  S  G    5
AGTATACCCTTGAGAGCTATGGGGCCTCCCTGCCAGCCTTGGTAATGGAAGCACTGACGT  540
 Q  Y  T  L  E  S  Y  G  A  S  L  P  A  L  V  M  E  A  L  T   25
TTGCTGACCGAAATGTGGAGATGTCAGCAGTTTTATCACAGTGTGGCTGGGCGTGGCTTG  600
 F  A  D  R  N  V  E  M  S  A  V  L  S  Q  C  G  W  A  W  L   45
TGTGTGGCCGACGTCTCTTAGTGTGGAGGTATAAAGTAGATGACTCACGACGCCTTGTCA  660
 V  C  G  R  R  L  L  V  W  R  Y  K  V  D  D  S  R  R  L  V   65
ACCACCAGTTCCGGGAACTTACTTTACCTCCATCAGACTTGGCACACAGAGCCCAGCTGG  720
 N  H  Q  F  R  E  L  T  L  P  P  S  D  L  A  H  R  A  Q  L   85
TGGTTGTATATGCCGCAAATGAAGGCCAGGTACCAGCGTGTGTAGCAGCATCACCCGAGG  780
 V  V  V  Y  A  A  N  E  G  Q  V  P  A  C  V  A  A  S  P  E  105
GGTTCGTCAGATTCTGGCCCAGTATTGCACATGAAGGCTCATATTTTGAAGTCAGCACTG  840
 G  F  V  R  F  W  P  S  I  A  H  E  G  S  Y  F  E  V  S  T  125
AACTACAGGGCCAAGAGTGTGACAGCCTGGTGTTCCTGGGGTCATCCTTAGGGTGTCTGC  900
 E  L  Q  G  Q  E  C  D  S  L  V  F  L  G  S  S  L  G  C  L  145
```

```
TGGCCACAACCACATCCACCACCCTGCTCATCCAACCAGGCCAGGGCTCTGGTGGGAACC  960
 L  A  T  T  T  S  T  T  L  L  I  Q  P  G  Q  G  S  G  G  N   165
AGACCATTAATGTGAGGCAGTTGAAGGTTCCTGCTGGACTCTTAGGGGGTATATCACGCA 1020
 Q  T  I  N  V  R  Q  L  K  V  P  A  G  L  L  G  G  I  S  R   185
GAGTTTCCTCACTAGTGTTTGGATCCACGCCCACGCAAGCCCCAGAAGCGCGCTTGGTCC 1080
 R  V  S  S  L  V  F  G  S  T  P  T  Q  A  P  E  A  R  L  V   205
GTGCAGTAGCAGTTCCTGGTGCAGAGTCAGATGAACGTTCAGTGCTGGTGCTGTCTGCCA 1140
 R  A  V  A  V  P  G  A  E  S  D  E  R  S  V  L  V  L  S  A   225
ACTCCTTGCAGAAGTGGTACTTGATCCCTAATGAACCTGACAAGCTTGTTTATGAGTGTA 1200
 N  S  L  Q  K  W  Y  L  I  P  N  E  P  D  K  L  V  Y  E  C   245
ATGTTGAAAAATACATCAGAGAAGGTTTTGTTGATCATGTTTGGGGCCGTGAGAGAGCAG 1260
 N  V  E  K  Y  I  R  E  G  F  V  D  H  V  W  G  R  E  R  A   265
GAGCGACACAGTTGAGGGTGTGGTTGGTTGATATGCAGCCGACCTCAGGGGTGTCATCAG 1320
 G  A  T  Q  L  R  V  W  L  V  D  M  Q  P  T  S  G  V  S  S   285
TCATGGTCCTGACAGCAGCAGTTAACCCTCAGGTCTCACAGCAGTTGGTTTACGCCCTTG 1380
 V  M  V  L  T  A  A  V  N  P  Q  V  S  Q  Q  L  V  Y  A  L   305
CCACAATGGATACTGGTGGCTCAGCTCCACCCCTTGCAGTTGGCAATTTCTCGGTATTAA 1440
 A  T  M  D  T  G  G  S  A  P  P  L  A  V  G  N  F  S  V  L   325
AGCATTCCGAATATTATCAAGACTCTGCGGAGAATGTGCTCCTCTCACAACGCTTCCTTC 1500
 K  H  S  E  Y  Y  Q  D  S  A  E  N  V  L  L  S  Q  R  F  L   345
TTTGTGCAAACACAGCTTATATTTACAACAAGAACACAGTCCTTTGTGTCTCAGCTGTGG 1560
 L  C  A  N  T  A  Y  I  Y  N  K  N  T  V  L  C  V  S  A  V   365
GCGAAGGGGACAGTGGGGATCGGGTGGAGTTCTCTGGAGCAGGGGATAGTATCCTGGGTG 1620
 G  E  G  D  S  G  D  R  V  E  F  S  G  A  G  D  S  I  L  G   385
TTGGCAGATCGCAGGGCTTGCCCCTCTTCTTCTCTGCAAACCATGGCATTGTGTCTATCA 1680
 V  G  R  S  Q  G  L  P  L  F  F  S  A  N  H  G  I  V  S  I   405
CGCCCAGTCAGCACCTGAACCAGTCAAGCATGTTGTTGAATGAGAGTGTGGCTGAAGATA 1740
 T  P  S  Q  H  L  N  Q  S  S  M  L  L  N  E  S  V  A  E  D   425
CGTCTCGGCTTTGTGAGGCTCTCAACATCAGCAGTGTGGGCCTGGAAGCTATCACATCCA 1800
 T  S  R  L  C  E  A  L  N  I  S  S  V  G  L  E  A  I  T  S   445
GCCAAGACCATACGGCCCGACTCCAGGCAGCCTTCCTGCATTTCAATAAGAACAACATTC 1860
 S  Q  D  H  T  A  R  L  Q  A  A  F  L  H  F  N  K  N  N  I   465
CACAGGCTCAGGCTCTTCTGGATGAGCTCTTCCCAGGGGCAGACAACTCCACTCTAGATG 1900
 P  Q  A  Q  A  L  L  D  E  L  F  P  G  A  D  N  S  T  L  D   485
CTACTGTCATCAGCCTTTCAACCAATCTGCTGGATGACTCTCCAGCAACAGATCCTCGCT 1960
 A  T  V  I  S  L  S  T  N  L  L  D  D  S  P  A  T  D  P  R   505
GGGCTGAGAGCAATGAGGCAGGTGGTACAGGTGCTCCGATGTCCCTCATTTTACAGAATC 2020
 W  A  E  S  N  E  A  G  G  T  G  A  P  M  S  L  I  L  Q  N   525
AGCTGAGAGACAAGACAACAGCTCATCAGTACTACATTAACTTTTTGCACCAGACTGGAC 2080
 Q  L  R  D  K  T  T  A  H  Q  Y  Y  I  N  F  L  H  Q  T  G   545
TATGGCAGCGCTTGAGTGTTGGTCAGGTAGAAGAAACCCGCGTCCTGACTCGAGTATTGC 2120
 L  W  Q  R  L  S  V  G  Q  V  E  E  T  R  V  L  T  R  V  L   565
TTGCAGAACATGCAGAACAGTTAGCATTTGCAACATCTCTACGCACAAGACATAATGACC 2180
 L  A  E  H  A  E  Q  L  A  F  A  T  S  L  R  T  R  H  N  D   585
ATCAGCATCTCATTGATGCTGCCATTAGGCATGTGCTAAATGAACGAGGAGAGACTCCAA 2240
 H  Q  H  L  I  D  A  A  I  R  H  V  L  N  E  R  G  E  T  P   605
AAGGGAAACTGACCCATGCAGACCTCTTCTATCGCCGTGTTAGCCAGATTGAAGGCATCA 2300
 K  G  K  L  T  H  A  D  L  F  Y  R  R  V  S  Q  I  E  G  I   625
TCTGGGGATTGTTACGTGCACAGAGGAGGTCTTGGCTGCTGATGTCGCACCTCGCGATGC 2360
 I  W  G  L  L  R  A  Q  R  R  S  W  L  L  M  S  H  L  A  M   645
TCCGGGCCACTATTCACTCGGTAAACAGCCTTGTCTTAGCACTGTTACAAAGTGCCAGGG 2420
 Y  T  Q  M  V  D  L  A  D  C  I  L  T  G  Y  Q  P  Q  L  R   665
TAGGCAGTGCATCCATTTCTGGGATCACAGGGACTGTTAACGATCCTCCTCTTGAACACA 2480
 L  R  A  T  I  H  S  V  N  S  L  V  L  A  L  L  Q  S  A  R   685
TACCTTGGACTGCCACACAGGGGTCCCGTGGAGTGCGAACCCTTCTGCTGGAGCAGCATA 2540
 V  G  S  A  S  I  S  G  I  T  G  T  V  N  D  P  P  L  E  H   705
ACACCACAGTGGAGGTTGGCCTAGCAAGAGCTGAGGATGGAGCAACCCGAGCTAAACTCT 2600
 I  P  W  T  A  T  Q  G  S  R  G  V  R  T  L  L  L  E  Q  H   725
```

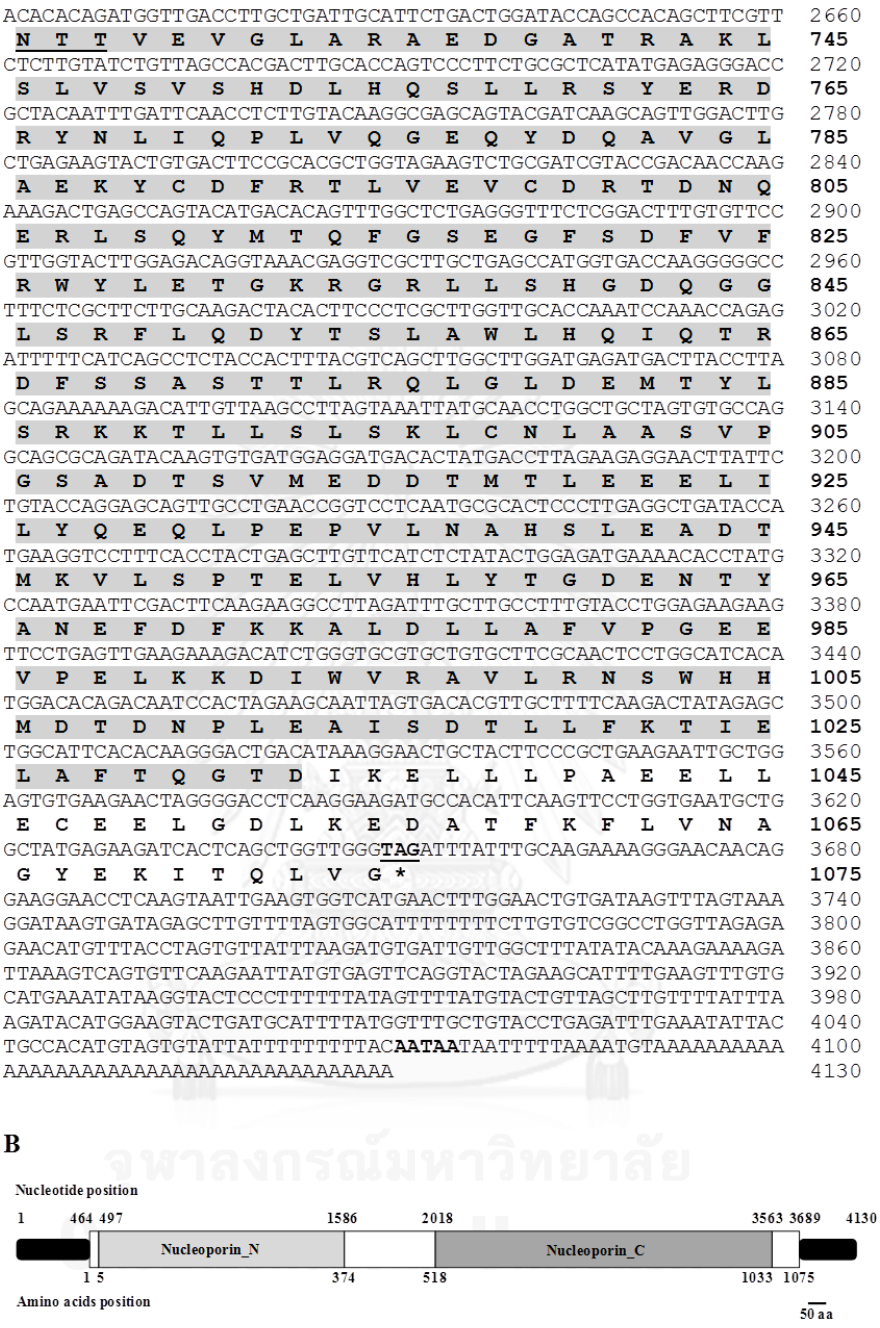


Figure 3.34 (A) The full-length cDNA and deduced protein sequences of *PmNPC133* of *P. monodon*. Start and stop codon are illustrated in boldfaced and underlined. The poly A additional signal (AATAA) is boldfaced. A putative *N*-linked-glycosylation site is highlighted and underlined. (B) Diagram illustrating the full-length cDNA of *PmNPC133*. The nucleoporin_N and nucleoporin_C domains were found in the deduced PmNPC133 protein. The scale bar is 50 amino acids in length.

3.3.7 *Semaphorin-2a*

An amplified fragment of approximately 1500 and 1050 bp were obtained from the primary 3′ and 5' RACE-PCR of *semaphorin-2a* (Figure 3.27). This fragment was cloned and sequenced. Nucleotide sequences of the original EST (OV-N-S01-0003-W) and 5' and 3′ RACE-PCR (Figure 3.35) were assembled.

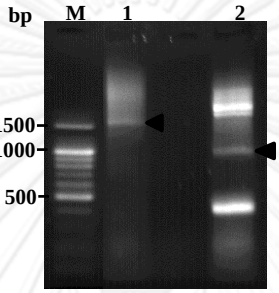


Figure 3.35 Agarose gel electrophoresis showing results from the primary 3′ (lane 1) and 5' (lane 2) RACE-PCR of *PmSema.* An arrowhead indicates a RACE-PCR product that was cloned and sequenced. Lanes M is a 100 bp DNA ladder.

The partial nucleotide sequence covering the 3′UTR and C-terminus portion of *P. monodon semaphorin-2a* (*PmSema*) were obtained. This transcript was significantly similar to *semaphorin-2a* of *Tribolium castaneum* (*E*-value = 7e-158). The Sema domain which is found in secreted and transmembrane proteins, was found in the deduced protein of this partial transcript (Figure 3.36).

```
CTTCCAGACAACGTGCTGACATTCCTCCAGTCGCATCCCCTGATGGACGAAGCAGTGGCT  60
 L  P  D  N  V  L  T  F  L  Q  S  H  P  L  M  D  E  A  V  A   20
CATGAACACAACAAGCCAGTGTTCTTCAAGCGGGATCTTATCTTCACACACCTGGTGGTA  120
 H  E  H  N  K  P  V  F  F  K  R  D  L  I  F  T  H  L  V  V   40
GATAAGGTTACTGGAGGCACTTATGGTCAAGAGAACACTTATACCGTTTATTATGCTGGT  180
 D  K  V  T  G  G  T  Y  G  Q  E  N  T  Y  T  V  Y  Y  A  G   60
TCATTGGAAGGCCGTGTCTATAAGGTGGTTGAGTGGTTGGATTCAGATGGAGTTTCTCAT  240
 S  L  E  G  R  V  Y  K  V  V  E  W  L  D  S  D  G  V  S  H   80
TCTGAACTTCTTGACGTGTATGAAGTTACAACTCCAGAACCCATTAGAGCTATTCAAATC  300
 S  E  L  L  D  V  Y  E  V  T  T  P  E  P  I  R  A  I  Q  I  100
TCCAAGAAGCACAAGTCACTGTATGTCTCATCGGATACACGAGTGCGCCAAGTGGATCTC  360
 S  K  K  H  K  S  L  Y  V  S  S  D  T  R  V  R  Q  V  D  L  120
TACATGTGTAAGGGGAGATATGACAACTGCCTTCGCTGCTCACATGACCCATACTGTGGA  420
 Y  M  C  K  G  R  Y  D  N  C  L  R  C  S  H  D  P  Y  C  G  140
TGGGACAAGGATGCTAATACCTGCAAACCTTATGAGCCAGGACTTCTGCAAGATGTCATG  480
 W  D  K  D  A  N  T  C  K  P  Y  E  P  G  L  L  Q  D  V  M  160
GGAACAACCCCTGGTCTCTGTGACGCGTGCATTGCGAAGAAGAAAATGGTAGTGACCTGG  540
 G  T  T  P  G  L  C  D  A  C  I  A  K  K  K  M  V  V  T  W  180
GGGCAGAGTATCCATTTAGGCTGTGCAGTGAAACTTCCCCGTCCGATTTCCCTCAAGGAT  600
 G  Q  S  I  H  L  G  C  A  V  K  L  P  R  P  I  S  L  K  D  200
ATTACTTGGCATCACTATTCTAAGGATAAGGGCAAATACCAGATTAGATACAGGCCAGAC  660
 I  T  W  H  H  Y  S  K  D  K  G  K  Y  Q  I  R  Y  R  P  D  220
AAATATATTGAGACTTCAGAACATGGCTTGGTGGTGATGAATGTAAATGAAGCTGATGCT  720
 K  Y  I  E  T  S  E  H  G  L  V  V  M  N  V  N  E  A  D  A  240
GGTCGTTATGACTGCAAGATGGGAGGAGATATTGTCTGCTCATACAACATTACTGTTGAT  780
 G  R  Y  D  C  K  M  G  G  D  I  V  C  S  Y  N  I  T  V  D  260
GCACACCGCTGCTCTGCACCTGCTCGCACTAATGATTTCCAGAAGGTGTATAGCGACTGG  840
 A  H  R  C  S  A  P  A  R  T  N  D  F  Q  K  V  Y  S  D  W  280
TGCCATGAATTTGAGAAATACAAGCTAGCCATGAAGACGTGGGAAAGGAAGCAAGCGCAA  900
 C  H  E  F  E  K  Y  K  L  A  M  K  T  W  E  R  K  Q  A  Q  300
TGTGCCAATAGGAATAACATCAGCCAGCAGAACAGCCATCCCAATGACATTTATCAGAGG  960
 C  A  N  R  N  N  I  S  Q  Q  N  S  H  P  N  D  I  Y  Q  R  320
AGCAATCCCTTCGTCTGATTACACCAAGAAAGTATAACTATTGTCAAGGAGTAGAAGGGT  1020
 S  N  P  F  V  *                                            325
CAGGAATTCTCCTTCCTCGAGATGGTCTAGAATTTGCCTGGTATTTTTAGTTCAGCTACT  1080
TAAATTATTACTACACACTGGAAACATGTATAGTGTTGACAATCACCATCTTCAGTGACA  1140
AGTGACTTACTTTACAACAAGTGAATTCATTAGCTCTGTCACAGAGAATACTGCCAAAAA  1200
CAGGCATGTAATAGTATTGATTTTTTTTTTTTTTTTACCAATTGTTTGTTTCCACATTAA  1260
GATCATATGAAATTTTAATGTAATCAATCATGGTGCCACCAAAAAAATGGGTGCTAGCAA  1320
GGACTGGTGCCATGTGCACAGAAAATGATCTCTTACAGTATATCTTATGCTAAAGTTCTG  1380
ACAGGCAACTTGACCCAAACTCCAGTAACAATCAGGGTACCATAAGCTATTGACTAATTC  1440
ATTTTAATTTACCAATCTTGGTAAAATCTTTTAACTGTGAAGGAGTTCATTAGGATCTAG  1500
TTCTTGCTCCTGGACAATCGCCTGTGGGCATTATTGCTTCTGCATTCAAAGAGCTTCAGT  1560
GAGCAGTGGATCTTAGAACTCC                                        1582
```

Figure 3.36 The partial nucleotide and deduced amino sequences of *PmSema.* The stop codon is illustrated in boldfaced and underlined.

3.4 RT-PCR and tissue distribution analysis of reproduction-related genes

Total RNA extracted from ovaries revealed predominated discrete bands along with smeared high molecular weight RNA (Figure 3.37A). The ratios of purified

RNA were 1.7-2.0 implying that the quality of extracted DNA was acceptable for further applications. The first cDNA synthesized from these total RNA covered the large product sizes (Figure 3.37B).

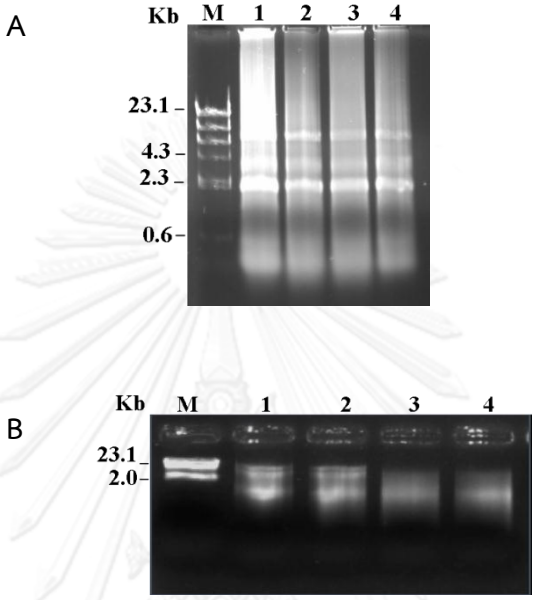


Figure 3.37 1.0% ethidium bromide-stained agarose gel showing the quality of total RNA (A) and corresponding first strand cDNA (B) from female broodstock of *P. monodon*. Lane M (A and B) = λ-*Hin*d III. Lanes 1-4 (A) = total RNA from ovaries of each *P. monodon* broodstock. (B). Lanes 1-4 (B) = the first strand cDNA from ovaries of each *P. monodon* broodstock.

Seven primer pairs were designed from nucleotide sequences of EST libraries previously established from ovaries (2 primers pairs), hemocyte (4 primers pairs) and hepatopancrease (1 primer pairs) of *P. monodon*. RT-PCR was carried out using an identical amplification conditions across all primers.

Initially, the first strand cDNA synthesized from ovaries and testes were subjected to RT-PCR and eletrophoretically analyzed. *PmVCP* was more abundantly expressed than in ovaries than testes in both juveniles and broodstock. Similarly the

expression of *PmPKC* and *PmSema* in ovaries was greater than that in testes of *P. monodon*. In contrast, *PmTmsb*, *PmcAMP-RPL* and *PmRacgap1* seemed to be non-differentiallyexpressed between ovaries and testes of *P. monodon* (Figure 3.38).

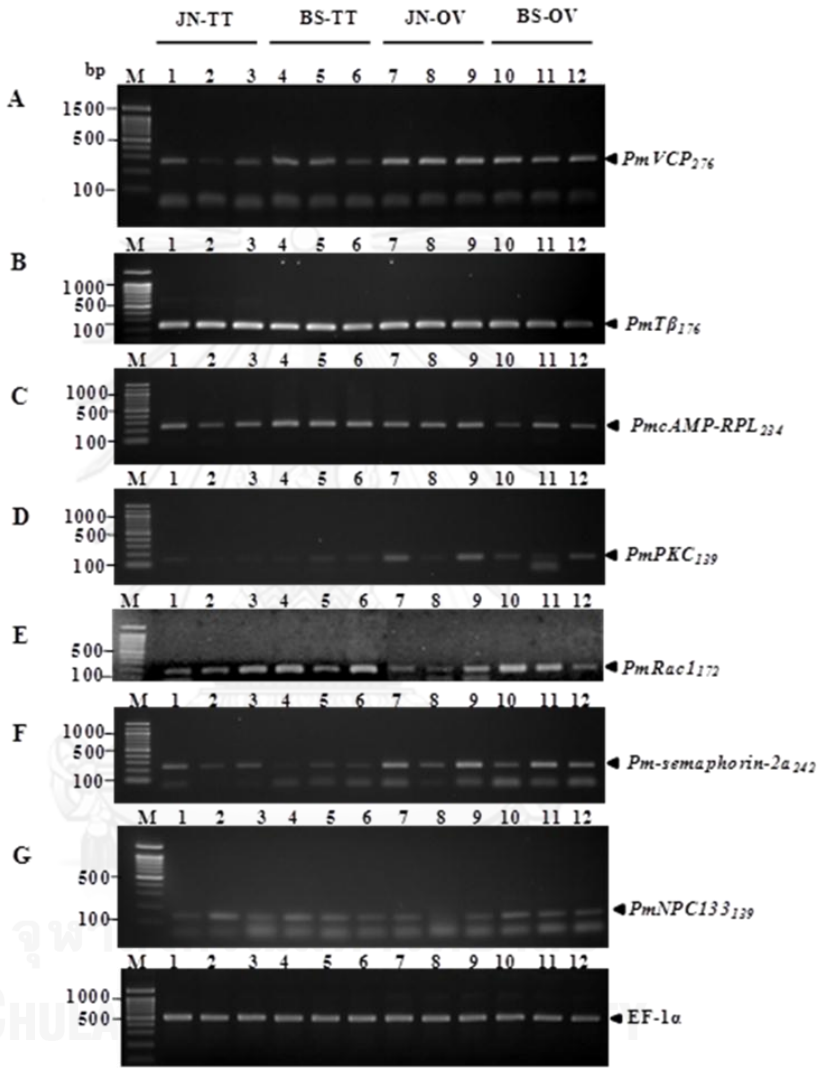


Figure 3.38 1.6% ethidium bromide-stained agrarose gels showing results from RT-PCR of *PmVCP* (A) *PmTmsb* (B) *cyclic AMP-regulated protein like protein* (*PmcAMP-RPL*) (C) *PmPKC* (D) *PmRacgap1* (E) *PmSema* (F), *PmNUP13* (G) and *EF-1α* (H) using first strand cDNA of testes and ovaries of juveniles and broodstocks. Lanes 1-3 = juvenile testes, lanes 4-6 = broodstock testes, lanes 7-9 = juveniles ovaries and Lanes 10-12 = broodstock ovaries. Lanes M are a 100 bp DNA ladder marker.

Tissue distribution analysis of these transcripts was carried out and it indicated that *PmVCP* was abundantly expressed in ovaries, the heart and lymphoid organ but less so in testes, the subcuticular epithelium and hepatopancreas. Based on RT-PCR, PmVCP mRNA was not found in eyestalks, gills, pleopods, the thoracic ganglion, the stomach, hemocytes or the intestine of wild female *P. monodon* (Figure 3.39A).

PmTmsb was constitutively expressed in all examined tissues of wild females (ovaries, intestine, heart, lymphoid organs, eyestalk, hepatopancrease, gill, stomach, pleopods, thoracic ganglion and hemocyte) and testes of wild males (Figure 3.39B).

PmcAMP-RPL was abundantly expressed in pleopods, thoracic ganglion, hemocyte, eyestalk and hepatopancrease. A lower expression was observed in testes, intestine, gills, heart and lymphoid organs. Limited expression of this transcript was observed in ovaries and stomach (Figure. 3.39C).

PmPKC was abundantly expressed in ovaries and lower expression was found in thoracic ganglion, heart and testis. Extremely low expression of this transcript was observed in eyestalk, intestine, stomach, gills, hemocytes, lymphoid organs, subcuticular epithelium, pleopods and antennal gland. Based on RT-PCR, it was not expressed in hepatopancrease (Figure 3.39D).

PmRacgap1 was highly expressed in ovaries, antennal gland, eyestalk and hemocytes. A lower expression was observed in testes, gill, heart and lymphoid organs. Based on RT-PCR, this transcript was limitedly expressed in subcuticular epithelium, hepatopancreas, intestine pleopods, stomach and thoracic ganglion (Figure. 3.39E).

PmSema was abundantly expressed in ovaries followed by stomach, gill and lymphoid organs. This transcript was expressed with very low levels extremely in eyestalk, intestine, subcuticular epithelium, hepatopancrease, heart, hemocytes, pleopods and antennal gland. Based on RT-PCR analysis, PmSema was not expressed in testes of male broodstock (Figure. 3.39F).

PmNUP133 was abundantly expressed in ovaries followed by tetes. A lower expression was observed in gills, heart and eyestalk and it was not expressed in subcuticular epithelium, intestine, hemocyte, thoracic ganglion, lymphoid organs, hepatopancrease, pleopods and antennal gland (Figure. 3.39H).

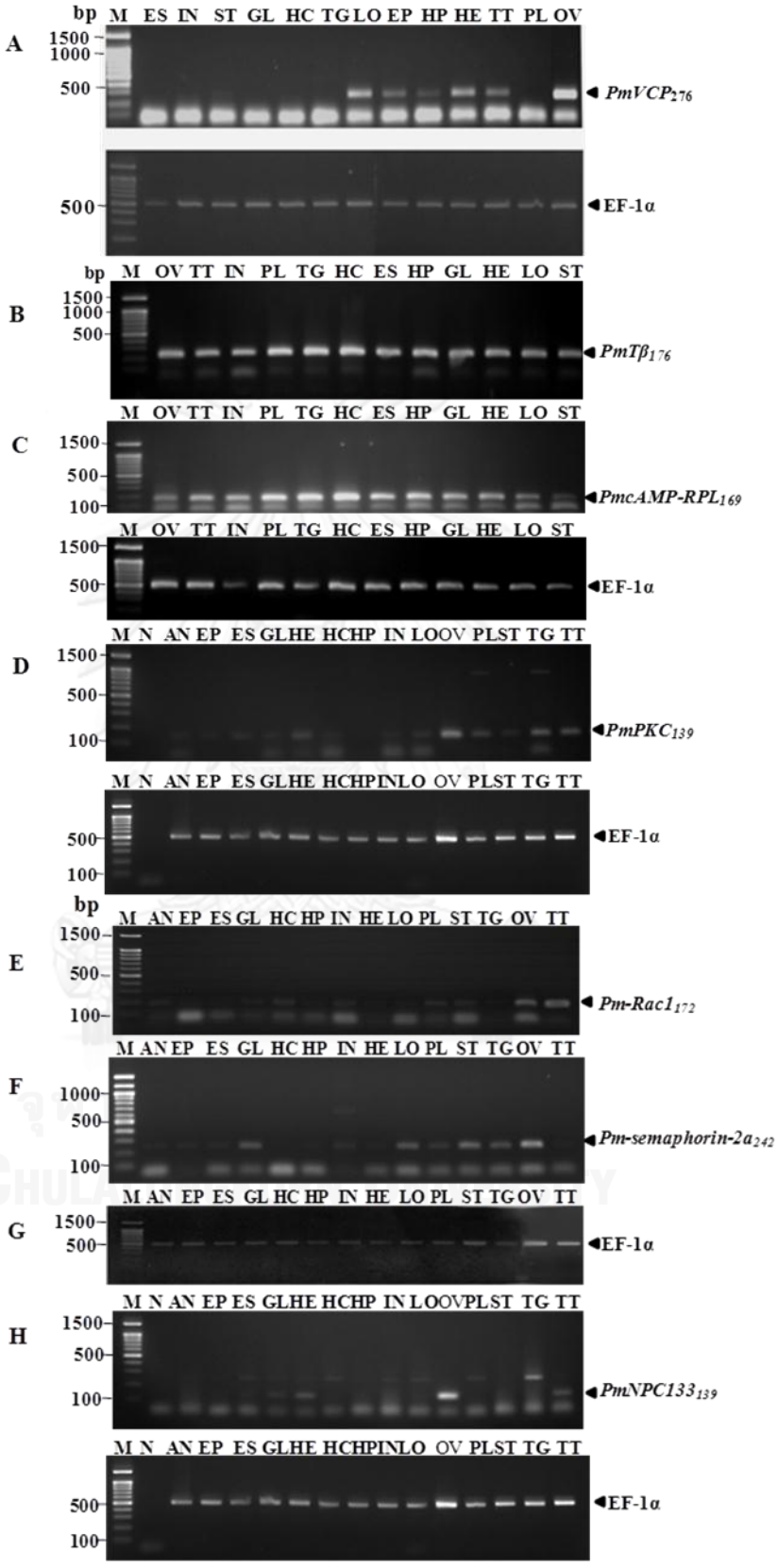

A
bp M ES IN ST GL HC TG LO EP HP HE TT PL OV
1500
1000
500
PmVCP276
500
EF-1α
B
bp M OV TT IN PL TG HC ES HP GL HE LO ST
1500
1000
500
PmTβ176
C
M OV TT IN PL TG HC ES HP GL HE LO ST
1500
500
100
PmcAMP-RPL169
M OV TT IN PL TG HC ES HP GL HE LO ST
1500
500
EF-1α
D
M N AN EP ES GL HE HC HP IN LO OV PL ST TG TT
1500
500
100
PmPKC139
M N AN EP ES GL HE HC HP IN LO OV PL ST TG TT
500
100
EF-1α
E
bp M AN EP ES GL HC HP IN HE LO PL ST TG OV TT
1500
500
100
Pm-Rac1172
F
M AN EP ES GL HC HP IN HE LO PL ST TG OV TT
1000
500
100
Pm-semaphorin-2a242
G
M AN EP ES GL HC HP IN HE LO PL ST TG OV TT
1500
500
EF-1α
H
M N AN EP ES GL HE HC HP IN LO OV PL ST TG TT
1500
500
100
PmNPC133139
M N AN EP ES GL HE HC HP IN LO OV PL ST TG TT
500
100
EF-1α

Figure 3.39 1.6% ethidium bromide-stained agrarose gels showing results from RT-PCR of *PmVCP* (A) *PmTmsb* (B) *PmcAMP-RPL* (C) *PmPKC* (D) *PmRacgap1* (E) *PmSema* (F), *PmNUP13* (G) using first strand cDNA of ovaries and various tissues of *P. monodon* broodstock and *EF-1α* was successfully amplified romthe same template. Lanes M are a 100 bp DNA ladder marker. AN= antennal gland, ES = eyestalk, IN = intestine, ST = stomach, GL = gill, HC = hemocytes, TG = thoracic ganglion, LO = lymphoid organ, EP = subcuticular epithelium, HP = hepatopancrease, HE = heart, TT = testes, PL = pleopod and OV = ovaries.

A summary on expression of these genes in various tissues was illustrated in Table 3.9.

Table 3.9 Expression of reproduction-related genes in different tissues of *P. monodon*

Gene homologues	Expected size (bp)	Tissue
1. *PmVCP*	276	OV, HE, LO, TT, EP, HP
2. *PmTmsb*	176	ES, IN, ST, GL, HC, TG, HE, HP, LO, TT, PL, OV, EP
3. *PmcAMP-RPL*	169	PL, TG, HC, ES, HP, TT, IN, GL, HE, LO, OV, ST
4. *PmPKC*	139	OV, TG. HE, TT, ES, IN, ST, GL, HC, LO, EP, PL, AN
5. *PmRac1gap1*	172	OV, AN, ES, HC, TT, GL, HE, LO, EP, HP, IN, PL, ST, TG
6. *PmSema*	242	OV, GL, LO, ST, AN, EP, ES, HC, IN, PL, HE
7. *PmNUP133*	139	OV, TT, ES, GL

3.5 Quantitative analysis of *PmVCP, PmTmsb, PmRacgap1, PmPKC and PmcAMP-RPL during ovarian devlopment of P. monodon*

The standard curves for real-time PCR analysis of *PmVCP, PmTmsb, PmRacgap1, PmPKC and PmcAMP-RPL* and EF-1α was constructed (Figure. 3.40). High amplification efficiency but low errors of each transcript were found.

3.5.1 *PmVCP*

Quantitative real-time PCR analysis revealed that the level of *PmVCP* expression in ovaries of juvenileswas significantly lower than in ovaries of broodstock ($P < 0.05$) (Figure 3.34). *PmVCP* mRNA was significantly higher in stages II and IV (mature) ovaries in wild intact broodstock of *P. monodon* ($P < 0.05$). By contrast, there was no statistically significant difference in PmVCP mRNA expression during ovarian development of eyestalk-ablated broodstock. The expression level of PmVCP mRNA in each ovarian stage (I–IV) of eyestalk-ablated broodstock seemed to be greater than that in intact broodstock but results were not significant owing to large standard errors among groups of samples (Figure 3.41).

3.5.2 *PmTmsb*

The expression level of *PmTmsb* in ovaries of juveniles was significantly lower than that in stages III, IV and post-spawning (V) ovaries of wild intact broodstock ($P < 0.05$). In intact broodstock, *PmTmsb* was not differentially expressed in different ovarian stages of *P. monodon* ($P > 0.05$). In unilateral eyestalk-ablated broodstock, its expression was significantly increased in vitellogenic (II) and mature (IV) ovaries ($P < 0.05$). Eyestalk ablation resulted in a significantly lower expression of *PmTmsb* in

previtelogenic (I) and late vitellogenic (III) ovaries compared to the same stages in intact broodstock ($P < 0.05$, Figure 3.42).

3.5.3 *PmcAMP-RPL*

Quantitative real-time PCR analysis shown that the mRNA levels of *PmcAMP-RPL* in stage IV (mature) ovaries was greater than that in premature ovaries of juveniles ($P < 0.05$). Nevertheless, this transcript was not differentially expressed during ovarian development in both intact and eyestalk-ablated broodstock ($P > 0.05$; Figure 3.43).

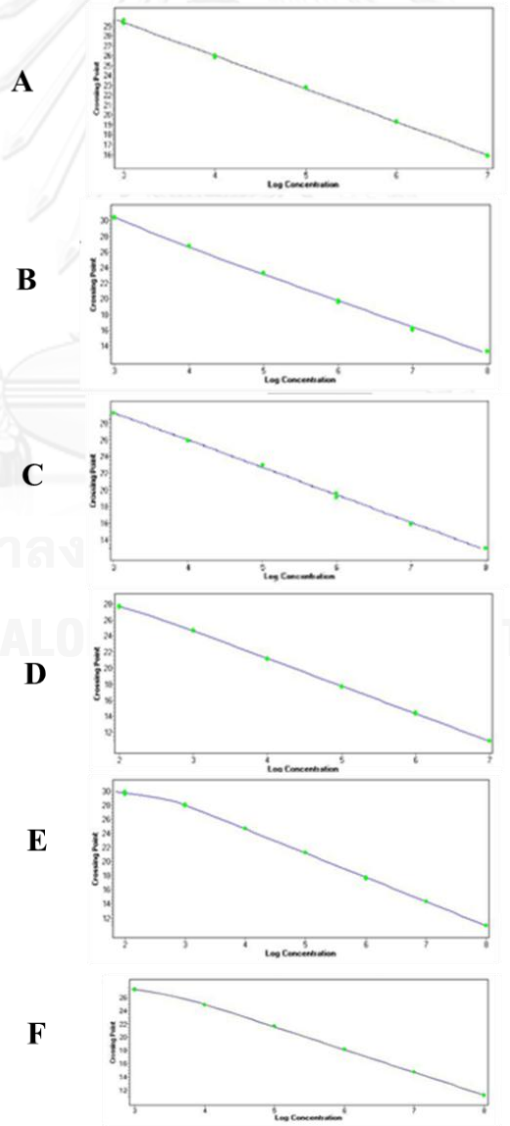


Figure 3.40 The standard amplification curve of various genes examined by real-time PCR analysis. The standard curve of *PmVCP* (A; r^2 for standard curve = 0.9930, efficiency for the amplification = 1.986), *PmTmsb* (B; r^2 for standard curve = 0.9820, efficiency for the amplification = 1.964), *PmcAMP-RPL* (C; r^2 for standard curve = 1.0035, efficiency for the amplification = 2.007), *PmPKC* (D; r^2 for standard curve = 0.9780, efficiency for the amplification = 1.956), *PmRacgap1* (E; r^2 for standard curve = 0.9790, efficiency for the amplification = 1.958) and *EF-1α* (F; r^2 for standard curve = 0.9805, efficiency for the amplification = 1.961). The abscissa reveals log copy number concentrations of each gene (10^3 to 10^8 copy, respectively).

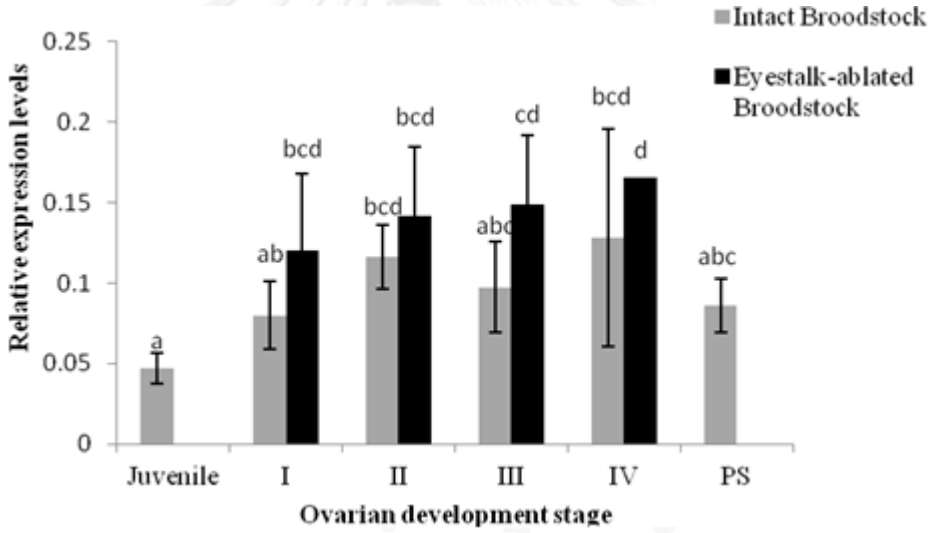


Figure 3.41 Histograms showing relative expression levels of *PmVCP* during ovarian development of intact broodstock and unilateral eyestalk-ablated of *P. monodon* broodstock. The same letters indicate non-significant differences between relative expression levels of different groups of samples.

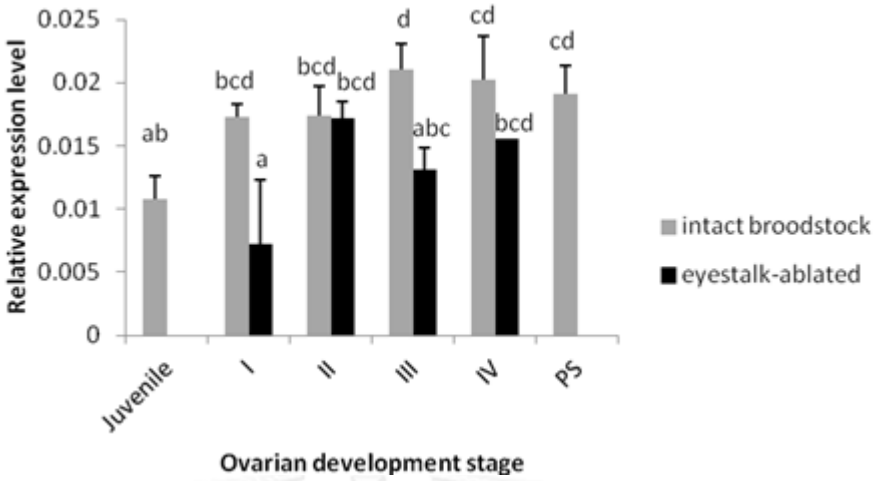


Figure 3.42 Histograms showing relative expression levels of *PmTmsb* during ovarian development of intact broodstock and unilateral eyestalk-ablated of *P. monodon* broodstock. The same letters indicate non-significant differences between relative expression levels of different groups of samples.

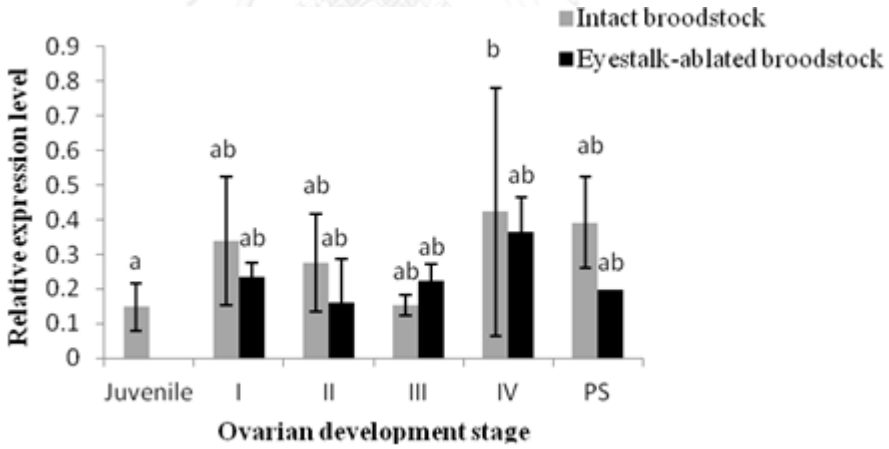


Figure 3.43 Histograms showing relative expression levels of *PmcAMP-RPL* during ovarian development of intact broodstock and unilateral eyestalk-ablated of *P. monodon* broodstock. The same letters indicate non-significant differences between relative expression levels of different groups of samples.

3.5.4 *Protein kinase C*

Result from quantitative real-time PCR indicated that the expression level of *PmPKC* in intact broodstock was significantly greater than that of juveniles ($P < 0.05$). Its expression level in different developmental stages in wild intact broodstock was comparable ($P > 0.05$). In eyestalk-ablated broodstock, the expression level of *PmPKC* was also not differentially expressed ($P > 0.05$). Eyestalk ablation resulted in significant reduction of *PmPKC* in stages I-IV ovaries ($P < 0.05$) (Figure 3.44).

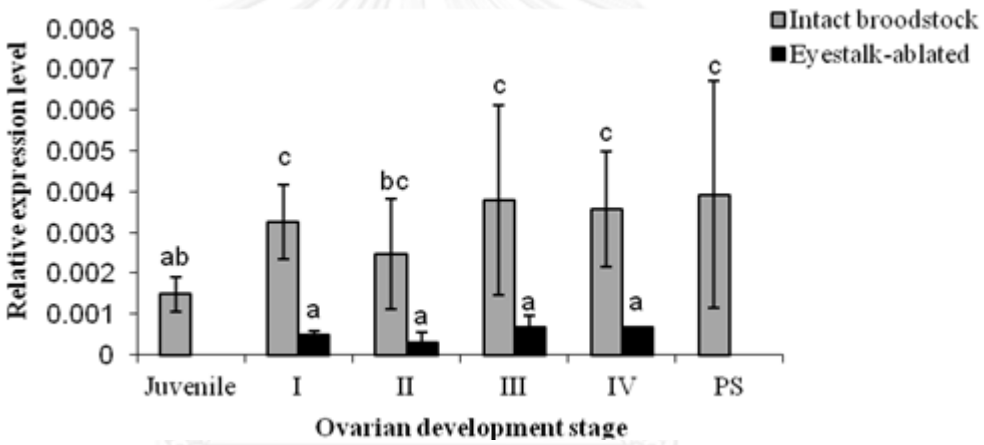


Figure 3.44 Histograms showing relative expression levels of *PmPKC* during ovarian development of intact broodstock and unilateral eyestalk-ablated of *P. monodon* broodstock. The same letters indicate non-significant differences between relative expression levels of different groups of samples.

3.5.5 *PmRacgap1*

The expression level of *PmRacgap1* in intact broodstock was significantly greater than that of juveniles ($P < 0.05$). Its expression was not significantly altered during ovarian development in intact broodstock ($P > 0.05$). The expression level of *PmRacgap1* I in eyestalk-ablated shrimp seemed to be lower than that in intact

broodstock but results were not statistically significant due to large standard deviations between groups of samples ($P > 0.05$, Figure 3.45).

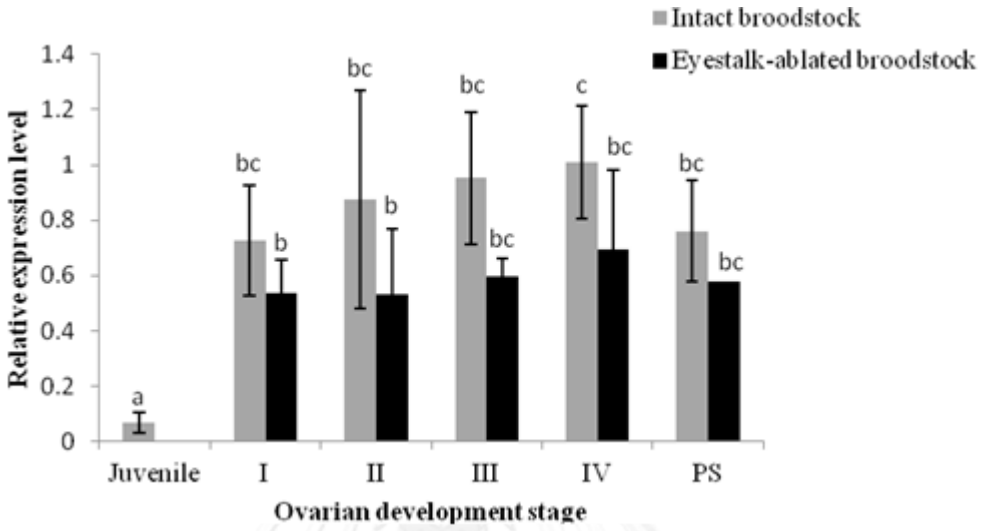


Figure 3.45 Histograms showing relative expression levels of *PmRacgap1* during ovarian development of intact broodstock and unilateral eyestalk-ablated of *P. monodon* broodstock. The same letters indicate non-significant differences between relative expression levels of different groups of samples.

3.6 Effects of progesterone and 5-HT administration on transcription of reproduction-related genes in ovaries of *P. monodon*

PmVCP, *PmcAMP-RPL*, *PmPKC, PmTsb* and *PmRacgap1* are involved in ovarian development and/or signal transduction pathway in ovaries of shrimp. To verify the regulatory effects of neurotransmitters and steroid hormones on expression of these genes, various groups of domesticated shrimp samples were treated with progreterone and 5-HT. The gene expression analysis was carried out

3.6.1 Effect of progrsterone administration on transcription of *PmVCP* in ovaries of domesticated 14-month-old broodstock

The effect of progesterone on expression of *PmVCP* in ovaries of 14-month-old of *P. monodon* was examined. The expression level of *PmVCP* in ovaries of 14-month-old shrimp was not significantly altered following progesterone injection ($P > 0.05$) (Figure 3.46).

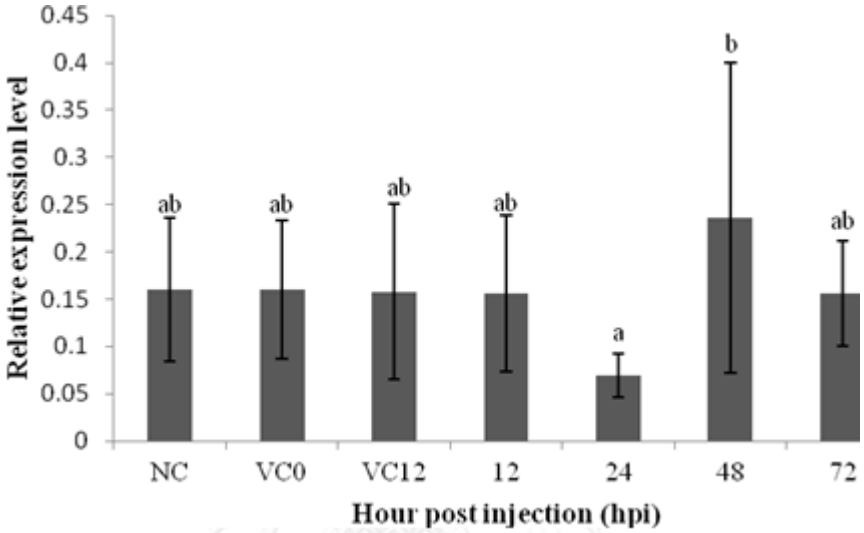


Figure 3.46 Time-course relative expression levels of *PmVCP* in ovaries of domesticated 14-month-old shrimp after progesterone injection (1 µg/g body weight) at 12, 24, 48 and 72 hours post injection (hpt; $N = 4$ for each stage). Shrimp injected with absolute ethanol at 0 hpi were included as the vehicle control.

3.6.2 Effects of 5-HT administration on transcription of *PmVCP*, *PmcAMP-RPL* and *PmPKC* in ovaries of domesticated 18-month-old broodstock

The effects of 5-HT on expression of *PmVCP*, *PmcAMP-RPL* and *PmPKC* in ovaries of 18-month-old *P. monodon* were examined. Exogenous 5-HT injection resulted in a rapid increase of ovarian *PmVCP* mRNA of 18-month-old shrimp at 6 and 24 h post-injection (hpi) ($P < 0.05$). Its expression level returned to non-significance level relative to the vehicle control at 48 and 72 hpi (Figure 3.47).

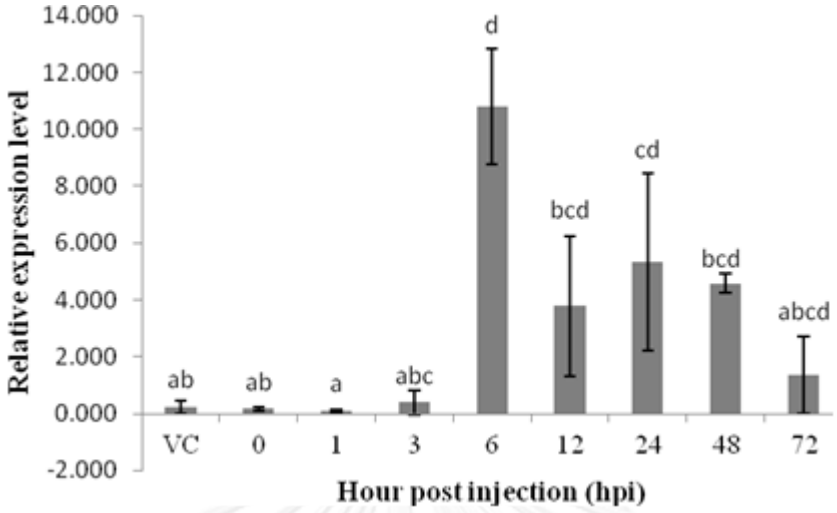


Figure 3.47 Time-course relative expression levels of *PmVCP* in ovaries of 18-month-old shrimp after serotonin injection (50 µg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hours post injection (hpt; *N* = 4 for each stage). Shrimp injected with 0.85% saline solution at 0 hpi were included as the vehicle control.

In contrast, 5-HT administration resulted in significant increasing of ovarian *PmPKC* expression for approximately 50-fold at 6 hpi ($P < 0.05$). Subsequently, its expression level was reduced but still significantly greater than that of the vehicle control at 12-48 hpi ($P < 0.05$) (Figure 3.48).

In addition, the expression level of *PmcAMP-RPL* was significantly increased at 6 h following the injection of 5-HT ($P < 0.05$). Its expression level peaked at 12 hpi ($P < 0.05$) and the expression level was reduced but still significantly greater than that of the vehicle control at 24-48 hpi ($P < 0.05$) (Figure 3.49).

In addition, exogenous 5-HT administration resulted in significant increasing of ovarian *PmRacgap1* expression for approximately 250-fold at 6 hpi ($P < 0.05$). Its

expression level was reduced but still significantly greater than that of the vehicle control for 50 fold at 12-48 hpi ($P < 0.05$) (Figure 3.50).

Unlike other transcripts, exogenous 5-HT injection resulted in a lower expression level of *PmTmsb* relative to the vehicle control at 6-72 hpi ($P < 0.05$) (Figure 3.51)

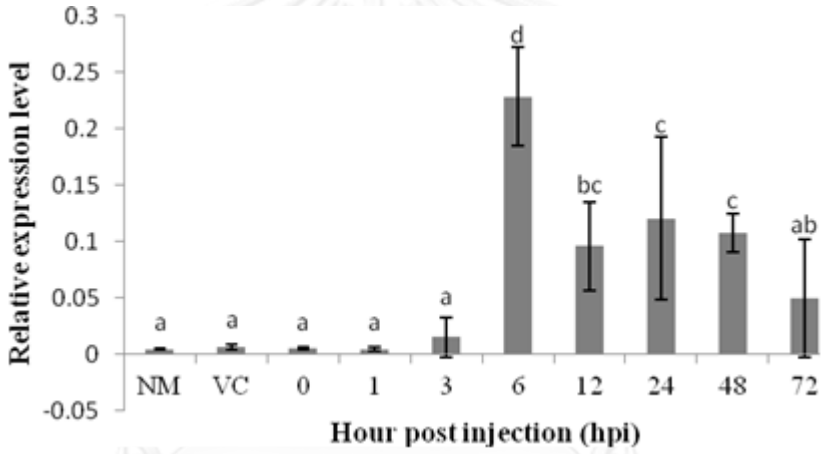


Figure 3.48 Time-course relative expression levels of *PmPKC* in ovaries of 18-month-old after serotonin injection (50 µg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hours post injection (hpt; $N = 4$ for each stage). Shrimp injected with 0.85% saline solution at 0 hpi were included as the vehicle control.

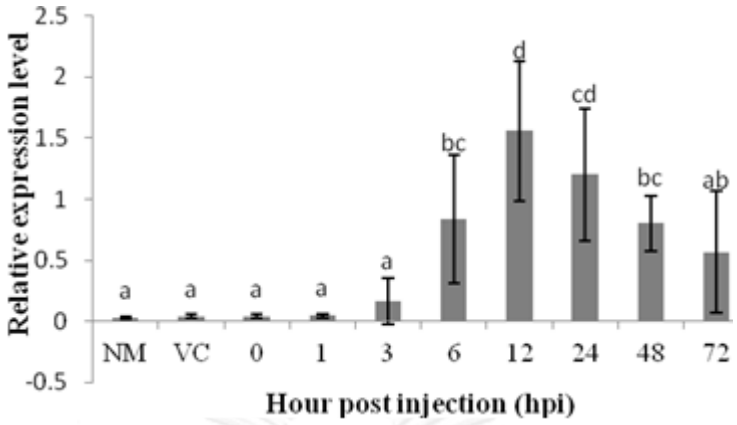


Figure 3. 49 Time-course relative expression levels of *PmcAMP-RPL* in ovaries of 18-month-old shrimp after serotonin injection (50 µg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hours post injection (hpt; *N* = 4 for each stage). Shrimp injected with 0.85% saline solution at 0 hpi were included as the vehicle control.

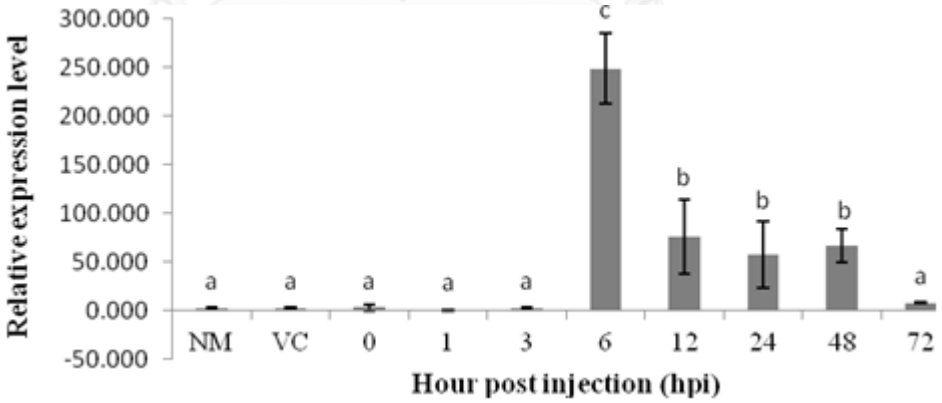


Figure 3.50 Time-course relative expression levels of *PmRacgap1* in ovaries of 18-month-old shrimp after serotonin injection (50 µg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hours post injection (hpt; *N* = 3 for each stage). Shrimp injected with 0.85% saline solution at 0 hpi were included as the vehicle control.

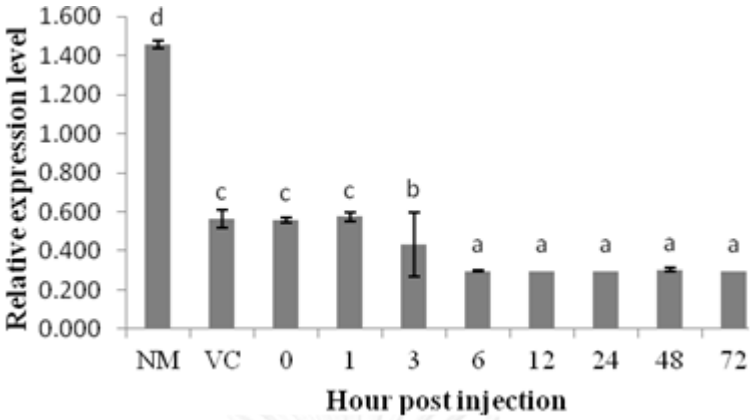


Figure 3.51 Time-course relative expression levels of *PmTmsb* in ovaries of 18-month-old after serotonin injection (50 µg/g body weight) at 1, 2, 3, 6, 12, 24, 48 and 72 hours post injection (hpt; *N* = 4 for each stage). Shrimp injected with 0.85% saline solution at 0 hpi were included as the vehicle control.

3.7 Localization of *VCP* genes in ovaries of *P. monodon* broodstock

3.7.1. Quantification of the cRNA probe

The sense and antisense cRNA probes were synthesized from an insert (600 bp) of the recombinant plasmid of *PmVCP* (Figure. 3.52A). The antisense and sense probes gave the positive signal at approximately 1 ng/µl. However, the amount of both cRNA probes of *PmVCP* was higher than 1 ng/µl (Figure 3.52B). An appropriate amount of the cRNA probe of each transcript was applied for examination of transcriptional localization using *in situ* hybridization.

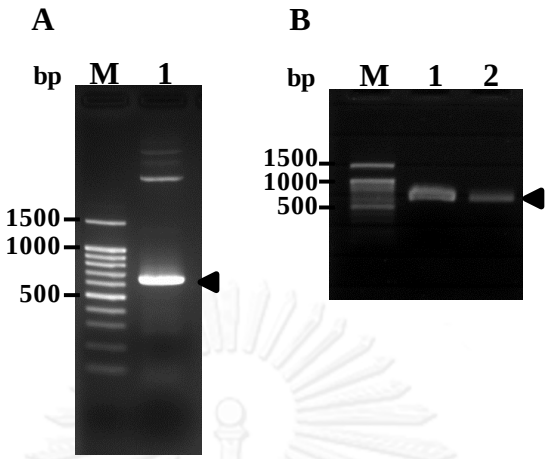


Figure 3.52 (A) The digested plasmid was used as the template for synthesis of the cRNA probe of *PmVCP* (lanes 1-3). (B) The antisense (lane 1) and sense (lane 2) were synthesized from the gel-eluted digested plasmid template. A 100 bp ladder (lanes M, A and B) was used as the DNA marker.

3.7.2 *In situ* hybridization (ISH)

Cellular localization of *PmVCP* transcripts in ovaries of *P. monodon* broodstock was determined by *in situ* hybridization. No signal was observed with the sense (control) probe (Figures 3.53A and 3.54A). The positive signal was observed when the tissue sections were hybridized with the antisense probe of *PmVCP*. The positive signals were observed in oogonia and ooplasm of previtellogenic and vitellogenic oocytes in different stages of ovaries of both intact and eyestalk-ablated broodstock (Figures. 3.53-3.54; B-E).

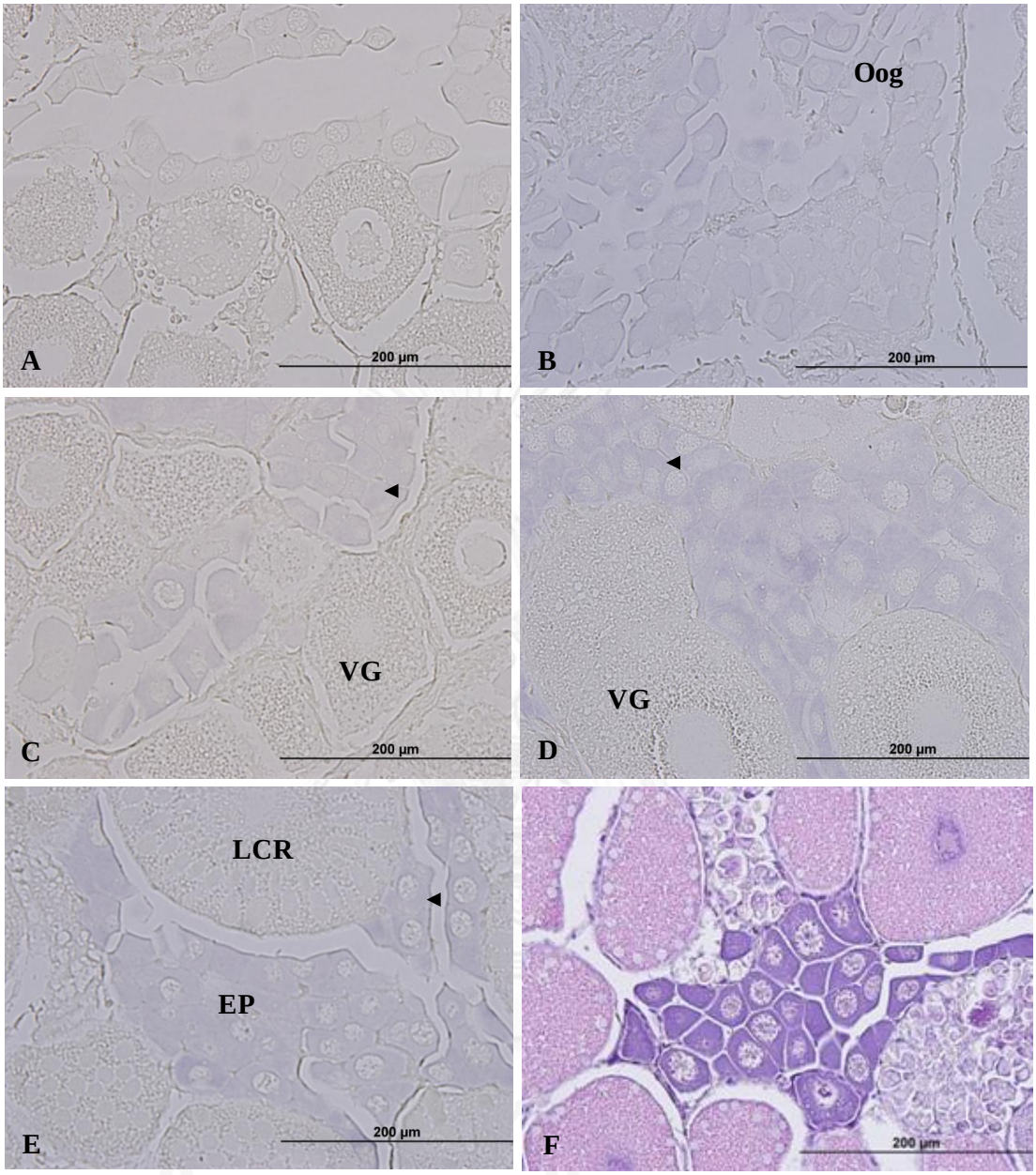


Figure 3.53 Localization of *PmVCP* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the antisense (B-E), sense (A) cRNA probes. No signal was observed from the sense cRNA probe. The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; Oog = oogonia; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte.

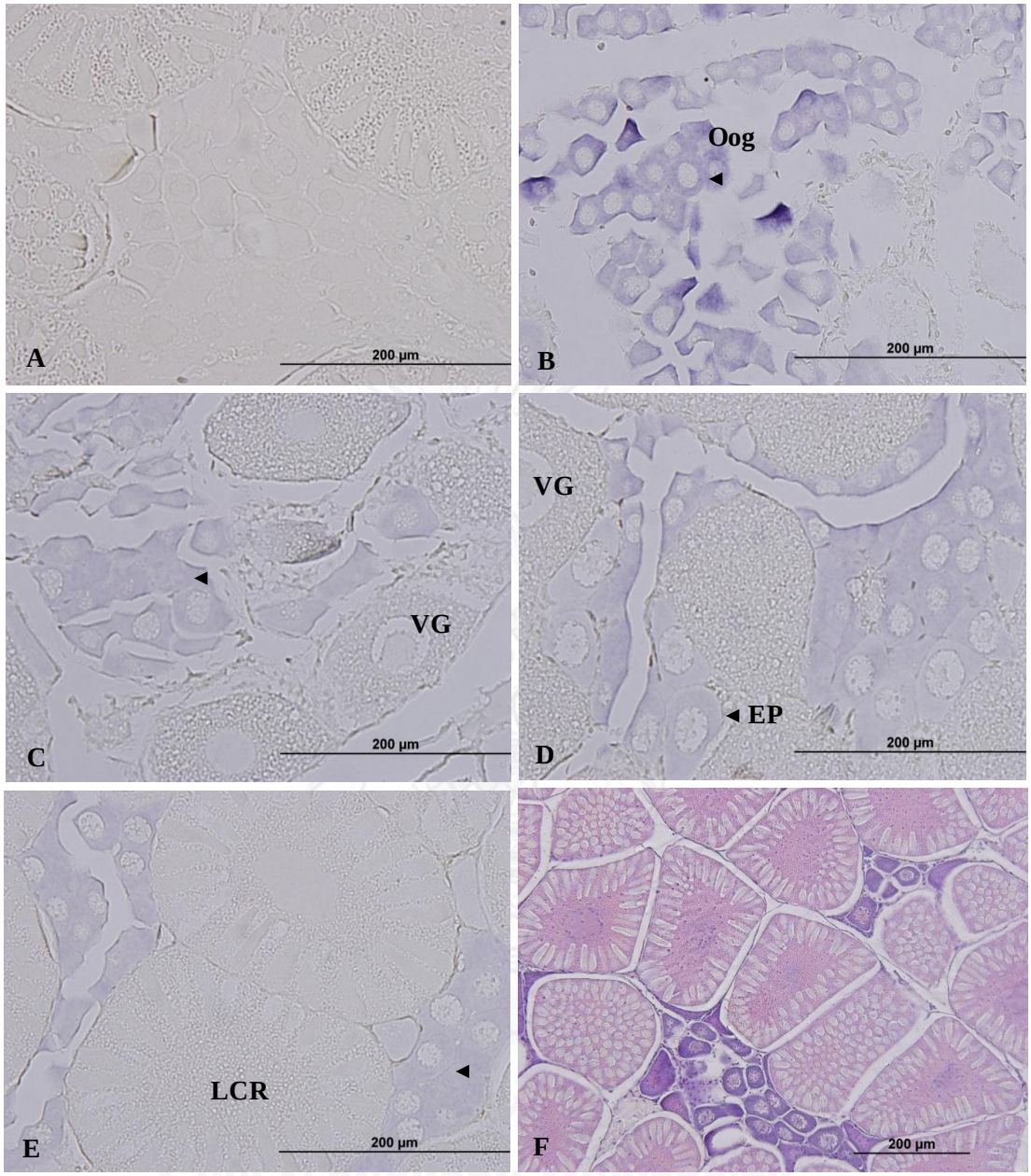


Figure 3.54 Localization of *PmVCP* transcript during ovarian development of eyestalk-ablated broodstock of *P. monodon* visualized by *in situ* hybridization using the antisense (B-E) and sense (A) cRNA probes. No signal was observed from the sense cRNA probe The conventional hematoxylin/eosin staining was carried out for identification of oocyte stages (F). EP = early previtellogenic oocytes; Oog = oogonia; LCR=late cortical rod oocytes; Vg = vitellogenic oocyte.

3.8 *In vitro* expression of reproduction-related proteins using the bacterial expression system

3.8.1 Construction of recombinant plasmids for cloning and expression of recombinant proteins

Two recombinant plasmids carrying the complete ORF of *PmRacgap1* and *PmTmsb* and three plasmids carrying the partial ORF of *PmVCP*, *PmNUP133* and *PmSema* covering Cdc48, nucleoporin_N and Sema domains, respectively, were constructed.

PmRacgap1 was initially identified from testis cDNA library and the full-length cDNA of this transcript in testes was characterized by RACE-PCR. The full-length cDNA of testis *Racgap1* was 2838 bp in length containing an ORF of 1812 bp deducing to 603 amino acids. This transcript significantly matched *Rac GTPase-activating protein 1* of *Crassostrea gigas* (*E*-value = 2e-127) (Leelatanawit, 2008). The ORF of the ovarian form was successfully amplified using primers designed from the testis form. It was 1881 bp inlength corresponding to 626 amino acids and significantly matched *Rac GTPase-activating protein 1* of *Crassostrea gigas* : E-value = 6e-117). The deduced amino acids of both isoform of *PmRacgap1* were aligned (Figures 3.55).

```
ORF-TT          MESLSAQFDDLMRQMQVLADPAEYKFLEFLDHEEKNRVQLRELEAEVSRLNEQAARYQKE
ORF-OV          MESLSAQFDDLMRQMQVLADPAEYKFLEFLDHEEKNRVQLRELEAEVSRLNEQAARYQKE
                ************************************************************
ORF-TT          IKSLEMKLKNAKHMLDVEKAKRITTEKEKNDLAGQIGLVMELLGRGQVNETRERLQQLQH
ORF-OV          IKSLEMKLKNAKHMLDVEKAKRITTEKEKNDLAGQIGLVMELLGRGQVNETRERLQQLQH
                ************************************************************
ORF-TT          SFTFSGTVTNQRRSTRDLSPGPLSTITEDNDTMGSILSVSDIDITEDDLEESRLRSGRSF
ORF-OV          SFTFSGTATNQRRSTRDLSPGPLSTITEDNDTMGSILSVSDIDITEDDLEESRLRSGRSF
                *******.****************************************************
ORF-TT          KRRSSPERQDSSKGKRRSGRRSEDMQTHEVKTQVTYYTQGEEIKKIHTETKVKPSAPPLS
ORF-OV          KRRSSPERQDSSKGKRRSGRRSEDMQTHEVKTQVTYYTQGDEIKKIHTETKVKPSAPPLS
                ****************************************:*******************
ORF-TT          TDEETEVSHLKKPTHGHTLNTPSTPHIPQTAYSPHFPNPITPQKQGTGQMYYTPTHNLVT
ORF-OV          TDEETEVSHLKKPTHGHTLNTPSTPHIPQTAYSPHFPNPITP--QGTGQMYYTPTHNLVT
                ************************************  ****************
ORF-TT          PVLRTHSSVTKINQRPHAFYTKTIYKTEHCQPCGKRIKFGKIALKCRDCRATCHP----E
ORF-OV          PVLRTHSSVTKINQRPHAFYTKTIYKTEHCQPCGKRIKFGKIALKCRDCRATCHLSVVNL
                ************************************************  
ORF-TT          CRESVPLPCVP--------------------TALVVHCTNEVENRGLSEVGIYRVPGAE
ORF-OV          CRFLVFLQLTLQLQRGNWEPLLTTHLVCPQWFQPLVVHCTNEVENRGLSEVGIYRVPGAE
                **  * *   .                    .*************************
ORF-TT          KDVKELKDQFLRGKGMPNLSQLDIHVVCGALKDFMRSLKEPLVTHLLWRDFTSAAEKSEA
ORF-OV          KDVKELKDQFLRGKGMPNLSQLDIHVVCGALKDFMRSLKEPLVTHLLWRDFTSAAEKSEA
                ************************************************************
ORF-TT          QDYLAALYQAISELPQPNRDTLAWIMTHLQRVAECPECKMPASNLAKVFGPTLVGYSVPE
ORF-OV          QDYLAALYQAISELPQPNRDTLAWIMTHLQRVAECPECKMPASNLAKVFGPTLVGYSVPE
                ************************************************************
ORF-TT          PDPATMLTETRQQQMVMEKLLEISTDYWNTFINVTDENVHQGVQQVPTLEGGTLLGGFPS
ORF-OV          PDPATMLTETRQQQMVMEKLLEISTDYWNTFINVTDENVHQGVQQVPTLEGGTLLGGFPS
                ************************************************************
ORF-TT          SNTRRRSILTRTPLTPRETPKNRYVFRK
ORF-OV          SNTRRRSILTRTPLTPRETPKNRYVFRK
                ****************************
```

Figure 3.55 Alignments of deduced amino acid sequences of *Racgap1* from testes (ORF-TT; Leelatanawit et al., 2008) and ovaries (ORF-OV) of *P. monodon.*

Primers overhang with appropriate restriction enzymes were designed to amplified the complete ORF of was designed to amplify cDNA of *PmRacgap1* and *PmTmsb* and *Cdc48* of *PmVCP*, *nucleoporin_N* of *PmNUP133* and *Sema* domains of *Pmsema*, *PmVCP* (called PmCdc48-VCP plasmid), *PmNUP133* (called PmNPC-NUP133) and *PmSema* (PmSema plasmid). The amplified fragment was ligated, cloned into pGEM-T easy vector and transformed into *E. coli* JM109. Plasmid DNA of the positive clone was re-sequenced to confirm the orientation and nucleotide sequence of a particular recombinant clone. Recombinant plasmid in the cloning vector was used as the template for amplification of the corresponding fragment of each transcript.

The amplification product of *PmRacgap1*, *PmTmsb* and *PmSema* were digested with *Nde* I and *BamH*I and ligated into pET17b while *PmCdc48-VCP* was digested with *EcoR* I and *BamH* I and ligated to pET29a. The *PmNPC-NUP133* fragment was digested with *Nde* I and *EcoR* I ligated to pET15b**.** All recombinant plasmids were transformed into *E. coli* BL21C+(DE3)RIPL.

3.8.2 *In vitro* expression of recombinant proteins

In vitro expression of five recombinants of PmRacgap1 (72 kDa), rPmTmsb (15.3 kDa), rPmCdc48-VCP (24 kDa), rPmNPC-NUP133 (41.37 kDa) and rPmSema (38.3 kDa) were carried out. After IPTG induction, the recombinant proteins were examined by SDS-PAGE and western blot analysis. In addition, soluble and insoluble protein fractions were also determined.

The rPmcdc48-VCP (containing cdc48_N and cdc48_2 domains) was expressed after induction by IPTG for 3 hr. The expected protein band of rPmCdc48-VCP (24 kDa) was observed when total proteins were incubated with Anti-6XHis tag. The expression level of rPmCdc48-VCP after indication for 6 hr was greater than that after IPTG induction for 3 hr (Figure 3.56). The rPmCdc48-VCP was expressed in both soluble and insoluble form when the recombinant clone was cultured at 15℃ for overnight. However, the expression in the inclusion bodies was greater than that in the soluble fraction (Figure. 3.57).

A recombinant clone of PmNPC-NUP133 was induced by IPTG for 0, 1, 2, 3, 6, 12 and 24 hr. The expected protein bands at 41.37 kDa of rPmNPC-NUP133 were observed at 1-3 hr post induction. The expression of this recombinant protein was reduced at 6 hr post induction and the positive band was not observed at 12 and 24

hr post induction (Fig 3.58). The rPmNPC-NUP133 was solely expressed in the inclusion bodies (Figure 3.59).

The rPmSema was stably expressed during 1-24 hr post induction with IPTG (Figure 3.60). It was entirely expressed as the insoluble protein when the recombinant clone was cultured at 37°C (Fig. 3.61).

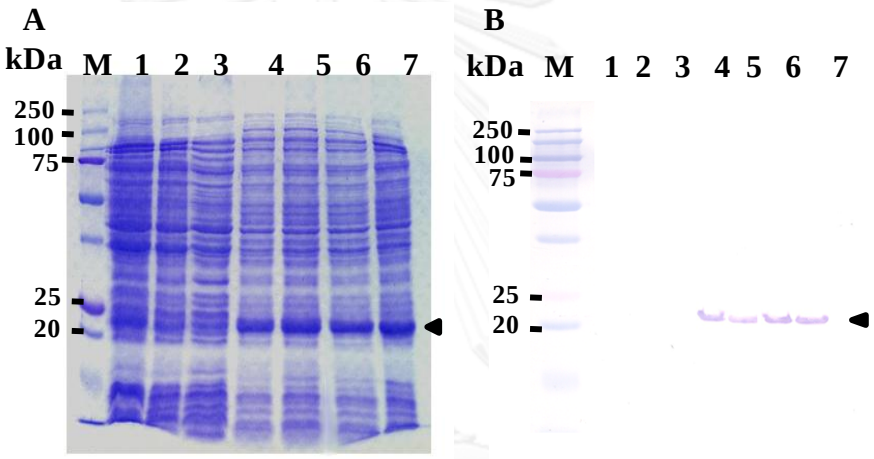


Figure 3.56 SDS-PAGE (A) and Western blot analysis (B) illustrating *in vitro* expression of two recombinant clones of PmCdc48-VCP after IPTG induction (1 mM) for 3 hr (lanes 4 and 6) and 6 hr (lanes 5 and 7). *E. coli* BL21C+(DE3)RIPL cells (lane 1), pET29a vector in *E. coli* BL21C+(DE3)RIPL cell (lane 2) and a recombinant clone at 0 hr after induction by 1 mM IPTG (lane 3) was included as the control.

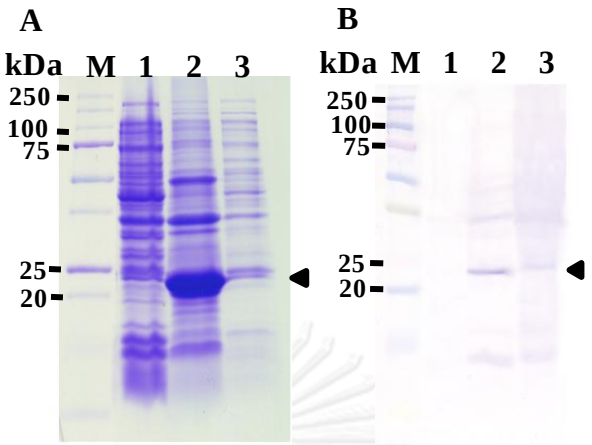


Figure 3.57 SDS-PAGE (A) and western blot analysis (B) showing expression of a recombinant clone of PmCdc48-VCP after the culture was induced by IPTG for 3 hr at 15°C. Lane 1= whole cells of *E. coli* BL21C+(DE3)RIPL, Lane 2 = an insoluble protein fraction (30 µg protein), and Lane 3 = a soluble protein fraction (30 µg protein).

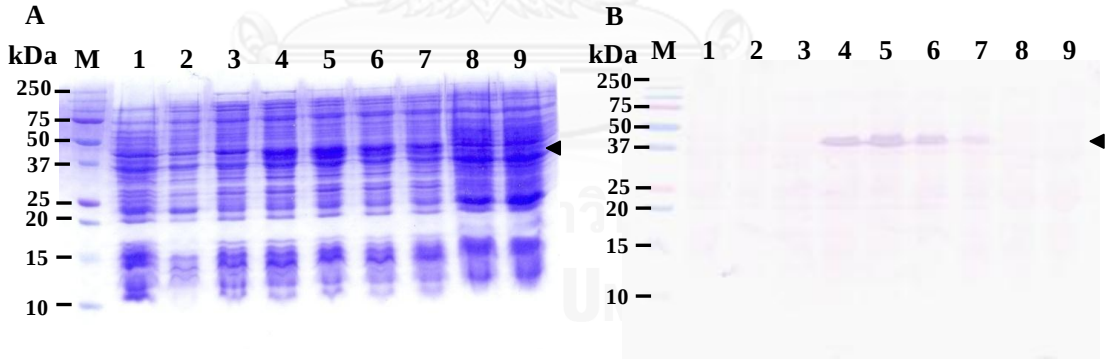


Figure 3.58 SDS-PAGE (A) and western blot analysis (B) of a recombinant clone of PmNPC-NUP133 after induction by 1 mM IPTG for 0, 1, 2, 3, 6, 12 and 24 hr (lanes 3-9). *E. coli* BL21C+(DE3)RIPL cells (lane 1) and pET15b in *E. coli* BL21C+(DE3)RIPL (lane 2) were included as the negative controls.

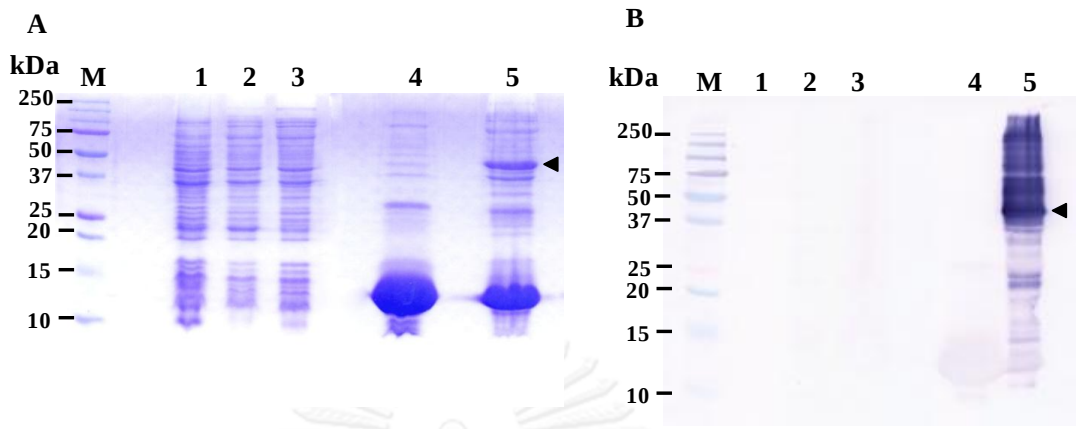


Figure 3.59 SDS-PAGE (A) and western blot analysis (B) showing expression of a recombinant clone of PmNPC-NUP133 after IPTG induction (1 mM) for 3 hr at 37°C. Lane 1 = *E. coli* BL21C+(DE3)RIPL. Lane 2 = pET15b in *E. coli* BL21C+ (DE3)RIPL. Lane 3 = whole cells after IPTG induction for 0 hr, lane 4 = soluble fraction (30 µg proteins) and lane 5 = inclusion bodies (30 µg proteins).

The expected protein band of 72 kDa was observed at 1-12 hr post induction with IPTG. However, the expression of rPmRacgap1 was quite low and it was not expressed after IPTG induction overnight (Figure 3.62). A protein band with a lower molecular weight of approximately 22 kDa was observed at 12 and 24 hr post induction. This suggested that rPmRacgap1 was not stable in the long culture period. Western blot analysis indicated that rPmRacgap1 was expressed in the insoluble form (Figure 3.63).

Like PmRacgap1, PmTmsb (15.3 kDa) was expressed at 1-12 hr post induction with IPTG. However, it was not expressed after induction with IPTG overnight (Figure 3.64). Western blot analysis indicated that rPmTmsb was expressed in the insoluble form (Figure 3.65).

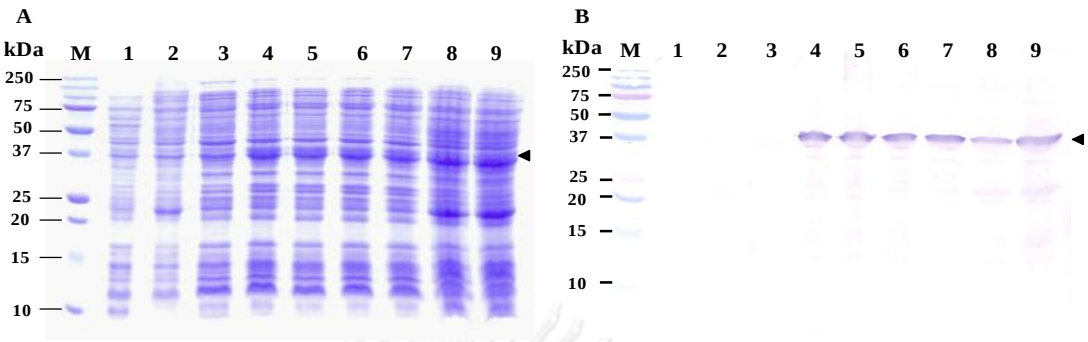


Figure 3.60 SDS-PAGE (A) and western blot analysis (B) of a recombinant clone of PmSema after induction by 1 mM IPTG for 0, 1, 2, 3, 6, 12 and 24 hr (lanes 3-9). *E. coli* BL21C+(DE3)RIPL (lane 1) and pET17b in *E. coli* BL21C+(DE3)RIPL (lane 2) were included as the negative controls.

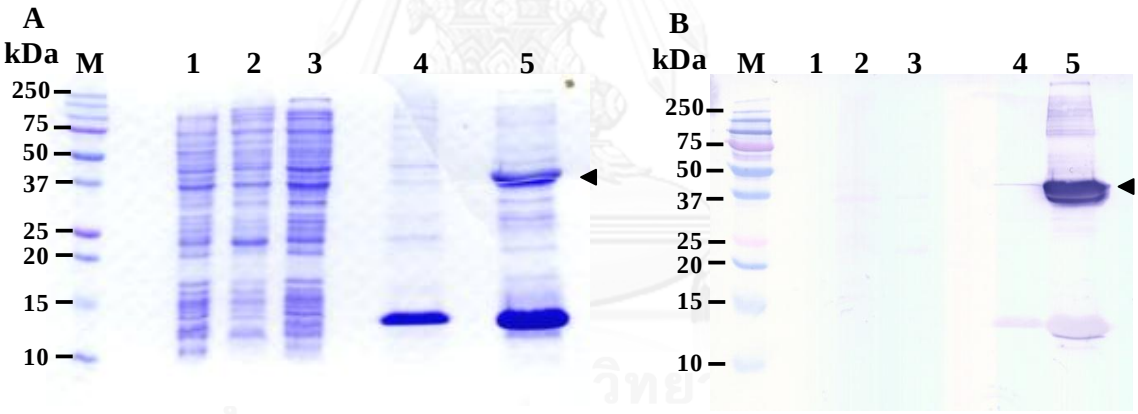


Figure 3.61 SDS-PAGE (A) and western blot analysis (B) showing expression of a recombinant clone of Pmsema after induction by 1 mM IPTG for 3 hr at 37°C. Lane 1 = *E. coli* BL21C+(DE3)RIPL. Lane 2 = pET17b in *E. coli* BL21C+(DE3)RIPL. Lane 3 = whole cell at 0 hr after induction with IPTG, lane 4 = soluble fraction (30 µg protein) and lane 5 = inclusion bodies (30 µg protein).

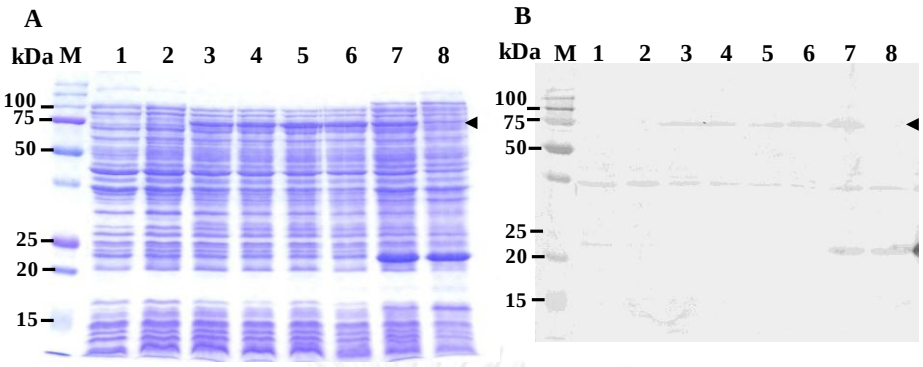


Figure 3.62 SDS-PAGE (A) and western blot analysis (B) of a recombinant clone of PmRacgap1 after induction by 1 mM IPTG for 0, 1, 2, 3, 6, 12 and 24 hr (lanes 3-8). *E. coli* BL21C+(DE3)RIPL (lane 1) was included the negative controls.

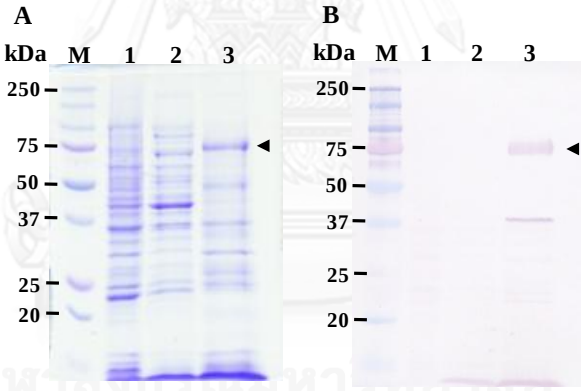


Figure 3.63 SDS-PAGE (A) and western blot analysis (B) showing expression of PmRacgap1 after a recombinant cloned was induced y IPTG (1 mM) for 3 hr at 37°C. Lane 1= *E. coli* BL21C+(DE3)RIPL. lane 2 = soluble fraction (30 µg protein) and lane 3 = inclusion bodies (30 µg protein).

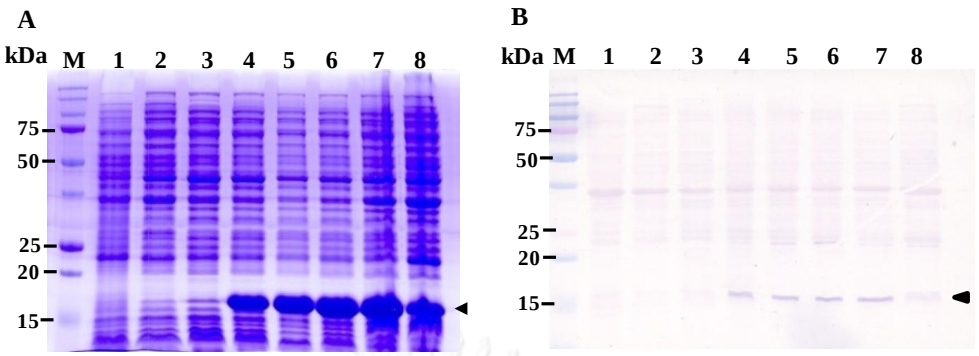


Figure 3.64 SDS-PAGE (A) and western blot analysis (B) of a recombinant clone of PmTmsb after induction by 1 mM IPTG for 0, 1, 2, 3, 6, and 12 (lanes 3-8). *E. coli* BL21C+(DE3)RIPL (lane 1) and pET17b in *E. coli* BL21C+(DE3)RIPL cell (lane 2) were included as the negative controls.

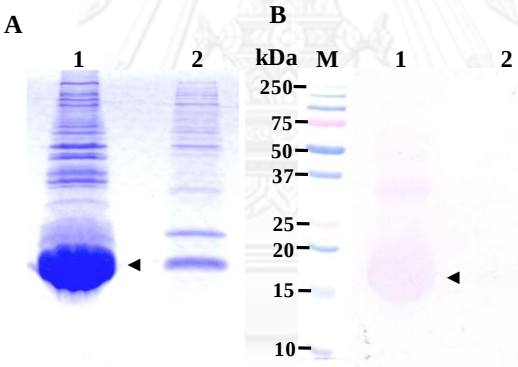


Figure 3.65 SDS-PAGE (A) and western blot analysis (B) showing expression of rPmTmsb after a recombinant PmTmsb was induced by 1 mM IPTG for 3 hr at 37°C. Lane 1 = soluble fraction (30 µg protein) and lane 3 = inclusion bodies (30 µg protein).

3.8.3 Purification of rPmTmsb, rPmCdc48-VCP, rPmRacgap1, rPmNPC-NUP133 and rPmSema

Recombinant proteins produced were further purified before used as the immunogens for the production of polyclonal antibodies. The soluble fractions of rPmTmsb, rPmCdc48-VCP proteins were purified under the non-denaturing conditions whereas rPmRacgap1, rPmNPC-NUP133 and rPmSema which were expressed as insoluble proteins were purified under the denaturing conditions. Washed and eluted fractions were analyzed by SDS-PAGE and western blot (Fig 3.66-3.70). After purification, eluted proteins were keeping at -20°C.

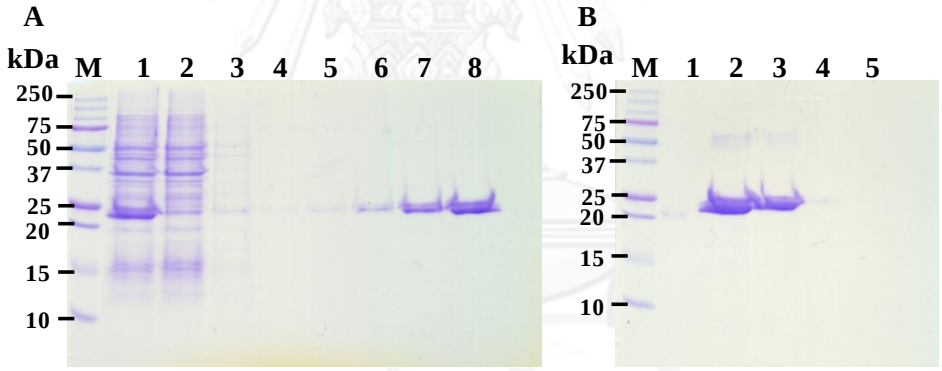


Figure 3.66 Purification of rPmCdc48-VCP (cultured at 15°C, overnight). Recombinant protein was examined by using SDS-PAGE (A-B). (A) lane 1 = the soluble protein fraction before pass through the column, lane 2 = the soluble protein fraction after pass through the column, lanes 3-4 = the first wash (40 mM imidazole) fractions 9 and 10, lanes 5-6 = the second wash (80 mM imidazole) fractions 9 and 10 and lanes 7-8 = the third wash (150 mM imidazole) fractions 3 and 5 respectively. (B): lanes 1-5 = eluted protein fractions 1-5, respectively.

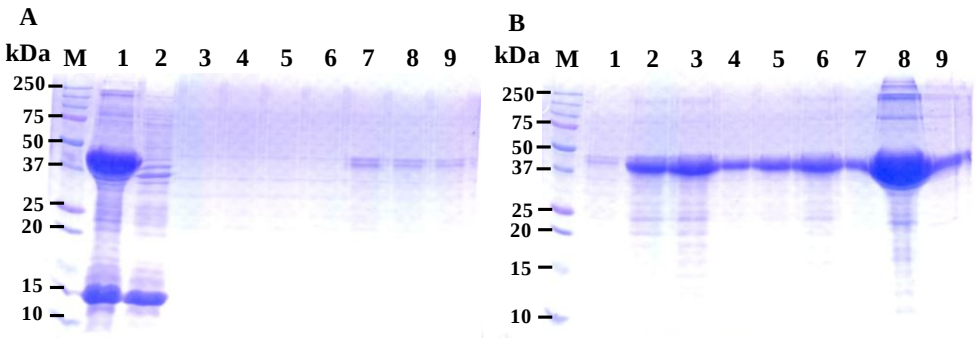


Figure 3.67 Purification of rPmNUP133 (cultured at 37°C, 3 hr). Recombinant protein was examined using SDS-PAGE (A-B). (A) lane 1 = the insoluble fraction before pass through the column, lane 2 = the insoluble fraction after pass through the column, lanes 3-6 = the first wash (20 mM imidazole) fractions 7, 8, 9 and 10, lanes 7-9 = the second wash (40 mM imidazole) fractions 8, 9 and 10 respectively. (B): lanes 1-3 = the third wash (80 mM imidazole) fractions 1, 3 and 5, lanes 4-6 = the last wash (150 mM imidazole) fractions 1, 3 and 5, lanes 7-9 = eluted protein fractions 1-3, respectively.

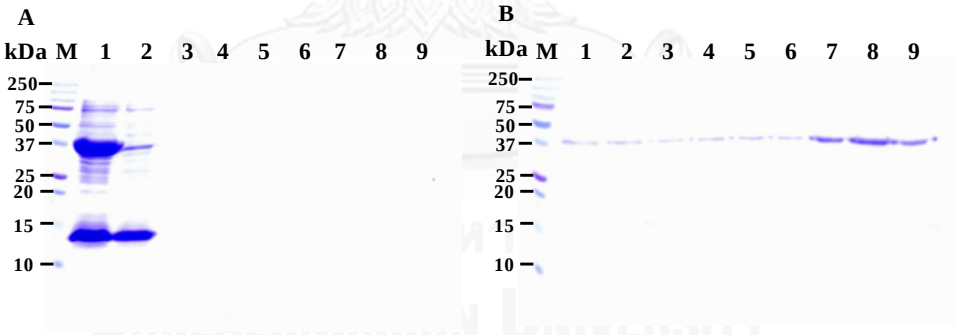


Figure 3.68 Purification of rPmSema (cultured at 37°C, 3 hr). Recombinant protein was examined using SDS-PAGE (A-B). (A) lane 1 = the insoluble fraction before pass through the column, lane 2 = the insoluble fraction after pass through the column, lanes 3-5 = the first wash (20 mM imidazole) fractions 8, 9 and 10, lanes 6-8 = the second wash (40 mM imidazole) fractions 8, 9 and 10. (A) lane 9 and (B) lanes 1-2 = the third wash (80 mM imidazole) fractions 1, 3 and 5, lanes 3-5 = the last wash (150 mM imidazole) fraction 1, 3 and 5, lanes 6-9 = eluted protein fractions 1-4, respectively.

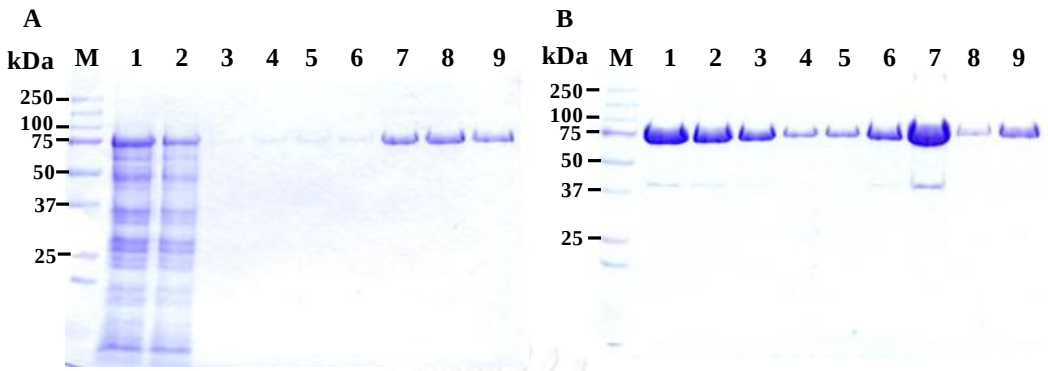


Figure 3.69 Purification of rPmRacgap1 (cultured at 37°C, 3 hr). Recombinant protein was examined using SDS-PAGE (A-B). (A) Lane 1 = the insoluble fraction before pass through the column, lane 2 = the soluble fraction after pass through the column, lanes 3-6 = the first wash (20 mM imidazole) fractions 7, 8, 9 and 10, lanes 7-9 = the second wash (40 mM imidazole) fractions 8, 9 and 10. (B) Lanes 1-3 = the third wash (80 mM imidazole) fractions 1, 3 and 5, lanes 4-6 = the last wash (150 mM imidazole) fraction 1, 3 and 5; lanes 7-9 = eluted protein fractions 1-3, respectively.

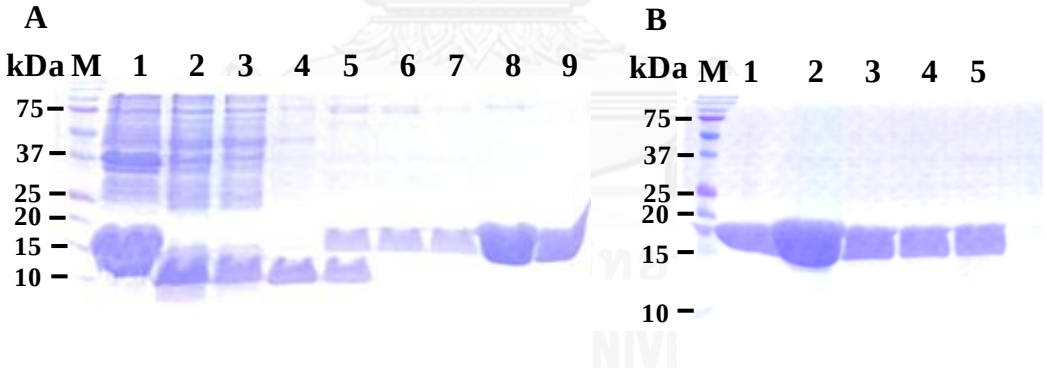


Figure 3.70 Purification of rPmTmsb (cultured at 37°C, 3 hr). Recombinant protein was examined using SDS-PAGE (A-B). (A) Lane 1 = the soluble fraction before pass through the column, lane 2 = the soluble fraction after pass through the column, lanes 3-5 = the first wash (40 mM imidazole) fractions 8, 9 and 10, lanes 6-7 = the second wash (80 mM imidazole) fractions 9 and 10 and lanes 8-9 = the third wash (150 mM imidazole) fractions 3 and 5, respectively. (B): lanes 1-5 = eluted protein fractions 1-5, respectively.

The eluted fractions of rPmCdc48-VCP, rPmNPC-NUP133, rPmSema, PmRacgap1 and PmTmsb proteins were poled and concentrated (*in vacuo* and ultrafiltration) (Figure 3.71) and subjected to the polyclonal production by Faculty of Medical Technology, Chiang Mai University.

Anti-rPmCdc48-VCP, anti-NPC-NUP133, PmSema, PmRacgap1 and PmTmsb polyclonal antibodies were successfully produced with a relatively high titer (1:32,000 with OD450 = 0.192, 0.653, 1.015, 0.163 and 0.294 against 1 μg of purified respective recombinant proteins) (Table 3.10).

3.9 Sensitivity and specificity of anti-rPmCdc48-VCP, PmrRacgap1 and rPmTmsb

The detection sensitivity for the produced antibody was tested. The positive reactions were observed with 0.1, 0.2 and 0.5 μg of rPmCdc48-VCP (Figure. 3.72A). In subsequent specificity tests with anti-rPmCdc48-VCP PAb, 0.2 μg of rPmCdc48-VCP was used. Anti-rPmCdc48-VCP PAb gave positive immunoreactive signals with the target but did not cross-react with non-target proteins including rPmCdc2 which contained an S_TKc domain, rPmRuvBL2 which contained an AAA domain, rPmRacGAP1 which contained a C1 domain, rPmCdc25 which contained a rhodanase domain, rPmDRK which contained the Src homolog domains SH2 and SH3, and rPmTHY-β which contained THY domains (Figure. 3.72B).

In addition, both 24 and 97 kDa bands were found when 0.2 μg of rPmCdc48-VCP was mixed with varying concentrations of total ovarian proteins (2.5, 5, 10 and 20 μg) and probed with purified anti-rPmCdc48-VCP PAb (Figure 3.72C).

Antigen-antibody competition assay was carried out to examine the specificity of the raised antibody and to determine whether positively reactive bands generated

from each antibody are the target protein. Importantly, a 97 kDa band reacted specifically with the purified antibody when different concentrations of rPmCdc48-VCP were used to compete with the antibody before probing total ovarian proteins. The positive immmunoreactive band was observed from 2.5, 5, 10 and 20 μg total ovarian proteins whether or not the purified antibody was used in competition with 1 μg rPmCdc48-VCP. Increasing competition from rPmCdc48-VCP to 2.5 μg resulted in the disappearance of the positive band in 2.5 μg total ovarian proteins. Only a faint positive band was observed with 10 and 20 μg ovarian proteins when the purified antibody was used in competition with 5 μg of rPmCdc48-VCP (Figure 3.73).

Table 3.10 Titers of polyclonal antibodies after rabbits were administrated by rCdc48-VCP, rNUP133, PmSema, PmRacgap1 and PmTmsb

Polyclonal antibody						
	rPmCdc48-VCP		PmNPC-NUP133		PmSema	
Dilution of serum	Pre-immunized serum	Immunized serum	Pre-immunized serum	Immunized serum	Pre-immunized serum	Immunized serum
Dilution of serum						
1:500	0.026	2.125	0.108	3.073	-	-
1:2000	0.009	1.449	0.069	2.641	0.086	2.567
1:8000	0.004	0.595	0.06	1.556	0.071	2.036
1:32000	0.002	0.192	0.056	0.653	0.069	1.015
	PmRacgap1		**PmTmsb**			
Dilution of serum						
1:500	0.030	1.977	0.035	2.327		
1:2000	0.020	1.351	0.017	1.703		
1:8000	0.009	0.554	0.016	0.858		
1:32000	0.007	0.163	0.011	0.294		

Pre-immunised serum = serum from normal rabbit; Immunised serum = serum from rabbit injected with the recombinant protein

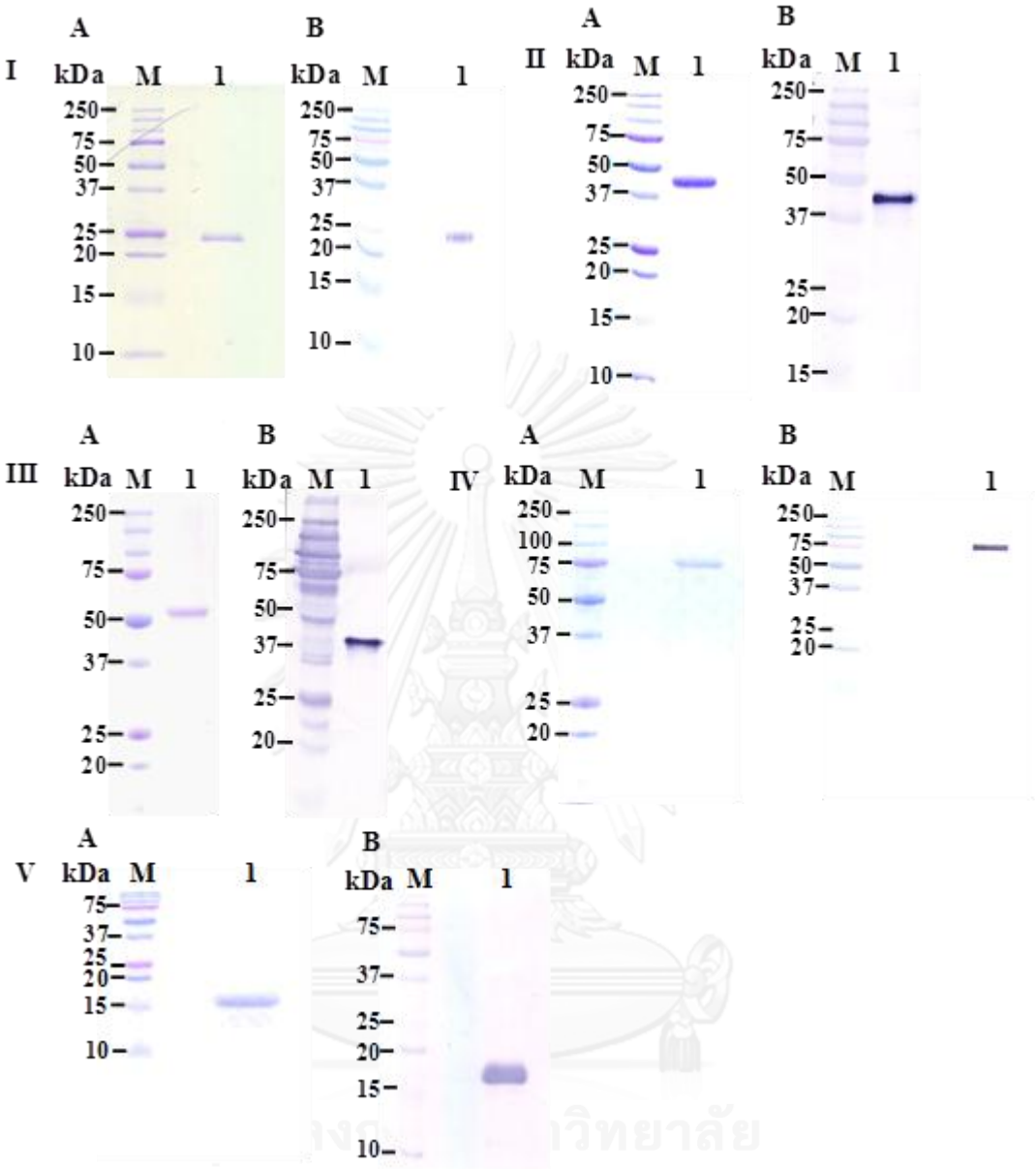


Figure 3.71 SDS-PAGE (A) and western blot analysis (B) showing electro-eluted rPmCdc48-VCP (I), rPmNUP133 (II), rPmSema (III), RacGAP 1 (IV) and rPmTmsb (V) of *P. monodon* (Figure 3.65C).

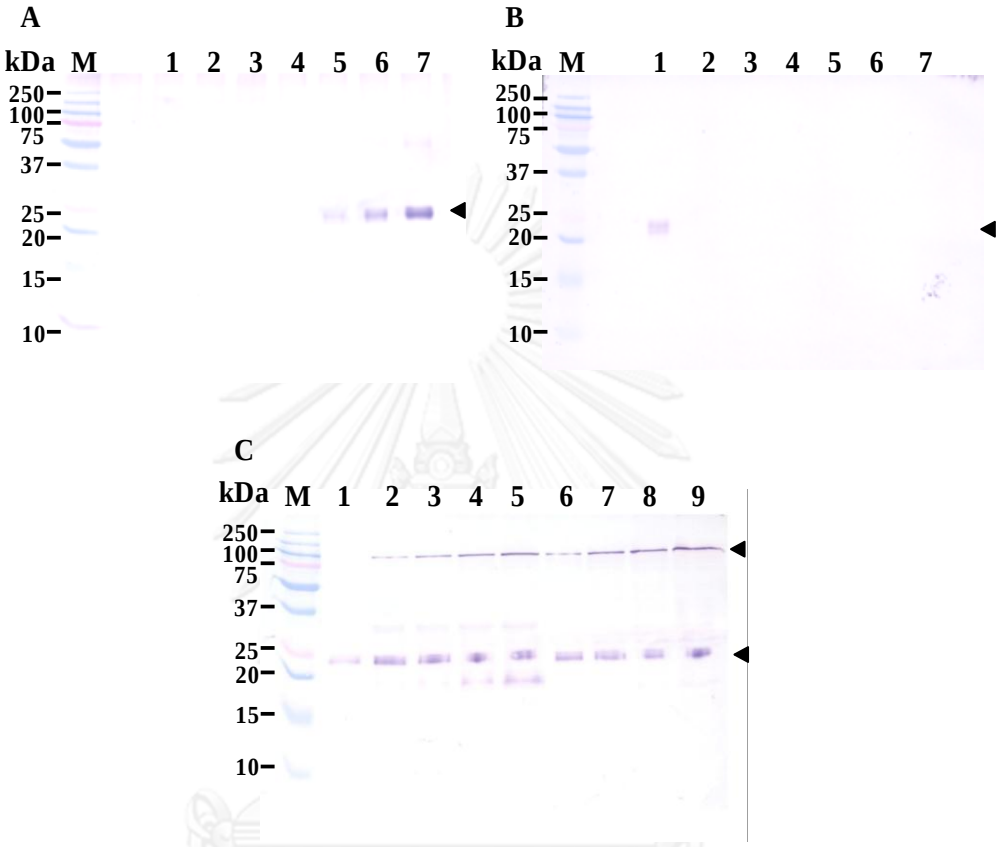


Figure 3.72 Sensitivity of anti-rPmCdc48-VCP PAb against varying amounts of rPmCdc48-VCP protein (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2 and 0.5 µg, lanes 1–8, respectively) (A) (B) Specificity of anti-rPmCdc48-VCP PAb tested against various recombinant proteins (0.2 µg) of *P. monodon* including rPmCdc48-VCP, rPmRacgap1, rPmRuvBL2, rPmCdc2, rPmCdc25, rPmDRK, and rPmTmsb (lanes 1–7, respectively), and showing binding only with rPmCdc48-VCP (lane 1). (C) Western blot analysis using anti-rPmCdc48-VCP PAb against rPmcdc48-VCP (lane 1) and varying concentrations of total ovarian proteins (2.5 µg, lanes 2 and 6; 5 µg, lanes 3 and 7; 10 µg, lanes 4 and 8 and 20 µg, lanes 5 and 9) pre-mixed with 0.2 µg of rPmCdc48-VCP.

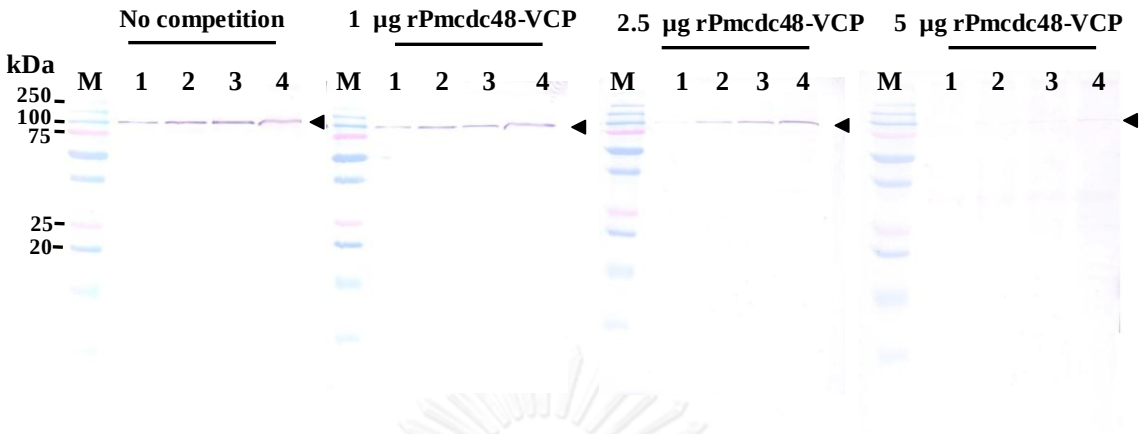


Figure 3.73 Competitive binding assays with rPmcdc48-VCP PAb. Western blot analysis of the purified anti-PmCdc48-VCP PAb (1:300) pre-mixed with 0, 1, 2.5 and 5 µg of rPmCdc48-VCP was carried out and used against 2.5, 5, 10 and 20 µg total ovarian proteins (lanes 1-4, respectively) of a female shrimp with vitellogenic ovaries (stage II) and showing complete competitive blocking at 5 µg rPmCdc48-VCP.

The detection sensitivity of the produced anti-rPmRacgap1 PAb was tested and positive reaction was observed with 0.5 and 1 µg of rPmRacgap1 (Figure. 3.74A). In the competitive binding assay, the positive immmunoreactive band of 34 kDa was observed from 2.5-20 µg ovarian membrane proteins while that of 100 kDa was observed from 5-20 µg ovarian membrane proteins when no competition of the purified antibody was carried out. In competition with 1 and 2.5 µg rPmRacGAP1, the 34 kDa band was still observed from 2.5-20 µg ovarian membrane proteins. However, the 100 kDa band was found from 5-20 µg and 10-20 µg, respectively. Increasing competition of rPmRacgap1 to 5 µg resulted in the disappearance of both positive bands in 2.5 and 5 µg ovarian membrane proteins. Only faint positive bands were observed from 20 µg ovarian membrane proteins when the purified antibody was used in competition with 10 µg rPmRacgap1 (Figure. 3.74B). This confirmed that both 35 and 100 kDa bands are specifically recognized by anti-rPmRacgap1 PAb.

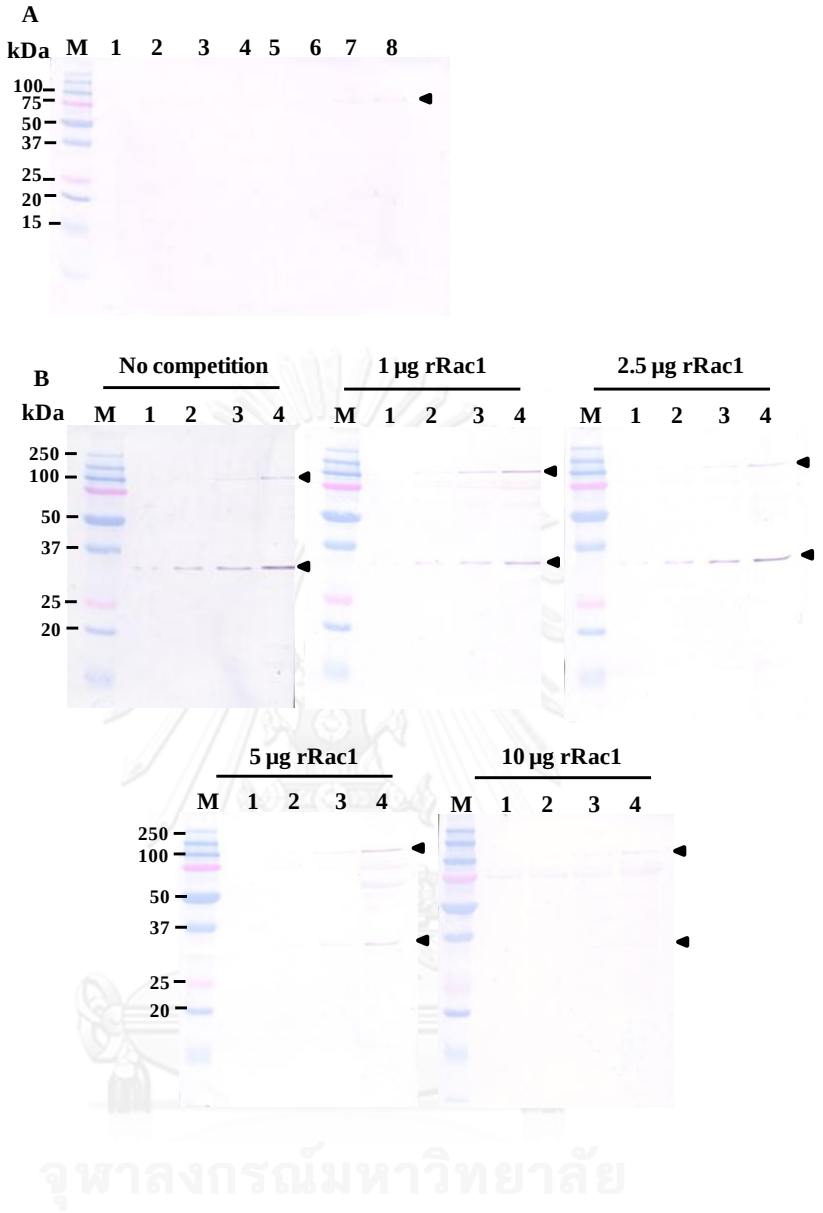


Figure 3.74 (A) Sensitivity of anti-rPmRacgap1 PAb against varying amounts of rPmRacgap1 proteins (0.001, 0.002, 0.005, 0.05, 0.1, 0.2, 0.5 and 1 µg corresponding to lanes 1-8, respectively). (B) Competitive binding assays with anti-rPmRacgap1 PAb. Western blot analysis of the purified anti-rPmRacgap1 PAb (1:100) pre-mixed with 0, 1, 2.5, 5 and 10 µg of rPmRacGAP 1 were carried out and used against 2.5, 5, 10 and 20 µg membran ovarian proteins (lanes 1-4, respectively) of a female shrimp with vitellogenic ovaries (stage II) and showing complete competitive blocking at 10 µg rPmRacgap1.

The positive signals of anti-rPmTmsb PAb were observed against 0.05, 0.1, 0.2, 0.5 and 1 µg of rPmTmsb (Figure 3.75A). For anti-PmTmsb, the positive immunoreactive bands of 22 and 28 kDa were observed from 5-20 µg and 10-20 µg total ovarian proteins whether or not the purified antibody was used in competition with 1 µg rPmTmsb. Nevertheless, these bands were disaapeared in the Ag-Ab competition test when anti-PmTmsb PAb competed with 2.5 or 5 µg rPmTmsb. Results from nanoESI-LC–MS/MS further indicated that these bands were closest similar to thymosin isoform 2 (EST clone HC-N-S01-0235-LF) and thymosin-β-repeated protein 2 (EST clone HC-H-S01-0682-LF) of *P. monodon* (http://pmonodon.biotec.or.th), respectively. Notably, a constant level of a 76 kDa band which significantly matches vitellogenin of *Fenneropenaeus chinensis* (gi|86129739) was observed in all examined samples (Figure 3.75B). Nevertheless, the vitellogenic band was not observed in subsequent Western blotting for expression analysis of ovarian PmTmsb during ovarian development of *P. monodon*.

3.9 Mass spectrometry analysis of immunoreactive bands and recombinant proteins

A discrete immunoreactive band of 97 KDa was observed when anti-PmCdc48-VCP PAb testes against total ovarian proteins of juveniles and wild intact and eyestalk-ablated broodstock. The positive protein band on a Western blotted PVDF membrane was trypsinized and further analyzed by nano ESI-LC-MS/MS. Internal peptide sequences of the immunoreactive protein were K.G-D-L-F-L-V-R.G, K.G-V-L-F-Y-G-P-P-G-C-G-K.T and K.V-T-H-G-F-S-G-A-D-L-T-E-I-C-Q-R.A which significantly matched valosin containing protein 1 of *Eisenia fetida* (score = 81; $P < 0.05$) and perfectly matched the deduced amino acid sequence obtained from *P. monodon*

EST (gi|000034674). The internal peptide of rPmTmsb were K.G-Q-L-E-G-F-S-A-V-N-L-K-.K, R.Q-G-I-E-G-F-D-H-A-A-L-K-.K, K.G-Q-L-E-G-F-S-A-V-N-L-K-K.T, K.G-Q-L-E-G-F-S-A-V-N-L-K-K.T, K.I-V-L-P-A-Q-E-D-I-E-T-E-K.T, K.I-H-L-P-N-R-E-D-V-E-A-E-K.K, K.V-Q-A-H-L-Q-A-V-E-G-F-N-T-A-Q-L-K.H and K.K-V-Q-A-H-L-Q-A-V-E-G-F-N- T-A-Q-L-K.H which significantly matched Tmsb of *Scylla paramamosain* (score 309; $P < 0.05$).

Results from nanoESI-LC–MS/MS further indicated that the positive molecular size 28 and 22 kDa generated from anti-PmTmsb PAb against total ovarian proteins of *P. monodon* were closest similar to thymosin-β-repeated protein 2 (EST clone HC-H-S01-0682-LF) and thymosin isoform 2 (EST clone HC-N-S01-0235-LF; http://pmonodon.biotec.or.th) of *P. monodon*, respectively.

3.10 Expression of PmVCP, PmRacgap1 and PmTmsb proteins during ovarian development of *P. monodon*

Western blot analysis revealed that PmVCP protein (97 kDa) was abundantly expressed in gonads (ovaries and testes). Limited expression of PmVCP was observed in the heart and lymphoid organ. PmVCP protein was not detected in the subcuticular epithelium and hepatopancreas (Figure 3.76A).

The molecular size of ovarian PmVCP (approximately 97 kDa) was greater than that predicted from the PmVCP cDNA suggesting that it was post-translationally modified by glycosylation. Female shrimp with the immunological signals of ovarian PmVCP were observed in juvenile ovaries and at all stages of ovarian development in both intact and eyestalk-ablated broodstock of wild *P. monodon* (Figure 3.76B).

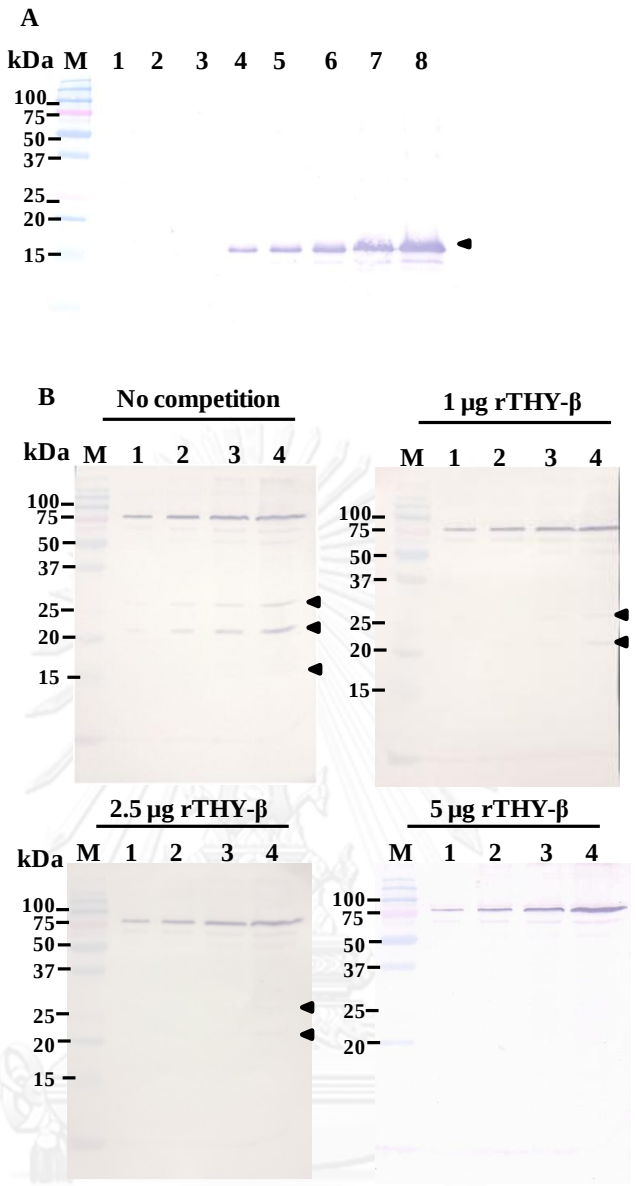


Figure 3.75 (A) Sensitivity of anti-rPmTmsb PAb against varying amounts of rPmtmsb protein (0.001, 0.002, 0.005, 0.05, 0.1, 0.2, 0.5 and 1 µg; lanes 1-8 respectively). (B) Competitive binding assays of anti-rPmTmsb PAb. Western blot analysis of the purified anti-rPmTmsb PAb (1:200) pre-mixed with 0, 1, 2.5, and 5 µg of rPmTmsb was carried out and used against 2.5, 5, 10 and 20 µg total ovarian proteins (lanes 1-4, respectively) of a late vitellogenic ovaries (stage III) and showing complete competitive blocking at 5 µg rPmTmsb.

It seemed to be expressed at comparable levels for all stages of ovarian development in both intact wild broodstock and eyestalk-ablated broodstock.

A discrete band of 34 kDa and a faint band of approximately 100 kDa (in stages I and II ovaries) were observed from western blot analysis of total soluble proteins against purified anti-rPmRacgap1 PAb. The expression level of PmRacgap1 reflected from a 34 kDa band seemed to be decreased in late stages of ovarian development (stages III and IV ovaries) in both intact and eyestalk-ablated broodstock (Figure 3.77). However, both intense 34 and 100 kDa bands were observed when membrane proteins of ovaries were analyzed.

The expression level of these bands was comparable during ovarian development in intact broodstock but it was decreased in mature ovaries in eyestalk-ablated broodstock (Figure 3.78).

Anti-rPmTmsb PAb gave positive immunoreactive signals of 22 and 28 kDa, respectively. The expression level of PmTmsb reflected from a positive 28 kDa band (thymosin-β-repeated protein 2) seemed to be decreased in mature (IV) ovaries in intact broodstock. In eyestalk-ablated broodstock, it was not expressed in late vitellogenic (III) and mature ovaries of wild *P. monodon* broodstock. A similar expression profile of a 22 kDa band (thymosin isoform 2) was found with the exception that the positive immunoreactive signal was not observed in mature ovaries (Figure 3.79).

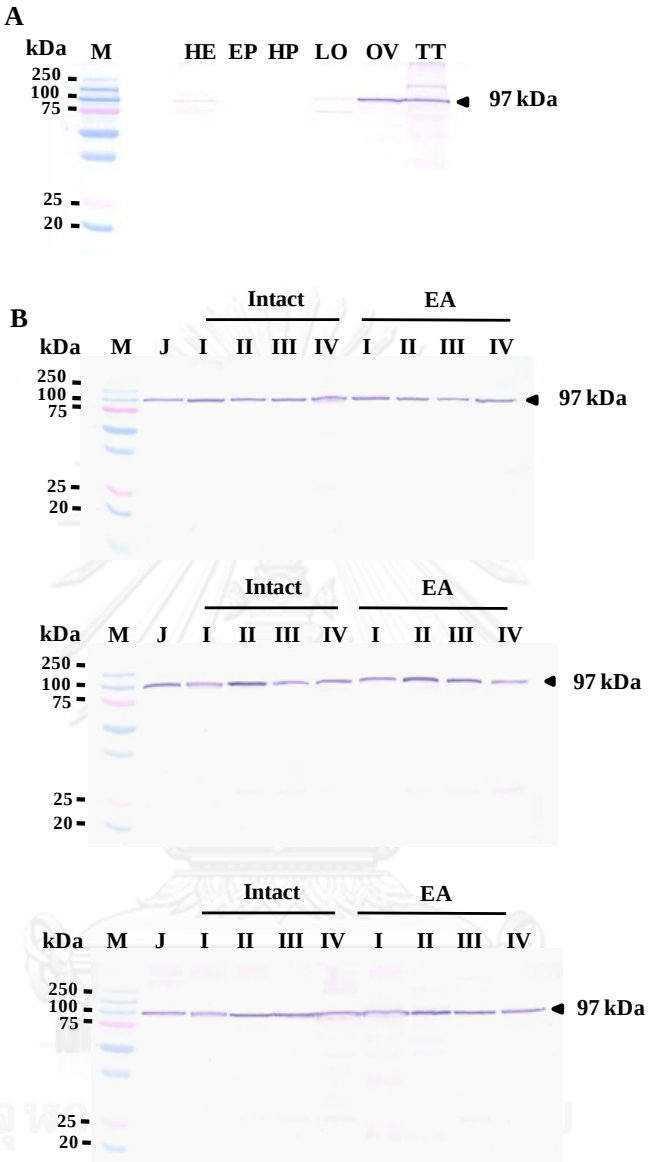


Figure 3.76 (A) Western blot analysis showing tissue expression analysis of the PmVCP protein in pooled hemocytes (HE), subcuticular epithelium (EP), the hepatopancreas (HP), lymphoid organ (LO),ovaries (OV) andtestes (TT) from 3 shrimp broodstock. (B) Western blot analysis of PmVCP in 20 µg total ovarian proteins from 3 sample sets consisting of juvenile shrimp (J, *N* = 3), intact broodstock (*N* = 3 each of stages I–IV) and eyestalk-ablated broodstock (EA, *N* = 3 each of stages I–IV). Lanes M = a protein standard marker.

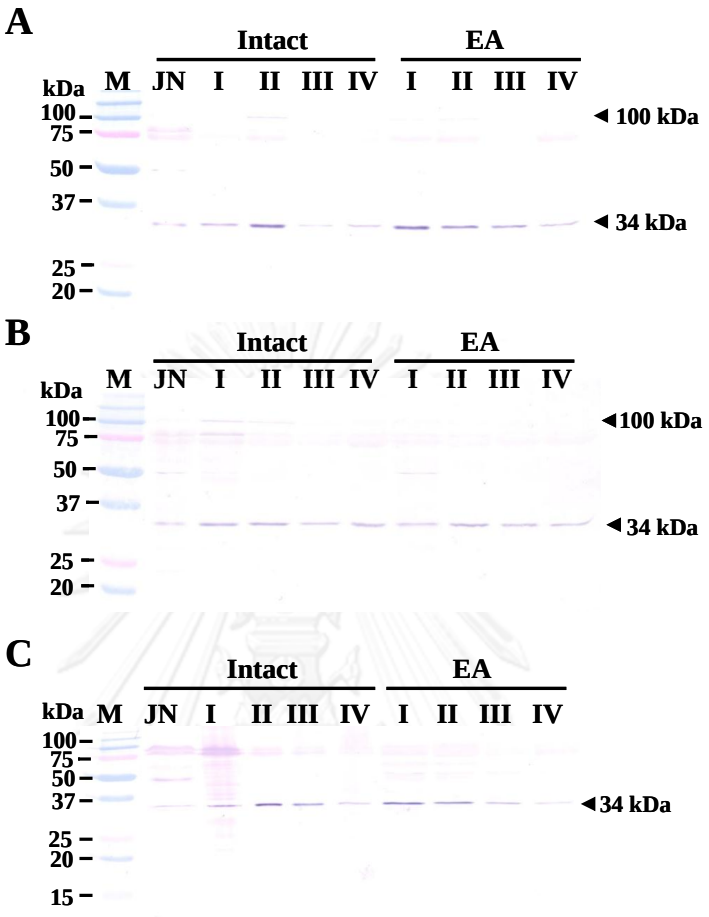


Figure 3.77 Western blot analysis of PmRacgap1 in 20 μg total ovarian proteins from 3 sample sets consisting of juvenile shrimp (J, *N* = 3), intact broodstock (*N* = 3 each of stages I–IV) and eyestalk-ablated broodstock (EA, *N* = 3 each of stages I–IV). Lanes M = a protein standard marker.

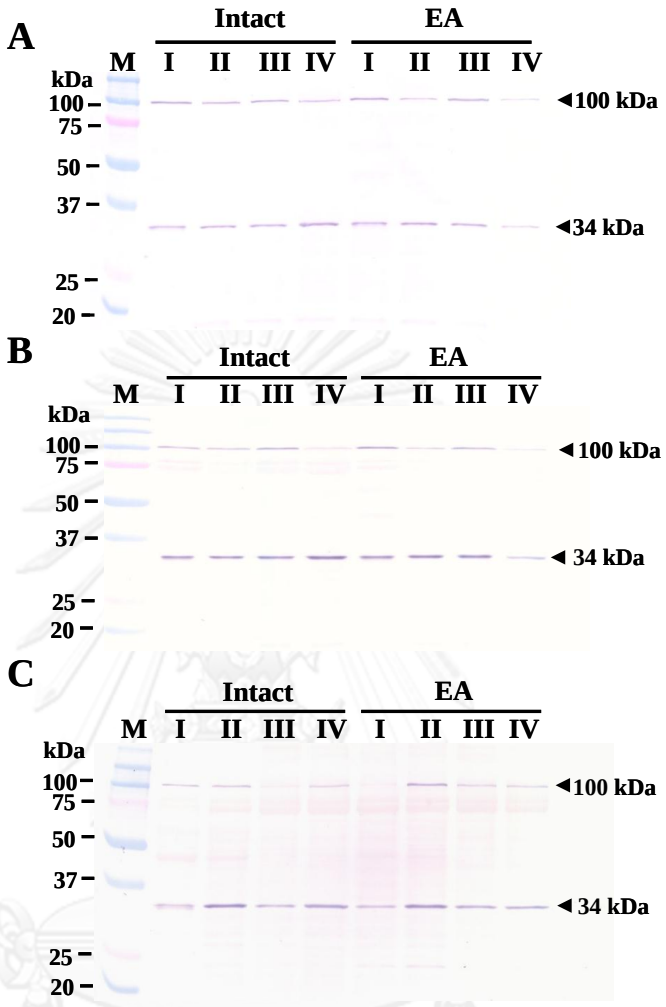


Figure 3.78 Western blot analysis of PmRacgap1 in 20 µg ovarian membrane proteins from 3 sample sets consisting of intact broodstock (*N* = 3 each of stages I–IV) and eyestalk-ablated broodstock (EA, *N* = 3 each of stages I–IV). Lanes M = a protein standard marker.

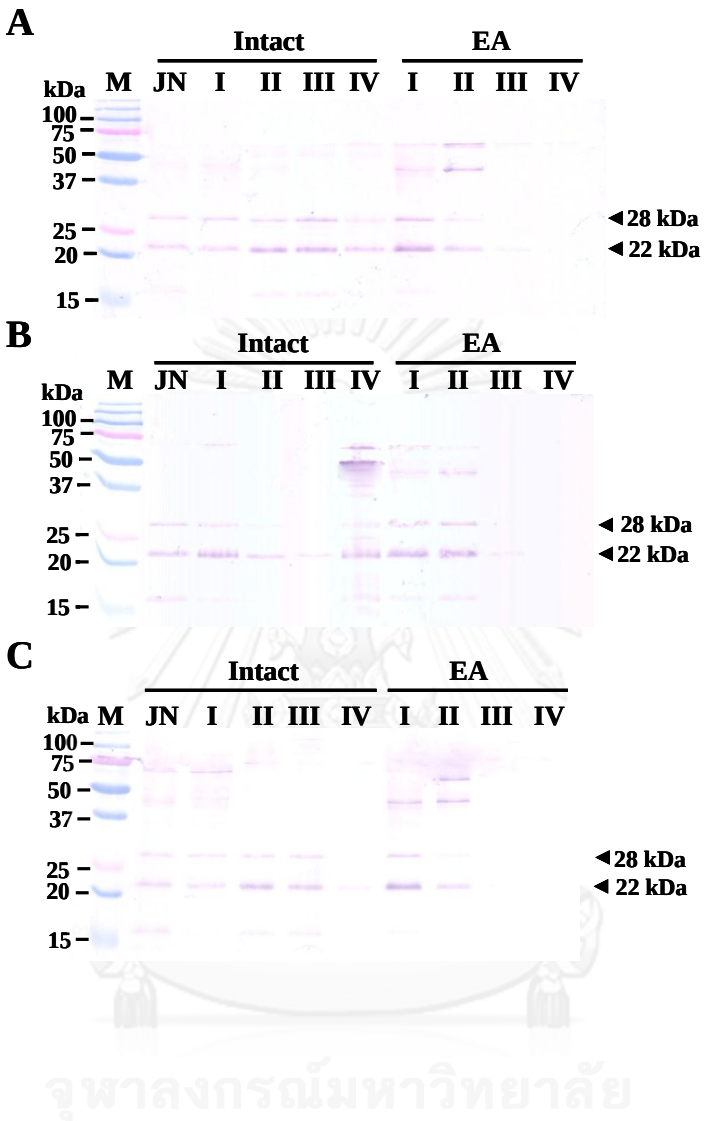


Figure 3.79 Western blot analysis of PmTmsb in 20 µg total ovarian proteins from 3 sample sets consisting of juvenile shrimp (J, *N* = 3), intact broodstock (*N* = 3 each of stages I–IV) and eyestalk-ablated broodstock (EA, *N* = 3 each of stages I–IV). Lanes M = a protein standard marker.

Anti-rPmNPC-NUP133 PAb generated a single discrete band against rPmNPC-NUP133 but non-specific signal were observed against ovarian membrane proteins (Figure 3.80A). Anti-rPmSema PAb also gave a single discrete band against rPmSema. Nevertheless, no positive signal was observed when tested against ovarian membrane proteins (Fig. 3.80B).

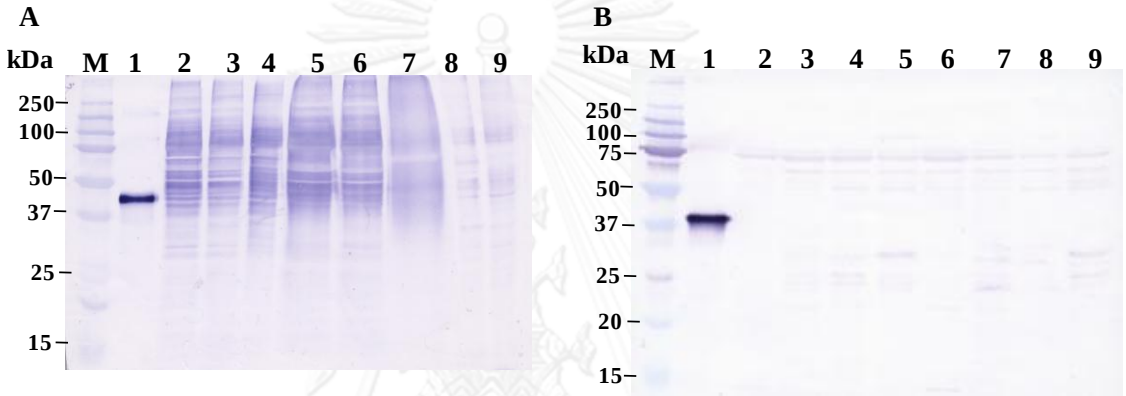


Figure 3.80 Western blot analysis of PmNPC-NUP133 (A) and PmSema (B) in 40 µg ovarian membrane proteins of intact broodstock (stages I–IV, lanes 2-5) and eyestalk-ablated broodstock (EA, stages I–IV, lanes 6-9). Lane 1 = rPmNPC-NUP133, A and rPmSema, B. Lanes M = a protein standard marker.

3.11 Localization of VCP protein in ovaries of *P. monodon* during ovarian development

Localization of PmVCP protein in oocytes at different developmental stages was examined by both immunohistochemistry (Figure 3.81-3.82) and immunofluorescence (Figure 3.83). Results from both methods were comparable but the latter provided clearer results. PmVCP protein was observed in the ooplasm of

previtellogenic oocytes and was translocated into the nucleus of vitellogenic oocytes. Interestingly, it was found in nucleo-cytoplasmic compartments, the cytoskeletal architecture and the plasma membrane in mature oocytes of both intact and eyestalk-ablated broodstock.

The immunoreactive signals of PmRacgap1 protein in ovaries of *P. monodon* were examined by immunohistochemistry (Fig 3.84-3.85). PmRacgap1 was observed in ooplasm of oogonia and all developmetal stages of oocytes (previtellognic, vitellogenic, early cortical rod and mature oocytes) in both intact and eyestalk-ablated broodstock. During vitellogenesis, it was also observed in the nucleus of vitellogenic oocytes. it was found in nucleo-cytoplasmic compartments, the cytoskeletal architecture and in cortical rods in more mature oocytes of both intact and eyestalk-ablated broodstock.

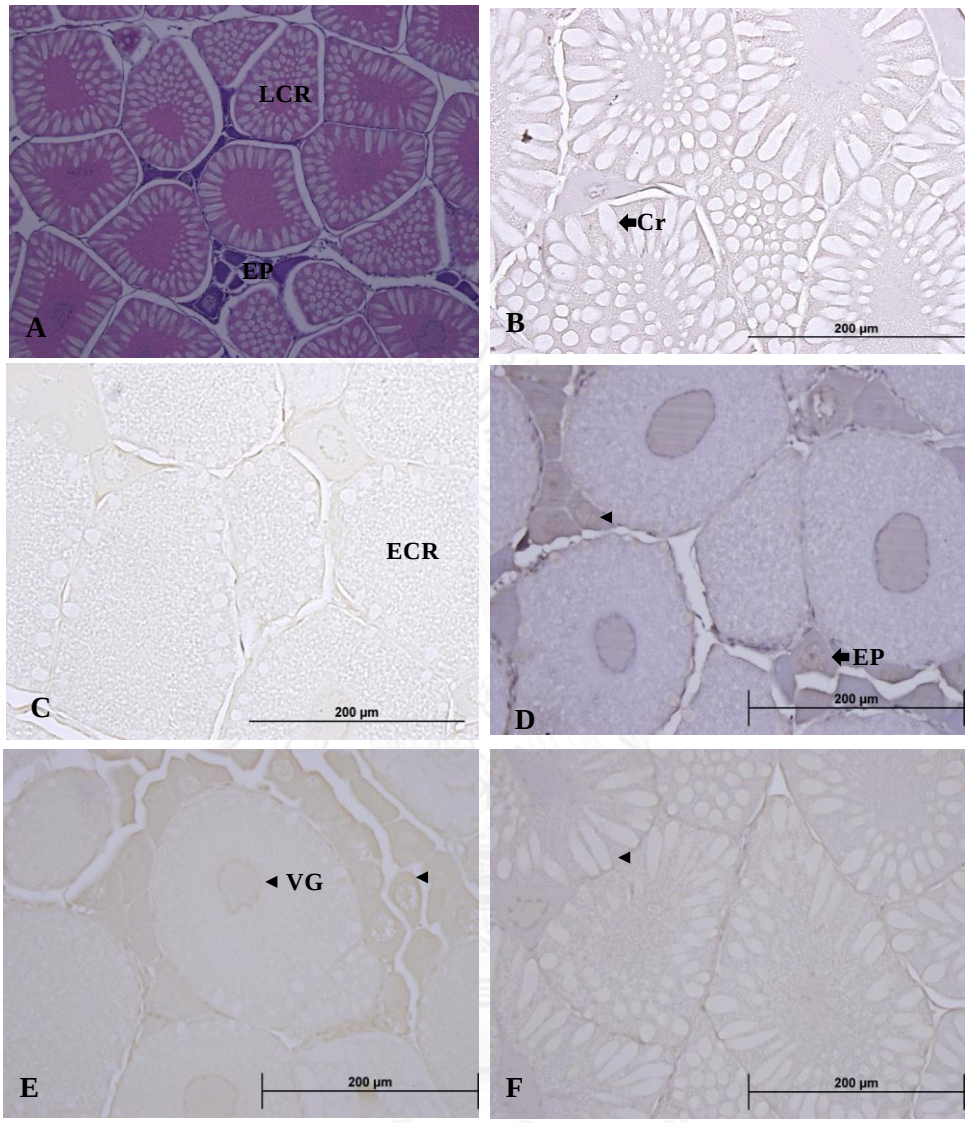


Figure 3.81 Localization of PmVCP protein revealed by immunohistochemistry using anti-rPmCdc48-VCP PAb against conventional tissue sections from intact wild *P.monodon* specimens (D-F). Hematoxylin and eosin staining (A) of tissue sections was carried out for classification of oocyte stages. Blocking solution (B) and the preimmune serum (C) was used as the negative control. EP = early previtellogenic oocytes; VG = vitellogenic oocytes; LCR = late cortical rod (mature) oocytes and Cr = cortical rods. Arrowheads indicate the immunologically positive signals.

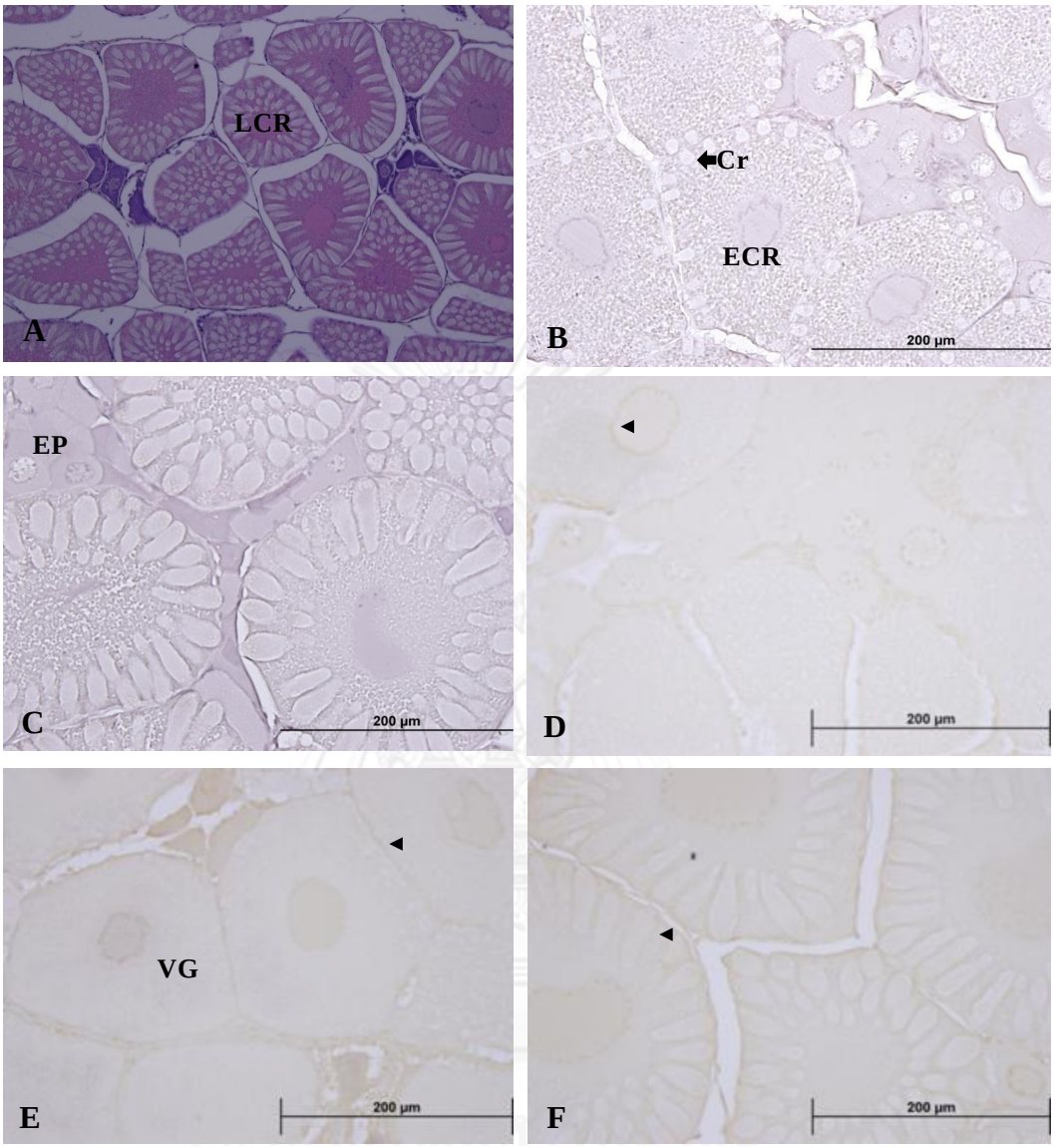


Figure 3.82 Localization of PmVCP protein revealed by immunohistochemistry using anti-rPmCdc48-VCP PAb against conventional tissue sections from intact wild *P.monodon* specimens (D-F). Hematoxylin and eosin staining (A) of tissue sections was carried out for classification of oocyte stages. Blocking solution (B) and the preimmune serum (C) was used as the negative control. EP = early previtellogenic oocytes; VG = vitellogenic oocytes; LCR = late cortical rod (mature) oocytes and Cr = cortical rods. Arrowheads indicate the immunologically positive signals.

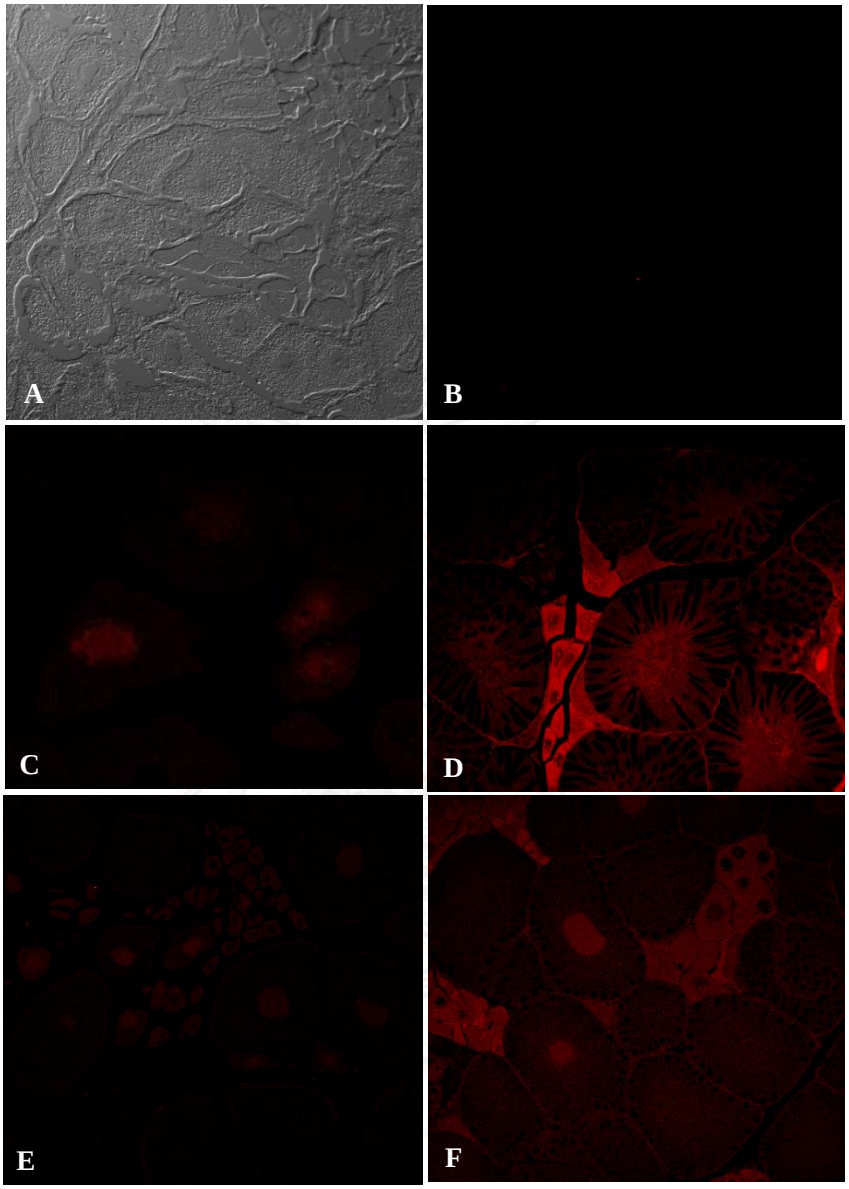


Figure 3.83 Localization of PmVCP protein revealed by immunofluorescence of anti-PmCdc48-VCP PAb against conventional ovarian tissue sections from intact wild *P. monodon* specimens (C–D) and eyestalk-ablated broodstock (E-F). Light microscopy of ovarian tissue section incubated with preimmune serum (A). Goat anti-rabbit IgG labeled with Alexa 635 was used as the second antibody. Ovarian tissue sections incubated with the preimmune serum were used as the negative control (B).

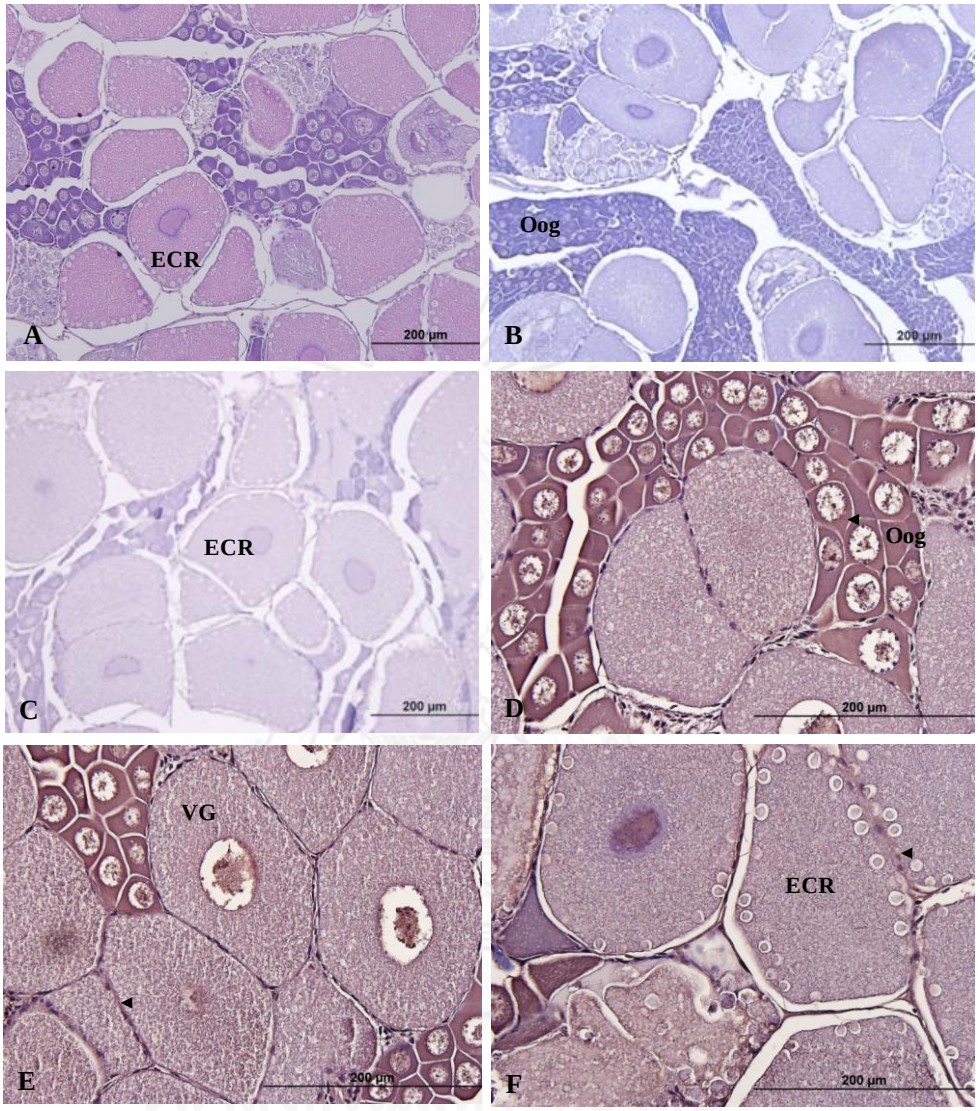


Figure 3.84 Localization of PmRacgap1 protein revealed by immunohistochemistry using anti-rPmRacgap1 PAb against conventional tissue sections from wild intact *P.monodon* (D-F). Hematoxylin and eosin staining (A) of tissue sections was carried out for classification of oocyte stages. Blocking solution (B) and the preimmune serum (C) was used as the negative controls. EP = early previtellogenic oocytes; VG = vitellogenic oocytes; LCR = late cortical rod (mature) oocytes and Cr = cortical rods. Arrowheads indicate the immunologically positive signals.

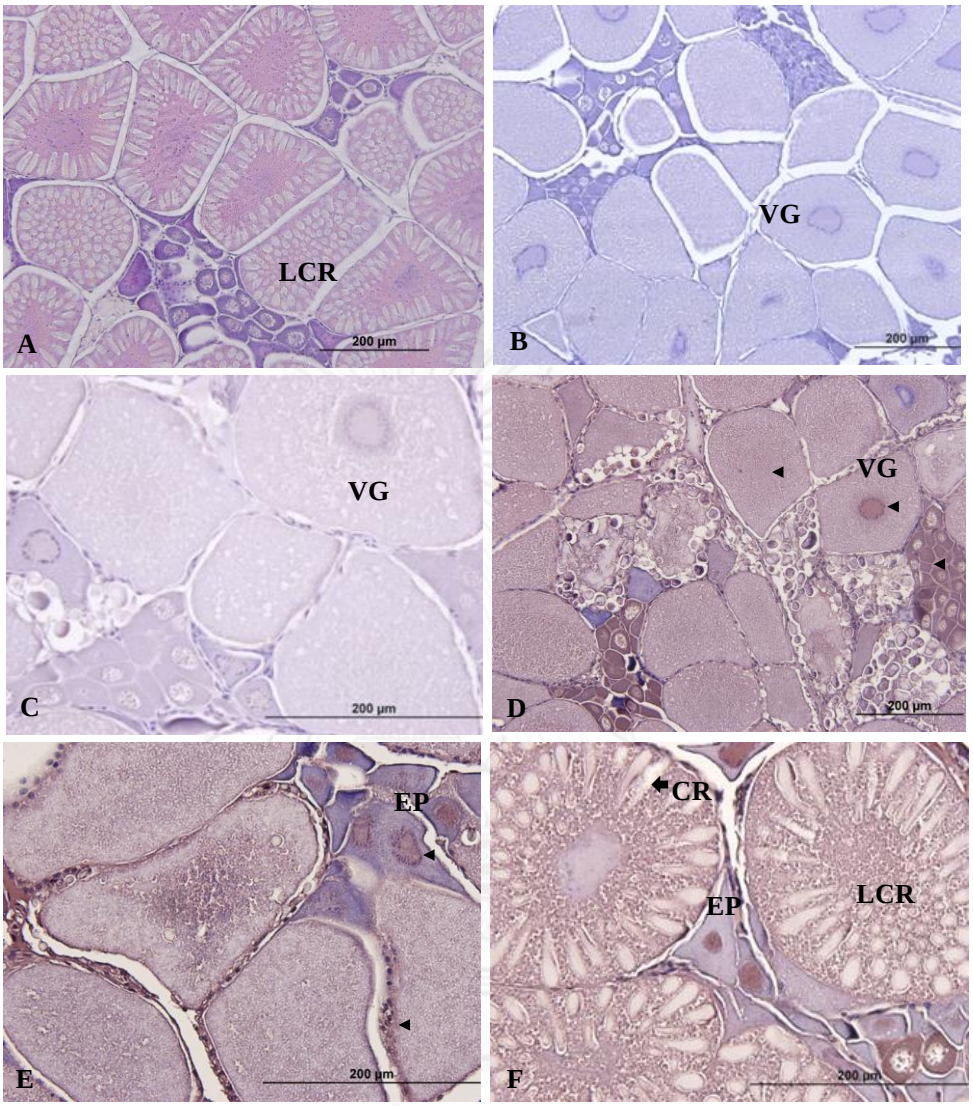


Figure 3.85 Localization of PmRacgap1 protein revealed by immunohistochemistry using anti-rPmRacgap1 PAb against conventional tissue sections from eyestalk-ablated wild *P.monodon* specimens (D-F). Hematoxylin and eosin staining (A) of tissue sections was carried out for classification of oocyte stages. Blocking solution (B) and the preimmune serum (C) was used as the negative controls. EP = early previtellogenic oocytes; VG = vitellogenic oocytes; LCR = late cortical rod (mature) oocytes and Cr = cortical rods. Arrowheads indicate the immunologically positive signals.

3.13 Interaction between rPmCdc48-VCP and rPmTmsb analyzed by a pull down assay

To identify the partner proteins that interact with recombinant proteins in this study, protein-protein interaction was examined using Sulfo-NHS-LC-Biotin kit (Pierce). The pull down assay of rPmCdc48-VCP and rPmTmsb was carried out. Initially, these soluble recombinant proteins were separately conjugated with biotin before immobilized with strevtavidin ligand column. In the control reaction, TBS buffer was incubated in the streptavidin ligand column before prey proteins was loaded into the column. After washed and eluted protein-protein interaction profiles was determined by SDS-PAGE and western blot analysis (Figure 3.86-3.87).

Partners of rPmVCP and PmTmsb were analyzed from the eluted fractions using nanoESI-LC-MS/MS.

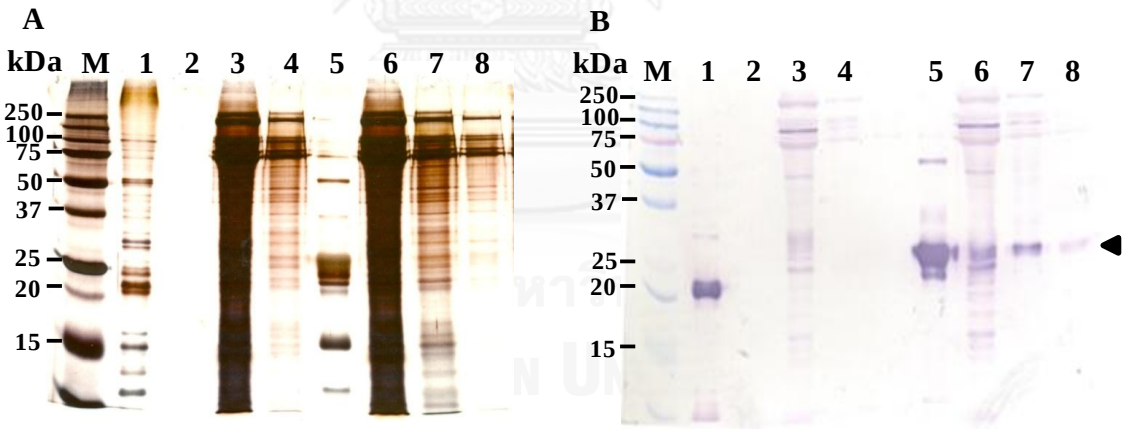


Figure 3.86 15% SDS-PAGE (A) and western blot analysis (B) showing protein profiles from the pull down assay of soluble rPmCdc48-VCP with ovarian proteins of *P. monodon*. Lane 1 is rPmCdc48-VCP and lanes 5-8 are rCdc48-VCP conjugated with biotin, prey protein flow through column, eluted fraction 1 and eluted fraction 2 of protein-protein interaction, respectively. Lanes 2-4 are immobilized ligand column with TBS buffer (prey protein flow through column and eluted fraction, respectively), as the control fraction.

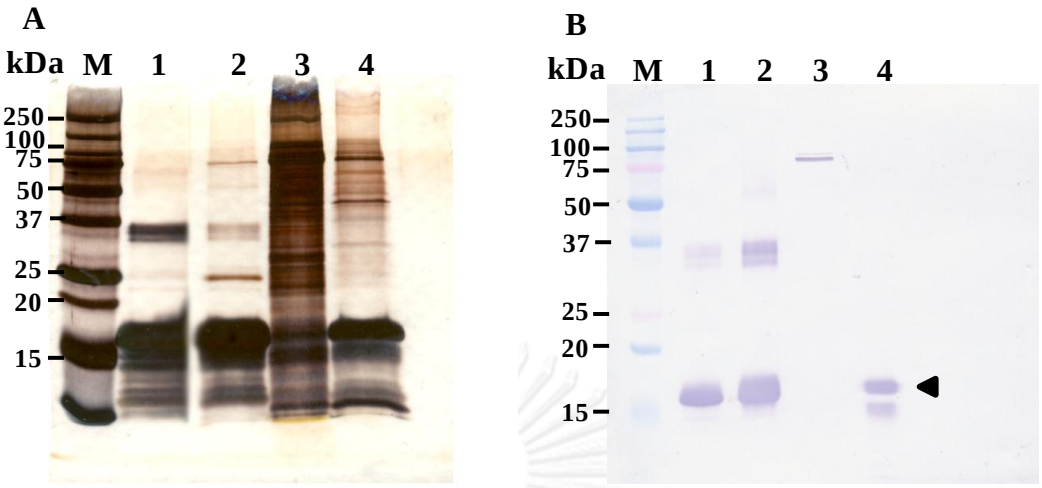


Figure 3.87 15% SDS-PAGE (A) and western blot analysis (B) showing protein profiles from the pull down assay of soluble rPmTmsb with ovarian proteins of *P. monodon*. Lane 1 is rTmsb of *P. monodon* and lanes 2-4 are rTmsb conjugated with biotin, prey protein flow through column, eluted 1 of protein-protein interaction, respectively.

The identified proteins from a pull down assay of rPmCdc48-VCP were those seinificantly matched Cadherin of *Ligia exotica*, Cysteine-rich receptor-like protein kinase 23-like of *Cicer arietinum*, NADPH oxidase 1 isoform X1 *Heterocephalus glaber*, acyl-CoA Delta(11) desaturase-like, partial *Apis florae* and Double WAP domain-containing protein and Receptor-type tyrosine-protein phosphatase T of *P. monodon* (Table 3.11).

In addition, a pull down assay of rPmTmsb was also carried out and results indicated that its candidate interacting partners were those significantly matched F-box and leucine-rich repeat protein 16 of *Clonorchis sinensis*, Acetylserotonin O-methyltransferase of *Felis catus* and 26S proteasome non-ATPase regulatory subunit 6, MFS transporter, Aldehyde dehydrogenase, and two unknown protein (clones no. HC-N-N01-0618-LF and OV-N-S01-0835-W) of *P. monodon* (Table 3.12).

Table 3.11 Proteins identified from a pull down assay of rPmCdc48-VCP with total ovarian protein of *P. monodon* analyzed by nanoESI-LC-MS/MS

Protein name	Accession no.	Clone no.	Score	Peptide
Cadherin *Ligia exotica*	gi|61162138	-	40	R.TMAIHYGILLLLCVISCR.S + Oxidation (M)
Cysteine-rich receptor-like protein kinase 23-like, *Cicer arietinum*	gi|502100240	-	36	K.CRSSLEGIIWYSHCLLR.Y
NADPH oxidase 1 isoform X1, *Heterocephalus glaber*	gi|512901969	-	35	K.SDEYFYTREILGTALAMAR.A
acyl-CoA Delta(11) desaturase-like, partial, *Apis florae*	gi|380020875	-	29	-.MPFVFELTVSGTCSAK.I
Double WAP domain-containing protein	gi|0026991	HC-N-N01-7369-LF	7.23	ATKSFA
Receptor-type tyrosine-protein phosphatase T	gi|0023772	HPa-N-N04-1092-LF	5.21	AVMRVDSCR

Table 3.12 Proteins identified from a pull down assay of rPmTmsb with total ovarian protein of *P. monodon* analyzed by nanoESI-LC-MS/MS

Protein name	Accession no.	Clone no.	Score	Peptide
F-box and leucine-rich repeat protein 16, *Clonorchis sinensis*	gi|358341784	-	33	R.IVLAHAQEEAMQK.S + Oxidation (M)
Acetylserotonin O-methyltransferase, partial, *Felis catus*	Gi|410988030	-	31	K.DPLPEAELYVLAR.V
26S proteasome non-ATPase regulatory subunit 6	Gi|0030169	OV-N-S01-1436-W	11.47	TGSTK
Unknown	Gi|0020350	HC-N-N01-0618-LF	4.86	TPNTQK
Unknown	Gi|0006847	OV-N-S01-0835-W	4.33	QMEVYHK
MFS transporter	Gi|0028539	OV-N-N01-0169-W	4.28	GSGTI
Aldehyde dehydrogenase	Gi|0022675	HPa-N-N04-1004-LF	1.43	AEDLVLHDLA

CHAPTER IV
DISCUSSION

4.1 Cellular proteomics of ovarian proteins in domesticated and wild *P. monodon* adults

An important step toward understanding molecular mechanisms of development and maturation of ovaries and oocytes in *P. monodon* is the identification and characterization of reproduction-related proteins expressed in ovaries of this economically important species. In this study, proteomic analysis based on SDS-PAGE and nanoESI-LC-MS/MS was further applied. The GeLC-MS/MS used in this study is more convenient and cost-effective than the typical approach based on 2-DE/mass spectrometry analysis. Differentially expressed proteins during ovarian development of *P. monodon* were inferred from the protein spectra. This readily resolves problems from non-quantitative examination of the protein intensity based on silver staining in our laboratory.

Functionally important proteins for meiotic maturation of oocytes including cyclin B, Cdc2, Cdc25 and 14-3-3 were identified. Of these, only cyclin B showed a differential expression profile among different groups of samples. Cyclin B forms a complex with Cdc2 (called the maturation-promoting factor, MPF) which control the meiotic maturation of animal oocytes (Okano-Uchida et al., 1998). Signal transduction pathways that activate Cyclin/Cdc2 complex and subsequent processes are the key pathways in meiotic maturation of eukaryotes oocytes. Cdc25 removes inhibitory phosphates (Thr14 and Tyr15) from cdc2 to activate the MPF allowing the progression to the M-phase (Nigg, 2001) ; (Voronina and Wessel, 2004).

Phosphorylated of Cdc25 by protein kinase A (PKA) results in binding of 14-3-3 and thereby, inhibits its phosphatase activity or interaction with positive regulators or with substrate (Oe et al., 2001) ; (Kishimoto, 2003).

Recently, the full-length cDNA of *P. monodon cyclin B* (*PmCyB*, three transcripts with an identical ORF of 1206 bp corresponding to 401 amino acids but three different 3′ UTR lengths of 416, 543 and 1117 bp, respectively; (Visudtiphole et al., 2009) and *Cdc2* (an ORF of 900 bp in length corresponding to a polypeptide of 299 amino acids; (Phinyo et al., 2013). Gene expression analysis suggested their functionally important roles in ovarian development of *P. monodon*.

Molecular mechanisms on promotion of development and maturation of oocytes/ovaries have received high attention and may lead to the possible ways to control reproductive maturation in shrimp. Although results in this study clearly illustrated the induction effects of 5-HT on transcription of reproduction-related genes in several pathways, for example, *P. monodon broad-complex* (*PmBr-c*; an early ecdysteroid responsive gene) and *adipose differentiation-related protein* (*PmADRP*; neutral lipid accumulation), *small androgen receptor-interacting protein* (*PmSARIP1*; sex steroid pathway) and *cell division cycle 2* (*PmCdc2*; meiotic signal transduction pathway) in domesticated shrimp, receptors for neurotransmitters have not been discovered based on EST (Leelatanawit et al., 2004) ; (Preechaphol et al., 2007) ; (Hiransuchalert et al., 2013) ; (Buaklin et al., 2013) and proteomic 2-DE (Talakhun et al., 2012) analyses of genes and proteins expressed in ovaries of *P. monodon*. Using a simple GeLC-MS/MS approach, both 5-HT receptor 6 and Alpha-2A adrenergic receptor (α_2-AR) were identified.

The full-length cDNA of 5-hydroxytryptamine (5-HT) receptor was isolated and characteized from *Metapenaeus ensis* (1675 bp containing an ORF of 1230 bp deducing to a protein of 409 amino acids; (Tiu et al., 2005) and *P. monodon* (2291 bp containing an ORF of 1776 bp encoding a deduced protein of 591 amino acids; (Ongvarrasopone et al., 2006). The deduced proteins contained seven hydrophobic transmembrane domains and shared high amino acid sequence homology to G protein-coupled receptors (GPCRs). ARs are also a member of GPCRs. They mediate the physiological effects of the biogenic amine hormones/neurotransmitters and control intracellular second messenger systems by activating guanine nucleotide–binding regulatory proteins (G-proteins) (Ruuskanen et al., 2004). However, a gene encoding α_2-AR and its expression profile have not been characterized and reported in *P. monodon*.

The ubiquitin-proteasome pathway involved in several control mechanisms of gametogenesis and sexual reproduction (Sutovsky et al., 1999) ; (Sutovsky et al., 2001) ; (Sakai et al., 2004). Ubiquitin-activating enzyme E1-domain containing 1 (UBE1), two UBE2 (ubiquitin-conjugating enzyme E2 variant 2 and ubiquitin-conjugating enzyme E2) and 2 UBE3 (ubiquitin protein ligase E3A isoform 2 and E3 ubiquitin-protein ligase Bre1) which play an important role in steroid receptor activity for growth and reproduction (Koshiyama et al., 2006) were identified. In starfish, a proteasome-associating complex plays the important role oocyte maturation (Sakai et al., 2004). The non-traditional extracellular functions of ubiquitin-proteasome system also participate in the elimination of paternal mitochondria in fertilized eggs (Sutovsky et al., 1999) ; (Sutovsky et al., 2001).

In this study, several types of proteasomes (26S proteasome regulatory complex ATPase RPT4, 26S proteasome non-ATPase regulatory subunit 4, 26S proteasome non-ATPase regulatory subunit 6, proteasome 26S subunit, non-ATPase 13, proteasome subunit alpha type 6, proteasome accessory factor PafA, proteasome subunit p58 and proteasome subunit beta type) were identified in ovaries of *P. monodon*. Functionally analysis of proteins in the ubiquitin-proteasome system should be further carried out for better understanding of their roles in ovarian development of female *P. monodon*.

The oocyte maturation process is accompanied by several organelle (e.g. cytoskeleton, yolk granules and cortical granules) transformations. Microfilaments form the scaffold for organelle movement during oocyte maturation. In starfish and sea urchin oocytes, actin polymerization takes place at the onset of maturation (Heil-Chapdelaine and Otto, 1996) ; (Wessel et al., 2002), and (Voronina and Wessel, 2004).

The intracellular calcium levels are second messengers linking to the activation of MPF (cyclinB/Cdc2 complex). An increase in the intracellular calcium level is required for the induction of oocyte maturation, or GVBD (Masui and Clarke, 1979) ; (Pesty et al., 1998) ; (Voronina and Wessel, 2004). In this study, main proteins involved in signaling and homeostasis of Ca^{2+} including calmodulin, calreticulin and calnexin were identified. The full-lemgth cDNA of *P. monodon calriticulin* (*PmCRT*, 1682 bp in length, containing an ORF of 1221 bp corresponding to a deduced protein of 406 amino acids) were identified. The expression level of *PmCRT* in ovaries of intact broodstock was significantly reduced at stage III ovaries ($P < 0.05$). Eyestalk ablation resulted in a significant greater level than that in stages I-II ovaries in intact broodstock ($P < 0.05$) (S. Klinbunga, unpublished data).

Previously, several reproduction-related proteins during ovarian development of wild intact and eyestalk-ablated adults of *P. monodon* were identified based on the conventional 2-DE followed by mass spectrometry (Talakhun et al., 2012). The most abundantly expressed protein in ovaries of *P. monodon* was the protein disulfide isomerase family (PDI, PDIA6 and PDIA3; 17 spots) that are functionally involved in chaperone activity and cell redox homeostasis. Cellular structure proteins such as β-actin (14 spots) and Tmsb (7 spots) were also abundantly expressed (Talakhun et al., 2012). Tmsb functions as the G-actin sequestering factors preventing actin polymerization to filaments and is functionally related to intracellular signaling through kinase activation (Zhang et al., 2011). Accordingly, PmTmsb gene and protein were further characterized in subsequent experiments.

4.2 Proteomic analysis of ovarian nuclear membrane proteins and nuclear proteins of wild *P. monodon* adults

Nuclear membrane proteins and nuclear proteins from ovaries of *P. monodon* were also studied for isolation the potential molecular markers functionally involved in GVBD of oocytes. In this study, proteins that are localized at nuclear membrane of the oocytes were found for example, DEAD/DEAH box helicase domain-containing protein, importin subunit alpha-2, inner-membrane translocator, nucleoporin 50 kDa, ran GTPase-activating protein, leucine-rich receptor-like protein kinase-like and cytochrome P450, nuclear pore complex protein NUP133 and semaphorin). The nuclear envelope forms the boundary of the nuclear compartment in eukaryotes. Its primary functions involve selective transport of macromolecules between the nucleus and the cytoplasm and organization of higher level nuclear architecture (Senior and Gerace, 1988).

DEAD box proteins are putative ATP-dependent RNA unwinding proteins whose primary biochemical function is the alteration of RNA secondary structure. They have been implicated in translation initiation, ribosome assembly, RNA splicing, and RNA stability (Goldbout et al., 2002). DEAD-box RNA helicases required for germ cell functions. The conserved motif, (V/I)-L-D-E-AD- X-(M/L)-L-X-X-G-F, observed in all members of the DEAD box protein family (Linder, 1989) ; (Tanner and Linder, 2001).

Previously, Talakhun (2008) isolated the full-length cDNA of *DEAD box ATP-dependent RNA helicase* (ORF of 1209 bp corresponding to a polypeptide of 402 amino acis), The deduced DEAD box ATP-dependent RNA helicase of *P. monodon* contained the conserved motif, (V/I)-L-D-E-AD- X-(M/L)-L-X-X-G-F, observed in all members of the DEAD box protein family was found. In addition, the predicted DEXDc and HELICc domains were found in the deduced DEAD box ATP-dependent RNA helicase protein of *P. monodon*

In the present study, genes encoding nuclearpore complex protein NUP133 (PmNUP133) and semaphorin (PmSema) of *P. monodon* were further characterized.

4.3 Isolation of full-length cDNA of *PmVCP*, *PmPKC*, *PmTmsb* and the complete ORF of *PmRacgap1*

In this study, the full length cDNA of *PmVCP* (2724 bp with an ORF 2481 deducing to 826 amino acids), *PmTmsb* (1084 bp, ORF of 387 bp, 128 amino acids) *PKC* (3404 bp, ORF of 2235 bp, 744 amino acids), *PmcAMP-RLP* (1272 bp, ORF of 435 bp, 144 amino acids), *PmNUP133* (4130 bp, ORF 3228 bp, 1085 amino acids) were successfully characterized by RACE-PCR. In addition, the complete ORF of

PmRacgap1 (1881 bp, 626 amino acids) was successfully characterized by amplification from the ORF of PmRacgap1 previously isolated in testes.

Valosin-containing protein (VCP) belongs to the ATPase-associated with diverse cellular activity (AAA) family of ATPase (Bug and Meyer, 2012). VCP is a highly conserved protein in eukaryotes. In humans, p97/VCP/Cdc48 has been reported to be required for the mitotic M-phase (Wójcik et al., 2004). In *Caenorhabditis elegans*, two VCP homologs were characterized and shown to be essential for embryogenesis (Yamanaka et al., 2004). Characterization of VCP-depleted embryos and oocytes revealed that this protein was required not only for progression of meiotic metaphase I but also for chromosome condensation at the diakinesis phase in meiotic prophase I (Sasagawa et al., 2007)

PKC is reported to be involved in oocyte maturation following the resumption of meiosis, and regulates spindle organization in meiosis-I and -II until fertilization and embryogenesis (Kalive et al., 2010). The existence of a conserved phosphorylation site for PKC suggested that this enzyme may be involved in the cAMP-mediated MAPK activity regulation (Fan and Sun, 2004) by phosphorylation.

Rac, a member of Rho family GTPase, regulates specific morphologic changes in the actin microfilament-based cytoskeleton. Reorganization of cellular cytoskeleton facilitates the dynamic changes necessary for cellular adhesion, growth, and motility (Moorman et al., 1999). The catalytic activity of small GTPases can be enhanced by GTPase-activating proteins (GAPs). However, relative little information concerning the functional roles of Racgap1 in ovary/oocyte development is known. Accordingly, PmVCP, PmPKC, PmTmsb and PmRacgap1 genes and proteins were further examined.

4.4 Expression analysis of *PmVCP*, *PmPKC*, *PmTmsb*, *PmRacgap1* and *PmSema* during ovarian development and under the induction with 5-HT

Gene expression and tissue distribution analysis are important and provide the basic information to set up the priority for further analysis of functional genes. Based on the fact that a particular genes may express in several tissues and possesses a different function in different tissues.

Tissue distribution analysis revealed constitutive expression of *PmTmsb* and *PmcAMP-RPL* in all examined tissues suggesting they conserved function in different tissues. The *PmVCP* transcript was more abundantly expressed in ovaries than other tissues suggesting that it may crucial role in ovary/oocyte development. VCP is present in all types of cellular components from human to yeast, suggest that VCP an important role in cellular activities (Song et al., 2003). *PmRacgap1* was more abundantly in gonads than other tissues. This suggested that *PmRacgap1* may play its important role in reproduction of *P. monodon*. Moreover, *PmPKC*, *Pmsema* and *PmNUP133* were highly expressed in ovaries than other tissues, the results indicated that these transcripts are functional involved in ovarian development of *P. monodon.*

Transcripts preferentially expressed at different stages of ovarian development can be used as indicators for reproductive maturation in *P. monodon*. Quantitative real-time PCR analysis further confirmed that the expression level of *PmTmsb* and *PmRacgap1* in juveniles was lower than that during ovarian development in either intact or eyestalk-ablated broodstock. Likewise, *PmVCP* was lower expressed in juveniles than that both of intact and eyestalk-ablated broodstock of *P. monodon*. At the transcriptional level, a single form of *PmTmsb*

transcript was identified and *PmTmsb* mRNA was not differentially expressed. Unilateral eyestalk ablation resulted in a significant reduction of this transcript in stages I and III ovaries. This suggested that the accumulated *PmTmsb* mRNA in oocytes at the early ovarian development stage was not sufficient for rapid translation of its protein during the later stages of ovarian development in intact shrimp. Similarly, non-differential expression of *PmRacgap1* was observed in intact broodstock of *P. monodon*. Its transcription level seemed to be decreased following eyestalk ablation. Nevertheless, results were not statistically different due to large standard variation of each group of samples.

PmVCP expression was significantly increased during vitellogenesis and final ovarian maturation in intact broodstock. Eyestalk ablation seemed to slightly increase ovarian *PmVCP* transcripts. On the other hand, *PmPKC* transcripts exhibited significantly lower expression levels in ovaries of eyestalk-ablated broodstock of *P. monodon,* relative to intact broodstock. This result suggested that, higher levels of *PmVCP* but lower levels of *PmPKC* affected ovarian development of *P. monodon.* In addition, the expression level of *PmcAMP-RPL* was not differently expressed in ovaries of both intact and eyestalk-ablated broodstock.

Ovarian maturation of penaeid shrimp results from rapid synthesis and accumulation of the major yolk protein, vitellin (Meusy and Payen, 1988). The differential expression of these reproduction-related genes during ovarian development in intact wild broodstock suggests that it has a functional role in *P. monodon* reproduction.

Estrogen-like compounds in invertebrates were first described in the ovaries of an echinoderm (Donahue and Jennings, 1937). Progesterone, P4, and its derivatives

(progestins) are sex steroid hormones that play important roles in gametogenesis (Miura et al., 2006). Progesterone stimulated ovarian maturation and yolk protein synthesis of penaeid shrimp (Kulkarni et al., 1996) ; (Yano, 1985) ; (Quackenbush, 2001). It also promoted spawning of *Metapenaeus ensis* (Yano, 1985).

Recently, progesterone and 17α-hydroxyprogesterone were extracted from the polychaetes. Their activity in comparison with the synthetic hormones (0.4, 0.7 and 1.0 ng/ml for P4 and 1.0, 2.0 and 3.0 ng/ml for 17α-OHP4) were in vitro tested against previtellogenic ovaries of *P. monodon* for 24 h. P4 was more effective in enhancing the final maturation of oocytes while 17α-OHP4 had more effects on vitellogenic oocytes. Interestingly, synthetic steroid hormones at equal hormone concentrations produced similar results to steroid hormones extracted from natural polychaetes (Meunpol et al., 2007).

In *P. monodon*, progesterone administration has been reported to promote expression of *progestin membrane receptor component 1* (*PmPgmrc1*) at 72 hpi (S. Klinbunga, unpublished data) and *broad-complex* (*PmBr-c*) at 48–72 hpi (Buaklin et al., 2013) in 14-month-old shrimp. Similarly, administration of progesterone also had a significant effects on the expression of *fenesoate O-methyltransferase* (*PmFAMeT*) in ovaries of *P. monodon*. The results further indicated that progesterone stimulates ovarian development in *P. monodon* through several pathways including the signal transduction, ecdysteroid biosynthesis and MF biosynthesis. The information implied that progesterone is functionally involved in ovarian development in *P. monodon*. The information from previous studies indicated that progesterone may directly enhance maturation through vitellogenesis stimulation or act as a precursor of the vitellogenin-stimulating ovarian hormone (VSOH) in shrimp (Fingerman et al., 1993) ;

(Yano and Hoshino, 2006). Overexpression of *PmPgmrc1* and *PmBr-c* also indicated that appropriate form(s) of progesterone can potentially induce oocyte/ovarian development and maturation of *P. monodon*.

In this study, exogenous injection of progesterone did not affect *PmVCP* expression, suggesting that progesterone may affect such things in the steroid receptor and ecdysteroid biosynthetic pathways but not the VCP pathway.

Effects of exogenous 5-HT on the reproductive performance of shrimp were reported (Vaca and Alfaro, 2000). Simultaneous injections of 5-HT (25 µg/g body weight) and dopamine antagonist, spiperone (1.5 or 5 µg/g body weight) induced ovarian maturation and spawning in wild *L. stylirostris* and pond-reared *L. vannamei* (Alfaro et al., 2004). 5-HT induced ovarian development of *P. monodon* (Wongprasert et al., 2006) and *M. rosenbergii* (Meeratana et al., 2006) dose dependently.

Exogenous 5-HT administration clearly promoted expression of *P. monodon* Ovarian-Specific Transcript (*Pm-OST1*) in ovaries of subadults (5-month-old). *Pm-OST1* was up-regulated at 12-78 hpi ($P < 0.05$), with the highest expression level observed at 48 hpi ($P < 0.05$) (Klinbunga et al., 2009). In addition, its effects on promoting the expression of *P. monodon adipose differentiation related protein* (*PmADRP*) in ovaries of this shrimp group was also reported (Sittikankaew et al., 2010).

The GSI values of domesticated broodstock vary between 0.4-1.5% where most shrimp possess less than 1.0% GSI. Therefore, their ovaries are regarded as stage I compared with wild broodstock. The effects of 5-HT on expression of *PmFAMeT* in ovaries of 18-month-old *P. monodon* were examined. The injection of

5-HT resulted in increasing of *PmFAMeT* expression for approximately 50-fold at 1 hpt.

In this study, the expression levels of *PmVCP*, *PmPKC*, *PmcAMP-RLP* and *PmRacgap1* was significantly induced by serotonin injection. The molecular effects of 5-HT on expression of various reproduction-related genes (*Pm-OST1*, *PmADRP*, *PmFAMeT*, *PmVCP*, *PmPKC*, *PmcAMP-RLP* and *PmRacgap1*) in stage I ovaries of domesticated shrimp suggested that high expression levels of this gene should stimulate the early stage of ovary/oocyte development in *P. monodon*. More importantly, serotonin administration showed similar effects on promoting expression of various genes in ovaries of *P. monodon*.

Serotonin administration reduced *PmTmsb* transcripts in ovaries of *P. monodon*. In the previous publication, two *Tmsb* transcripts (*HaTHY1* and *HaTHY2*) encoding 19.0 and 14.5 kDa polypeptides was isolated in *Helicoverpa armigera*. *HaTHY1* was mainly transcribed in the integument and midgut, while *HaTHY2* was principally presented in the fat body and haemocytes. These transcripts were up-regulated following the ecdysteroid, 20-hydroxyecdysone (Zhang et al., 2011). In crustaceans, ecdysteroids recognized is the molting hormones as in other arthropods (Huberman, 2000) ; (Okumura and Sakiyama, 2004). Crustacean ecdysteroids are also synthesized in ovaries and testes of crustaceans under the negative and positive regulation of molt-inhibiting hormone (MIH) and methyl farnesoate (MF), respectively (Styrishave et al., 2008) ; (Brown et al., 2009). Accordingly, it is speculated that *PmTmsb* should play the important role in reproductive development and maturation in *P. monodon*.

4.5 Expression and localization of reproduction-related proteins

A partial PmVCP covering two Cdc48 domains (PmrCdc48-VCP approximately 24 kDa) was successfully expressed *in vitro* and an anti-rPmCdc48-VCP PAb was successfully produced in a rabbit. Antigen-antibody competition experiments revealed that the anti-rPmCdc48-VCP PAb was specific for a 97 kDa protein identified as valosin-containing protein by nanoESI-LC–MS/MS. The level of PmVCP protein was comparable during ovarian development of naturally maturing broodstock. By contrast, the PmVCP mRNA in intact shrimp was up-regulated in stages II and IV ovaries. This suggested that the accumulated PmVCP mRNA in oocytes at the early ovarian development stage was not sufficient for rapid translation of the PmVCP protein during the later stages of ovarian development in non-ablated *P. monodon*. In eyestalk-ablated broodstock, relatively stable levels of the PmVCP transcript and protein were found, suggesting that a steady state amount of this mRNA was sufficient to maintain all stages of ovarian development.

Immunoreactive signals for PmVCP protein were observed in the ooplasm of previtellogenic oocytes in *P. monodon*. There was a subsequent translocation of PmVCP to the germinal vesicle during vitellogenesis. After GVBD, the protein was observed in the nucleo-cytoplasmic compartment, the cytoskeletal architecture and the plasma membrane of oocytes.

Similarly, p97/VCP in *Ciona* was reported to be localized first in the endoplasmic reticulum (ER) region and cortical region of oocytes, later in the germinal vesicle (GV) and finally in the cortical ER region after GVBD (Kondoh et al., 2008). The p97/VCP/Cdc48 protein is involved in several biological functions. These include membrane fusion, protein folding and activation of membrane-bound transcription factors in association with various cofactors (Meyer et al., 2000). In

addition, p97/VCP/Cdc48 plays an important role in degradation of misfolded secretory proteins via the ER-associated degradation pathway (Madsen et al., 2011). The temporal changes in PmVCP location suggested that it also facilitates protein degradation during oogenesis in *P. monodon*.

Likewise, rPmRacgap1 and rPmTmsb and their polyclonal antibodies were successfully produced. At the protein level, a slightly lower level of PmRacgap1 (34 kDa) was found in late stages of ovarian development when total soluble proteins of ovaries were analyzed. The reduction of membrane PmRacgap1 (both 34 and 100 kDa bands) protein in stage IV ovaries in both intact and eyestalk-ablated broodstock further suggested its roles on prevention of cytoskeleton formation during the late stages of oocyte development.

Immunoreactive signal of PmRacgap1 was observed in ooplasm of oogonia and all developmetal stages of oocytes (previtellognic, vitellogenic, early cortical rod and mature oocytes) in both intact and eyestalk-ablated broodstock. During vitellogenesis, it was also observed in the nucleus of vitellogenic oocytes. its was found in nucleo-cytoplasmic compartments, the cytoskeletal architecture and in cortical rods in more mature oocytes of both intact and eyestalk-ablated broodstock. This result indicated that PmRacgap1 may be involved in translocation of cortical granules during meiotic maturation of *P. monodon*.

The expression level of PmTmsb protein was concordant to mRNA level. The reduction of expression level of PmTmsb (both 22 and 28 kDa) protein during late stage of ovarian development and also suggested that a decreased level of PmTmsb protein is required for cytoskeleton proliferation during ovarian maturation in *P. monodon*.

In this study, a large number of soluble proteins nuclear membrane and nuclear proteins expressed in ovaries of *P. monodon* ovaries were identified by 1-DE (SDS-PAGE) followed by mass spectrometry. The expression levels of *PmVCP*, *PmTmsb*, *PmRacgap1*, *PmcAMP-RLP* and *PmPKC* mRNAs during ovarian development in intact and eyestalk-ablated shrimp were examined. Additionally, the expression levels of PmVCP, PmTmsb and PmRacgap1 protein were examined and indicated the functionally important role of PmTmsb, PmRacgap1 and PmPKC as the negative effectors while PmVCP seemed to be the positive effectors on the maturation of ovary/oocyte development in *P. monodon*. For further applications of the basic knowledge found in this thesis, RNA interference (RNAi) of *PmTmsb*, *PmRacgap1* and *PmPKC* should be carried out to determine whether the inhibition of these genes (separately or simultaneously) affects ovarian development of *P. monodon*. At the protein level, anti-rPmTmsb and anti-rPmRacgap1 PAb may also be used for determination of their *in vivo* effects in ovarian development of *P. monodon*. In addition, rPmCdc48-VCP was expressed in both soluble and insoluble forms. Accordingly, the purified rPmCdc48-VCP could be used to determine its stimulation effects on ovarian development of *P. monodon in vivo*.

CHAPTER V
CONCLUSION

1. A total of 1638 proteins were identified from cellular proteomics of ovarian proteins of domesticates and wild broodstock of *P. monodon*. Of these 514 proteins (354 known proteins, 73 hypothetical proteins and 87 unknown proteins) were differentially expressed in between examined samples.

2 A total of 724 proteins were identified from proteomic analysis of nuclear membrane proteins and nuclear proteins of ovaries of wild *P. monodon* broodstock. Of these, 89 proteins were localized at integral to membrane or nuclear membrane and 99 proteins were recognized as nuclear proteins.

3. The full-length cDNAs of a *PmVCP*, *PmTmsb*, *PmPKC*, *PmcAMP-RPL* and *PmNup133* and the complete ORF of *PmRacgap1* were characterized.

4. The expression level of *PmTmsb* and *PmcAMP-RPL* was not differential expressed in ovaries of intact and eyestalk-ablated broodstock of *P. monodon*. However, the expression level of *PmVCP*, *PmPKC* and *PmRacgap1* were affected by eyestalk ablation.

5. Progesterone did not induce the expression of *PmVCP*. 5-HT injection did not affect the expression of *PmTmsb* but promoted the expression of *PmVCP*, *PmRacgap1*, *PmcAMP-RPL* and *PmPKC* in ovaries of 18-month-old shrimp.

6. Localization of PmVCP mRNA was examined and the positive signals were found in oogonia and ooplasm of previtellogenic and vitellogenic oocytes in different stages of ovaries in both intact and eyestalk-ablated broodstock.

7. Expression profiles of PmVCP, PmRacgap1 PmRacgap1 proteins suggested their functionally important roles during ovarian development of *P. monodon*.

8. PmVCP and PmRacgap1 proteins were localized in the ooplasm of previtellogenic oocytes. These proteins were translocated into the nucleus of vitellogenic oocytes and were found in nucleo-cytoplasmic compartments and the cytoskeletal architecture of mature ooytes. The former was also localized at the plasma membrane while the latter was also found in cortical rods in mature oocytes of both intact and eyestalk-ablated broodstock.

REFERENCES

Alfaro, J., Zúñiga, G., and Komen, J. (2004). Induction of ovarian maturation and spawning by combined treatment of serotonin and a dopamine antagonist, spiperone in *Litopenaeus stylirostris* and *Litopenaeus vannamei*. *Aquaculture* **236**, 511-522.

Bailey-Brock, J. H., and Moss, S. M. (1992). "Penaeid taxonomy, biology and zoogeography," Elsevier Science Publisher B.V, Netherlands.

Benzie, J. A. (1998). Penaeid genetics and biotechnology. *Aquaculture* **164**, 23-47.

Bourchookarn, A., Havanapan, P.-O., Thongboonkerd, V., and Krittanai, C. (2008). Proteomic analysis of altered proteins in lymphoid organ of yellow head virus infected *Penaeus monodon*. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **1784**, 504-511.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72**, 248-254.

Brown, M. R., Sieglaff, D. H., and Rees, H. H. (2009). Gonadal ecdysteroidogenesis in Arthropoda: occurrence and regulation. *Annual review of entomology* **54**, 105-125.

Buaklin, A., Sittikankaew, K., Khamnamtong, B., Menasveta, P., and Klinbunga, S. (2013). Characterization and expression analysis of the *Broad-complex* (*Br-c*) gene of the giant tiger shrimp *Penaeus monodon*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **164**, 280-289.

Bug, M., and Meyer, H. (2012). Expanding into new markets–VCP/p97 in endocytosis and autophagy. *Journal of structural biology* **179**, 78-82.

Chang, E. S., Prestwich, G. D., and Bruce, M. J. (1990). Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. *Biochemical and biophysical research communications* **171**, 818-826.

Chung, A. C.-K., Durica, D. S., and Hopkins, P. M. (1998). Tissue-Specific Patterns and Steady-State Concentrations of Ecdysteroid Receptor and Retinoid-X-Receptor mRNA during the Molt Cycle of the Fiddler Crab, *Uca pugilator*. *General and comparative endocrinology* **109**, 375-389.

Clark, W. H. J., Yudin, A. I., Lynn, J. W., Griffin, F. J., and Pillai, M. C. (1990). Jelly layer formation in Penaeoidean shrimp eggs. *Biological Bulletin* **178**, 295-299.

Clifford, H., and Preston, N. (2006). Genetic improvement. *Operating Procedures for Shrimp Farming: Global Shrimp OP Survey Results and Recommendations", Global Aquaculture Alliance, St. Louis*, 73-77.

Coman, G., Arnold, S., Peixoto, S., Crocos, P., Coman, F., and Preston, N. (2006). Reproductive performance of reciprocally crossed wild-caught and tank-reared *Penaeus monodon* broodstock. *Aquaculture* **252**, 372-384.

Cottrell, J. S., and London, U. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551-3567.

Cui, J., Wu, L. T., and Chu, K. H. (2014). Comparative proteomic profiling during ovarian development of the shrimp Metapenaeus ensis. *Molecular biology reports* **41**, 519-528.

Donahue, J. K., and Jennings, E. D. (1937). The occurrence of estrogenic substances in the ovaries of echinoderms. *Endocrinology* **21**, 690-691.

Fan, H.-Y., and Sun, Q.-Y. (2004). Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biology of reproduction* **70**, 535-547.

Fingerman, M. (1997). Roles of neurotransmitters in regulating reproductive hormone release and gonadal maturation in decapod crustaceans. *Invertebrate Reproduction & Development* **31**, 47-54.

Fingerman, M., Nagabhushanam, R., and Sarojini, R. (1993). Vertebrate-type hormones in crustaceans: localization, identification and functional significance. *Zoological Science* **10**, 13-29.

Harrison, K. E. (1990). The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. *J. Shellfish Res* **9**, 1-28.

Heil-Chapdelaine, R. A., and Otto, J. J. (1996). Characterization of changes in F-actin during maturation of starfish oocytes. *Developmental biology* **177**, 204-216.

Hiransuchalert, R., Yocawibun, P., Klinbunga, S., Khamnamtong, B., and Menasveta, P. (2013). Isolation of cDNA, genomic organization and expression of *small*

androgen receptor-interacting protein 1(*PmSARIP1*) in the giant tiger shrimp *Penaeus monodon*. *Aquaculture* **412**, 151-159.

Huberman, A. (2000). Shrimp endocrinology. A review. *Aquaculture* **191**, 191-208.

Huberman, A., Aguilar, M. B., Brew, K., Shabanowitz, J., and Hunt, D. F. (1993). Primary structure of the major isomorph of the crustacean hyperglycemic hormone (CHH-I) from the sinus gland of the Mexican crayfish *Procambarus bouvieri* (Ortmann): Interspecies comparison. *Peptides* **14**, 7-16.

Hui, J. H. L., Tobe, S. S., and Chan, S.-M. (2008). Characterization of the putative farnesoic acid *O*-methyltransferase (LvFAMeT) cDNA from white shrimp, *Litopenaeus vannamei:* Evidence for its role in molting. *Peptides* **29**, 252-260.

Jalabert, B. (2005). Particularities of reproduction and oogenesis in teleost fish compared to mammals. *Reproduction Nutrition Development* **45**, 261-279.

Johansson, C., Samskog, J., Sundström, L., Wadensten, H., Björkesten, L., and Flensburg, J. (2006). Differential expression analysis of Escherichia coli proteins using a novel software for relative quantitation of LC-MS/MS data. *Proteomics* **6**, 4475-4485.

Kalive, M., Faust, J. J., Koeneman, B. A., and Capco, D. G. (2010). Involvement of the PKC family in regulation of early development. *Molecular reproduction and development* **77**, 95-104.

Kegel, G., Reichwein, B., Tensen, C. P., and Keller, R. (1991). Amino acid sequence of crustacean hyperglycemic hormone (CHH) from the crayfish, *Orconectes limousus*: Emergence of a novel neuropeptide family. *Peptides* **12**, 909-913.

Kegel, G., Reichwein, B., Weese, S., Gaus, G., Peter-Katalinić, J., and Keller, R. (1989). Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*. *FEBS letters* **255**, 10-14.

Kenway, M., Macbeth, M., Salmon, M., McPhee, C., Benzie, J., Wilson, K., and Knibb, W. (2006). Heritability and genetic correlations of growth and survival in black tiger prawn *Penaeus monodon* reared in tanks. *Aquaculture* **259**, 138-145.

Kim, Y. K., Kawazoe, I., Jasmani, S., Ohira, T., Wilder, M. N., Kaneko, T., and Aida, K. (2007). Molecular cloning and characterization of cortical rod protein in the giant freshwater prawn Macrobrachium rosenbergii, a species not forming cortical rod structures in the oocytes. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **148**, 184-191.

Kim, Y. K., Tsutsui, N., Kawazoe, I., Okumura, T., Kaneko, T., and Aida, K. (2005). Localization and developmental expression of mRNA for cortical rod protein in kuruma prawn Marsupenaeus japonicus. *Zoological science* **22**, 675-680.

Kishimoto, T. (2003). Cell-cycle control during meiotic maturation. *Current opinion in cell biology* **15**, 654-663.

Klinbunga, S., Sittikankaew, K., Yuvanatemiya, V., Preechaphol, R., Prasertlux, S., Yamano, K., and Menasveta, P. (2009). Molecular cloning and expression analysis of *ovary-specific transcript 1* (*Pm-OST1*) of the giant tiger shrimp, Penaeus monodon. *Zoological science* **26**, 783-790.

Kondoh, E., Konno, A., Inaba, K., Oishi, T., Murata, M., and Yoshida, M. (2008). Valosin-containing protein/p97 interacts with sperm-activating and sperm-attracting factor (SAAF) in the ascidian egg and modulates sperm-attracting activity. *Development, growth & differentiation* **50**, 665-673.

Koshiyama, A., Hamada, F. N., Namekawa, S. H., Iwabata, K., Sugawara, H., Sakamoto, A., Ishizaki, T., and Sakaguchi, K. (2006). Sumoylation of a meiosis-specific RecA homolog, Lim15/Dmc1, via interaction with the small ubiquitin-related modifier (SUMO)-conjugating enzyme Ubc9. *Febs Journal* **273**, 4003-4012.

Kruevaisayawan, H., Vanichviriyakit, R., Weerachatyanukul, W., Magerd, S., Withyachumnarnkul, B., and Sobhon, P. (2007). Biochemical characterization and physiological role of cortical rods in black tiger shrimp, *Penaeus monodon*. *Aquaculture* **270**, 289-298.

Kruevaisayawan, H., Vanichviriyakit, R., Weerachatyanukul, W., Withyachumnarnkul, B., Chavadej, J., and Sobhon, P. (2010). Oogenesis and formation of cortical rods in the black tiger shrimp, *Penaeus monodon*. *Aquaculture* **301**, 91-98.

Kulkarni, J., de Castella, A., Smith, D., Taffe, J., Keks, N., and Copolov, D. (1996). A clinical trial of the effects of estrogen in acutely psychotic women. *Schizophrenia research* **20**, 247-252.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature* **227**, 680-685.

Laufer, H., Biggers, W. J., and Ahl, J. S. (1998). Stimulation of Ovarian Maturation in the Crayfish *Procambarus clarkii* by Methyl Farnesoate. *General and comparative endocrinology* **111**, 113-118.

Leelatanawit, R. (2008). Identification and Characterization of Genes Functionally Related to Growth and Reproduction of The Ginat Tiger Shrimp *Penaeus monodon*, Chulalongkorn University, Thailand.

Leelatanawit, R., Klinbunga, S., Aoki, T., Hirono, I., Valyasevi, R., and Menasveta, P. (2008). Suppression subtractive hybridization (SSH) for isolation and characterization of genes related to testicular development in the giant tiger shrimp *Penaeus monodon*. *BMB Rep* **41**, 796-802.

Leelatanawit, R., Klinbunga, S., Puanglarp, N., Tassanakajon, A., Jarayabhand, P., Hirono, I., Aoki, T., and Menasveta, P. (2004). Isolation and characterization of differentially expressed genes in ovaries and testes of the giant tiger shrimp (*Penaeus monodon*). *Mar Biotechnol* **6**, S506-S510.

Linder, P. (1989). Birth of the DEAD box. *Nature* **337**, 121-122.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J biol chem* **193**, 265-275.

Madsen, L., Kriegenburg, F., Vala, A., Best, D., Prag, S., Hofmann, K., Seeger, M., Adams, I. R., and Hartmann-Petersen, R. (2011). The tissue-specific Rep8/UBXD6 tethers p97 to the endoplasmic reticulum membrane for degradation of misfolded proteins. *PloS one* **6**, e25061.

Martin, G., Jaros, P., Besse, G., and Keller, R. (1984). The hyperglycemic neuropeptide of the terrestrial isopod, *Porcellio dilatatus*. II. Immunocytochemical demonstration in neurosecretory structures of the nervous system. *General and comparative endocrinology* **55**, 217-226.

Masui, Y., and Clarke, H. J. (1979). "Oocyte maturation," Academic Press.

Medina, A., Vila, Y., Mourente, G., and Rodríguez, A. (1996). A comparative study of the ovarian development in wild and pond-reared shrimp, *Penaeus kerathurus* (Forskål, 1775). *Aquaculture* **148**, 63-75.

Meeratana, P., Withyachumnarnkul, B., Damrongphol, P., Wongprasert, K., Suseangtham, A., and Sobhon, P. (2006). Serotonin induces ovarian maturation in giant freshwater prawn broodstock, *Macrobrachium rosenbergii* de Man. *Aquaculture* **260**, 315-325.

Meunpol, O., Iam-Pai, S., Suthikrai, W., and Piyatiratitivorakul, S. (2007). Identification of progesterone and 17α-hydroxyprogesterone in polychaetes (*Perinereis sp*.)

and the effects of hormone extracts on penaeid oocyte development in vitro. *Aquaculture* **270**, 485-492.

Meusy, J.-J., and Payen, G. G. (1988). Female reproduction in malacostracan Crustacea. *Zoological Science* **5**, 217-265.

Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (2000). A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *The EMBO journal* **19**, 2181-2192.

Mishra, A., and Joy, K. P. (2006a). 2-Hydroxyestradiol-17α-induced oocyte maturation in catfish (Heteropneustes fossilis) involves protein kinase C and its interaction with protein phosphatases. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **144**, 416-422.

Mishra, A., and Joy, K. P. (2006b). 2-Hydroxyestradiol-17α-induced oocyte maturation: Involvement of cAMP-protein kinase A and okadaic acid-sensitive protein phosphatases, and their interplay in oocyte maturation in the catfish *Heteropneustes fossilis*. *Journal of Experimental Biology* **209**, 2567-2575.

Mishra, A., and Joy, K. P. (2006c). HPLC-electrochemical detection of ovarian estradiol-17Î² and catecholestrogens in the catfish *Heteropneustes fossilis*: Seasonal and periovulatory changes. *General and Comparative Endocrinology* **145**, 84-91.

Mishra, A., and Joy, K. P. (2006d). Involvement of mitogen-activated protein kinase in 2-hydroxyestradiol-17α-induced oocyte maturation in the catfish *Heteropneustes fossilis* and a note on possible interaction with protein phosphatases. *General and Comparative Endocrinology* **147**, 329-335.

Miura, T., Higuchi, M., Ozaki, Y., Ohta, T., and Miura, C. (2006). Progestin is an essential factor for the initiation of the meiosis in spermatogenetic cells of the eel. *Proceedings of the National Academy of Sciences* **103**, 7333-7338.

Moorman, J. P., Luu, D., Wickham, J., Bobak, D. A., and Hahn, C. S. (1999). A balance of signaling by Rho family small GTPases RhoA, Rac1 and Cdc42 coordinates cytoskeletal morphology but not cell survival. *Oncogene* **18**.

Nigg, E. A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. *Nature reviews Molecular cell biology* **2**, 21-32.

Oe, T., Nakajo, N., Katsuragi, Y., Okazaki, K., and Sagata, N. (2001). Cytoplasmic Occurrence of the Chk1/Cdc25 Pathway and Regulation of Chk1 in *Xenopus* Oocytes. *Developmental biology* **229**, 250-261.

Ohira, T., Tsutsui, N., Nagasawa, H., and Wilder, M. N. (2006). Preparation of two recombinant crustacean hyperglycemic hormones from the giant freshwater prawn, Macrobrachium rosenbergii, and their hyperglycemic activities. *Zoological science* **23**, 383-391.

Okano-Uchida, T., Sekiai, T., Lee, K.-s., Okumura, E., Tachibana, K., and Kishimoto, T. (1998). *In Vivo* Regulation of Cyclin A/Cdc2 and Cyclin B/Cdc2 through Meiotic and Early Cleavage Cycles in Starfish. *Developmental biology* **197**, 39-53.

Okumura, T. (2004). Perspectives on hormonal manipulation of shrimp reproduction. *Japan Agricultural Research Quarterly* **38**, 49-54.

Okumura, T., and Aida, K. (2000). Hemolymph vitellogenin levels and ovarian development during the reproductive and non-reproductive molt cycles in the giant freshwater prawn *Macrobrachium rosenbergii*. *Fisheries Science* **66**, 678-685.

Okumura, T., Kim, Y. K., Kawazoe, I., Yamano, K., Tsutsui, N., and Aida, K. (2006). Expression of vitellogenin and cortical rod proteins during induced ovarian development by eyestalk ablation in the kuruma prawn, *Marsupenaeus japonicus*. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **143**, 246-253.

Okumura, T., and Sakiyama, K. (2004). Hemolymph levels of vertebrate-type steroid hormones in female kuruma prawn *Marsupenaeus japonicus* (Crustacea: Decapoda: Penaeidae) during natural reproductive cycle and induced ovarian development by eyestalk ablation. *Fisheries Science* **70**, 372-380.

Ongvarrasopone, C., Roshorm, Y., Somyong, S., Pothiratana, C., Petchdee, S., Tangkhabuanbutra, J., Sophasan, S., and Panyim, S. (2006). Molecular cloning and functional expression of the *Penaeus monodon* 5-HT receptor. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* **1759**, 328-339.

Palacios, E., Racotta, I. S., and Villalejo, M. (2003). Assessment of ovarian development and its relation to mating in wild and pond-reared *Litopenaeus*

vannamei shrimp in a commercial hatchery. *Journal of the World Aquaculture Society* **34**, 466-477.

Patio, R., Yoshizaki, G., Thomas, P., and Kagawa, H. (2001). Gonadotropic control of ovarian follicle maturation: The two-stage concept and its mechanisms. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **129**, 427-439.

Peixoto, S., Wasielesky Jr, W., Martino, R. C., Milach, Â., Soares, R., and Cavalli, R. O. (2008). Comparison of reproductive output, offspring quality, ovarian histology and fatty acid composition between similarly-sized wild and domesticated *Farfantepenaeus paulensis*. *Aquaculture* **285**, 201-206.

Pesty, A., Avazeri, N., and Lefèvre, B. (1998). Nuclear calcium release by InsP3-receptor channels plays a role in meiosis reinitiation in the mouse oocyte. *Cell Calcium* **24**, 239-251.

Phinyo, M., Visudtiphole, V., Roytrakul, S., Phaonakrop, N., Jarayabhand, P., and Klinbunga, S. (2013). Characterization and expression of *cell division cycle 2* (*Cdc2*) mRNA and protein during ovarian development of the giant tiger shrimp *Penaeus monodon*. *General and comparative endocrinology* **193**, 103-111.

Preechaphol, R., Klinbunga, S., Khamnamtongand, B., and Menasveta, P. (2010). Isolation and characterization of genes functionally involved in ovarian development of the giant tiger shrimp *Penaeus monodon* by suppression subtractive hybridization (SSH). *Genetics and molecular biology* **33**, 676-685.

Preechaphol, R., Leelatanawit, R., Sittikankeaw, K., Klinbunga, S., Khamnamtong, B., Puanglarp, N., and Menasveta, P. (2007). Expressed sequence tag analysis for identification and characterization of sex-related genes in the giant tiger shrimp *Penaeus monodon*. *Journal of biochemistry and molecular biology* **40**, 501-510.

Qiu, G.-F., and Yamano, K. (2005). Three forms of cyclin B transcripts in the ovary of the kuruma prawn *Marsupenaeus japonicus*: Their molecular characterizations and expression profiles during oogenesis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **141**, 186-195.

Qiu, G. F., Yamano, K., and Unuma, T. (2005). Cathepsin C transcripts are differentially expressed in the final stages of oocyte maturation in kuruma prawn *Marsupenaeus japonicus*. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **140**, 171-181.

Quackenbush, J. (2001). Computational analysis of microarray data. *Nature Reviews Genetics* **2**, 418-427.

Quackenbush, L. (1991). Regulation of vitellogenesis in penaeid shrimp. *Developments in aquaculture and fisheries science*.

Rattanarojpong, T., Wang, H. C., Lo, C. F., and Flegel, T. W. (2007). Analysis of differently expressed proteins and transcripts in gills of *Penaeus vannamei* after yellow head virus infection. *Proteomics* **7**, 3809-3814.

Ruuskanen, J. O., Xhaard, H., Marjamäki, A., Salaneck, E., Salminen, T., Yan, Y.-L., Postlethwait, J. H., Johnson, M. S., Larhammar, D., and Scheinin, M. (2004). Identification of duplicated fourth α2-adrenergic receptor subtype by cloning and mapping of five receptor genes in zebrafish. *Molecular biology and evolution* **21**, 14-28.

Sakai, N., Sawada, M. T., and Sawada, H. (2004). Non-traditional roles of ubiquitin–proteasome system in fertilization and gametogenesis. *The international journal of biochemistry & cell biology* **36**, 776-784.

Sambrook, J., and Russell, D. W. (2001). "Molecular Cloning," Cold Spring Harbor Laboratory Press, New York, USA.

Sarojini, R., Nagabhushanam, R., and Fingerman, M. (1995). Mode of action of the neurotransmitter 5-hydroxytryptamine in stimulating ovarian maturation in the red swamp crayfish, *Procambarus clarkii*: An in vivo and in vitro study. *Journal of Experimental Zoology* **271**, 395-400.

Sasagawa, Y., Yamanaka, K., Nishikori, S., and Ogura, T. (2007). *Caenorhabditis elegans* p97/CDC-48 is crucial for progression of meiosis I. *Biochemical and biophysical research communications* **358**, 920-924.

Senior, A., and Gerace, L. (1988). Integral membrane proteins specific to the inner nuclear membrane and associated with the nuclear lamina. *The Journal of cell biology* **107**, 2029-2036.

Senthilkumaran, B., and Joy, K. P. (2001). Periovulatory changes in catfish ovarian oestradiol-17β, oestrogen-2-hydroxylase and catechol-O-methyltransferase

during GnRH analogue-induced ovulation and in vitro induction of oocyte maturation by catecholoestrogens. *Journal of Endocrinology* **168**, 239-247.

Shui, Y., Guan, Z.-B., Xu, Z.-H., Zhao, C.-Y., Liu, D.-X., and Zhou, X. (2012). Proteomic identification of proteins relevant to ovarian development in the red swamp crayfish *Procambarus clarkii*. *Aquaculture* **370**, 14-18.

Sithigorngul, P., Panchan, N., Vilaivan, T., Sithigorngul, W., and Petsom, A. (1999). Immunochemical analysis and immunocytochemical localization of crustacean hyperglycemic hormone from the eyestalk of *Macrobrachium rosenbergii*. *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology* **124**, 73-80.

Sittikankaew, K., Hiransuchalert, R., Yocawibun, P., Yamano, K., and Klinbunga, S. (2010). Identification, characterization and expression of *adipose differentiation-related protein* (*ADRP*) gene and protein in ovaries of the giant tiger shrimp *Penaeus monodon*. *Aquaculture* **308**, S91-S99.

Somboonwiwat, K., Chaikeeratisak, V., Wang, H.-C., Fang Lo, C., and Tassanakajon, A. (2010). Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after Vibrio harveyi infection. *Proteome science* **8**, 39-49.

Song, C., Wang, Q., and Li, C.-C. H. (2003). ATPase activity of p97-valosin-containing protein (VCP) D2 mediates the major enzyme activity, and D1 contributes to the heat-induced activity. *Journal of Biological Chemistry* **278**, 3648-3655.

Soyez, D., Le Caer, J., Noel, P., and Rossier, J. (1991). Primary structure of two isoforms of the Vitellogenesis Inhibiting Hormone from the lobster *Homarus americanus*. *Neuropeptides* **20**, 25-32.

Styrishave, B., Lund, T., and Andersen, O. (2008). Ecdysteroids in female shore crabs *Carcinus maenas* during the moulting cycle and oocyte development. *Journal of the Marine Biological Association of the UK* **88**, 575-581.

Sun, Y. D., Zhao, X. F., Kang, C. J., and Wang, J. X. (2006). Molecular cloning and characterization of Fc-TSP from the Chinese shrimp *Fennerpenaeus chinensis*. *Molecular Immunology* **43**, 1202-1210.

Sutovsky, P., Moreno, R., Ramalho-Santos, J., Dominko, T., Thompson, W. E., and Schatten, G. (2001). A putative, ubiquitin-dependent mechanism for the

recognition and elimination of defective spermatozoa in the mammalian epididymis. *Journal of Cell Science* **114**, 1665-1675.

Sutovsky, P., Moreno, R. D., Ramalho-Santos, J., Dominko, T., Simerly, C., and Schatten, G. (1999). Development: Ubiquitin tag for sperm mitochondria. *Nature* **402**, 371-372.

Talakhun, W. (2008). Identification of genes and proteins related to ovarian development the giant tiger shrimp *Penaeus monodon*, Chulalongkorn University, Thailand.

Talakhun, W., Roytrakul, S., Phaonakrop, N., Kittisenachai, S., Khamnamtong, B., Klinbunga, S., and Menasveta, P. (2012). Identification of reproduction-related proteins and characterization of the *protein disulfide isomerase A6* cDNA in ovaries of the giant tiger shrimp *Penaeus monodon*. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* **7**, 180-190.

Tanner, N. K., and Linder, P. (2001). DExD/H box RNA helicases: from generic motors to specific dissociation functions. *Molecular cell* **8**, 251-262.

Tensen, C. P., Kleun, D. P., and Herp, F. (1991). Cloning and sequence analysis of cDNA encoding two crustacean hyperglycemic hormones from the lobster Homarus americanus. *European journal of biochemistry* **200**, 103-106.

Thorsell, A., Portelius, E., Blennow, K., and Westman-Brinkmalm, A. (2007). Evaluation of sample fractionation using micro-scale liquid-phase isoelectric focusing on mass spectrometric identification and quantitation of proteins in a SILAC experiment. *Rapid communications in mass spectrometry* **21**, 771-778.

Tiu, S. H., He, J.-G., and Chan, S.-M. (2005). Organization and expression study of the shrimp (*Metapenaeus ensis*) putative 5-HT receptor: Up-regulation in the brain by 5-HT. *Gene* **353**, 41-52.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences* **76**, 4350-4354.

Treerattrakool, S., Panyim, S., Chan, S. M., Withyachumnarnkul, B., and Udomkit, A. (2008). Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. *FEBS journal* **275**, 970-980.

Tsukimura, B., Nelson, W., and Linder, C. (2006). Inhibition of ovarian development by methyl farnesoate in the tadpole shrimp, *Triops longicaudatus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **144**, 135-144.

Udomkit, A., Chooluck, S., Sonthayanon, B., and Panyim, S. (2000). Molecular cloning of a cDNA encoding a member of CHH/MIH/GIH family from *Penaeus monodon* and analysis of its gene structure. *Journal of experimental marine biology and ecology* **244**, 145-156.

Udomkit, A., Treerattrakool, S., and Panyim, S. (2004). Crustacean hyperglycemic hormones of *Penaeus monodon*: cloning, production of active recombinant hormones and their expression in various shrimp tissues. *Journal of Experimental Marine Biology and Ecology* **298**, 79-91.

Vaca, A. A., and Alfaro, J. (2000). Ovarian maturation and spawning in the white shrimp, *Penaeus vannamei*, by serotonin injection. *Aquaculture* **182**, 373-385.

Visudtiphole, V., Klinbunga, S., and Kirtikara, K. (2009). Molecular characterization and expression profiles of *cyclin A* and *cyclin B* during ovarian development of the giant tiger shrimp *Penaeus monodon*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **152**, 535-543.

Voronina, E., and Wessel, G. M. (2004). The regulation of oocyte maturation. *Current topics in developmental biology* **58**, 53-110.

Wessel, G. M., Conner, S. D., and Berg, L. (2002). Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte. *Development* **129**, 4315-4325.

Wójcik, C., Yano, M., and DeMartino, G. N. (2004). RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis. *Journal of cell science* **117**, 281-292.

Wongprasert, K., Asuvapongpatana, S., Poltana, P., Tiensuwan, M., and Withyachumnarnkul, B. (2006). Serotonin stimulates ovarian maturation and spawning in the black tiger shrimp *Penaeus monodon*. *Aquaculture* **261**, 1447-1454.

Yamanaka, K., Okubo, Y., Suzaki, T., and Ogura, T. (2004). Analysis of the two p97/VCP/Cdc48p proteins of *Caenorhabditis elegans* and their suppression of

polyglutamine-induced protein aggregation. *Journal of structural biology* **146**, 242-250.

Yamano, K., Qiu, G.-F., and Unuma, T. (2004a). Molecular cloning and ovarian expression profiles of thrombospondin, a major component of cortical rods in mature oocytes of penaeid shrimp, *Marsupenaeus japonicus*. *Biology of reproduction* **70**, 1670-1678.

Yamano, K., Qiu, G. F., and Unuma, T. (2004b). Molecular cloning and ovarian expression profiles of thrombospondin, a major component of cortical rods in mature oocytes of penaeid shrimp, *Marsupenaeus japonicus*. *Biology of Reproduction* **70**, 1670-1678.

Yano, I. (1985). Induced ovarian maturation and spawning in greasyback shrimp, *Metapenaeus ensis*, by progesterone. *Aquaculture* **47**, 223-229.

Yano, I. (1988). Oocyte development in the kuruma prawn *Penaeus japonicus*. *Marine Biology* **99**, 547-553.

Yano, I. (1995). Final oocyte maturation, spawning and mating in penaeid shrimp. *Journal of experimental marine biology and ecology* **193**, 113-118.

Yano, I., and Hoshino, R. (2006). Effects of 17 β-estradiol on the vitellogenin synthesis and oocyte development in the ovary of kuruma prawn (*Marsupenaeus japonicus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **144**, 18-23.

Yodmuang, S., Udomkit, A., Treerattrakool, S., and Panyim, S. (2004). Molecular and biological characterization of molt-inhibiting hormone of *Penaeus monodon*. *Journal of Experimental Marine Biology and Ecology* **312**, 101-114.

Zhang, F. X., Shao, H. L., Wang, J. X., and Zhao, X. F. (2011). β-thymosin is upregulated by the steroid hormone 20-hydroxyecdysone and microorganisms. *Insect molecular biology* **20**, 519-527.

APPENDIX

Appendix A

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon*

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Hemocyanin	113	Oxygen transport	gi|16612121	1	0.94	0.97	1.06	1.12	0.042288803
RNA-binding protein squid	77	mRNA export from nucleus (mRNA 3'-UTR binding)	SQD_DROME	1	0.95	1.02	1.09	1.18	0.00445696
Thrombospondin *Fenneropenaeus chinensis* OV-N-N01-0896-W	40	cell adhesion (calcium ion binding)	gi|000015205	1	0.91	1.02	1.11	1.00	0.04124319
Signal peptide peptidase SppA, 67K type	32	signal peptide processing (peptidase activity)	gi|113970247	1	1.00	1.00	1.16	1.25	0.004596717
Predicted protein	30	-	gi|222869979	1	1.04	0.97	1.03	1.13	0.004848374
tRNA uridine 5-Carboxymethylaminomethyl modification enzyme GidA	30	tRNA methylation (flavin adenine dinucleotide binding)	gi|33866912	1	1.02	1.19	1.10	1.10	0.006317882
FCH domain only 2, partial	27	-	gi|115743065	1	1.00	1.00	1.16	1.25	0.004596717
SMC protein	27	DNA recombination (ATP binding)	gi|28375555	1	0.91	0.95	1.03	0.00	0.006471426
Amidohydrolase	27	nitrogen compound metabolic process (N-acetyltransferase activity)	gi|269926591	1	1.02	1.03	1.13	1.16	0.021946657
AKT1-like potassium channel	26	Ion transport (voltage-gated potassium channel activity)	gi|17887457	1	1.02	1.03	1.13	1.16	0.021946657
V-type ATP synthase subunit D	26	ATP biosynthetic process (proton-transporting ATPase activity, rotational mechanism)	gi|87302997	1	0.91	0.95	1.08	0.00	7.22E-08
Hypothetical protein Oter_3272	26	-	gi|182415085	1	1.02	1.03	1.13	1.16	0.021946657
Pol protein	26	DNA integration (DNA binding)	gi|156140948	1	1.07	1.05	1.24	1.30	0.002572325
Glutamyl-tRNA synthetase 1	25	-	SYE1_NEOSM	1	0.91	1.00	1.10	1.17	0.001112917
Squalene-hopene cyclase	25	hopanoid biosynthetic process (lyase activity)	gi|16519641	1	1.00	1.05	1.09	1.22	0.001687306

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Glyceraldehyde-3-phosphate dehydrogenase 1	24	glycolysis (NAD binding)	G3P1_KLUMA	1	1.01	1.03	1.13	1.17	0.002175479
Glyceraldehyde-3-phosphate dehydrogenase, muscle	24	glycolysis, Apoptosis (NAD binding)	G3P1_JACOR	1	1.01	1.03	1.13	1.17	0.002175479
Hypothetical protein	24	-	gi|296417753	1	1.02	1.19	1.10	1.10	0.006317882
Hypothetical protein LOC100410715	24	-	gi|296209385	1	0.94	0.97	1.05	1.09	9.00E-04
Phosphotyrosine protein phosphatase	24	Peptidyl-tyrosine dephosphorylation (protein tyrosine phosphatase activity)	gi|149188172	1	1.02	1.19	1.10	1.10	0.006317882
Hypothetical protein NAEGRDRAFT_58086	23	-	gi|290988356	1	0.97	1.00	1.09	1.10	0.001991721
Potential zinc-binding dehydrogenase	23	Oxidation-reduction (oxidoreductase activity)	gi|68485591	1	1.00	1.00	1.06	1.11	0.001378242
Ribonuclease PH	23	tRNA processing (tRNA nucleotidyltransferase activity)	gi|109900580	1	0.99	1.02	1.01	1.07	0.019285565
O-sialoglycoprotein endopeptidase	22	(metalloendopeptidase activity)	GCP_DECAR	1	1.01	1.02	1.11	1.24	0.010371256
Retinal dehydrogenase 1	22	response to oxidative stress (retinal dehydrogenase activity)	AL1A1_RABIT	1	0.69	0.00	0.99	1.08	0.004355756
Acetate kinase	22	organic acid metabolic process (ATP binding)	gi|296445450	1	1.02	1.19	1.10	1.10	0.006317882
Diaminopimelate decarboxylase	22	lysine biosynthetic process via diaminopimelate	gi|118602155	1	1.04	0.97	1.03	1.13	0.004848374
Kinesin heavy chain	22	axon cargo transport (plus-end-directed microtubule motor activity)	gi|51316437	1	1.02	1.19	1.10	1.10	0.006317882
ATP-binding region ATPase domain protein	21	peptidyl-histidine phosphorylation (ATP binding)	gi|268680200	1	0.90	0.97	0.97	1.08	0.036500726

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Galactose/methyl galactoside import ATP-binding protein MglA	21	Sugar transport (ATP binding)	MGLA_YERPA	1	1.02	1.03	1.06	1.08	0.027235862
LIM domain containing protein	21	cell migration (zinc ion binding)	gi|170588449	1	0.91	0.90	0.94	1.03	0.037243493
Hypothetical protein	21	-	gi|145497294	1	0.93	1.02	1.07	1.11	0.001118945
Hypothetical protein SELMODRAFT_187364	21	-	gi|300138864	1	0.00	0.93	0.00	1.08	0.015990637
C4-dicarboxylate transport sensor protein	20	peptidyl-histidine phosphorylation (ATP binding)	gi|167568606	1	1.04	1.14	1.10	1.10	0.02556746
DNA mismatch repair protein	20	DNA repair (ATP binding)	gi|56479127	1	0.90	0.97	0.97	1.08	0.036500726
DNA replication factor Cdt1, putative	20	-	gi|242010543	1	1.06	1.08	1.23	1.28	0.013457094
TraG *Comamonas sp. CNB-1*	20	unidirectional conjugation	gi|190572004	1	0.91	0.95	1.08	0.00	7.22E-08
Conjugal transfer protein TrbG	20	conjugation	gi|163858945	1	1.04	1.14	1.10	1.10	0.02556746
Succinate-semialdehyde dehydrogenase	20	gamma-aminobutyric acid catabolic process (succinate-semialdehyde dehydrogenase (NAD+) activity)	gi|254452417	1	0.97	1.03	1.09	1.14	0.005710353
Sugar transport system permease protein	20	carbohydrate transport (transporter activity)	gi|291459418	1	1.02	1.01	1.13	1.24	1.71E-04
DNA-directed RNA polymerase, beta subunit/140 kD subunit	19	Transcription (DNA binding)	gi|294085951	1	0.94	0.97	1.06	1.12	0.042288803
Fis family GAF modulated sigma54 specific transcriptional regulator	19	Transcription regulation	gi|114321883	1	0.97	1.00	1.09	1.10	0.001991721
30S ribosomal protein S3	18	translation (SSU rRNA binding)	gi|72163039	1	1.09	1.00	1.09	1.10	0.022005359

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Voltage-dependent L-type calcium channel subunit alpha-1S	18	Calcium transport (voltage-gated calcium channel activity)	CAC1S_CHICK	1	0.90	0.97	0.97	1.08	0.036500726
Pyrrolo-quinoline quinone	17	Oxidation-reduction (oxidoreductase activity, acting on CH-OH group of donors)	gi|196232348	1	0.91	1.02	1.11	1.00	0.04124319
SH3 and PX domain-containing protein 2A	17	cell communication (phosphatidylinositol binding)	gi|157820515	1	0.96	0.94	1.01	1.14	0.002949992
Sphingomyelinase C	17	cytolysis (sphingomyelin phosphodiesterase activity)	gi|229095326	1	0.98	1.02	1.08	1.12	0.015446262
Vacuolar protein sorting 26, putative	17	vacuolar transport	gi|159107953	1	1.07	1.05	1.24	1.30	0.002572325
Acetyl-coenzyme A synthetase	17	acetyl-CoA biosynthetic process (AMP binding)	gi|114326929	1	0.91	1.02	1.11	1.00	0.04124319
Ammelide aminohydrolase	17	(hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds)	gi|12644702	1	1.06	1.08	1.23	1.28	0.013457094
Copper ion binding / methyltransferase	17	-	gi|30697060	1	1.06	1.08	1.23	1.28	0.013457094
Disco-interacting protein, putative	17	Metabolic process (catalytic activity)	gi|240973890	1	1.01	1.01	1.04	1.22	9.43E-05
Peptidase M24 family protein	17	cellular process (metalloexopeptidase activity)	gi|66812452	1	0.96	0.78	1.14	0.00	0.022774031
Predicted protein	17	-	gi|168042089	1	0.98	0.98	1.07	1.22	0.001830104
Biotin synthase	16	biotin biosynthetic process (2 iron, 2 sulfur cluster binding)	BIOB_NITHX	1	0.95	0.98	1.07	1.07	0.037908323
Chaperone protein htpG	16	protein folding (ATP binding)	HTPG_ECOHS	1	1.05	1.09	1.05	1.12	0.001031452
Lysine exporter protein (LYSE/YGGA)	16	amino acid transport	gi|269125145	1	1.02	1.19	1.10	1.10	0.006317882

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
MFS monocarboxylate transporter (Mct), putative	16	transmembrane transport (transporter activity)	gi|119482369	1	0.91	1.02	1.11	1.00	0.04124319
Protein kti12	16	Transcription regulation (ATP binding, chromatin binding)	gi|254567335	1	1.00	1.00	1.16	1.25	0.004596717
TCP protein	16	-	gi|37726698	1	1.02	1.04	1.09	1.14	0.008524377
Trigger factor	16	de novo' cotranslational protein folding (peptidyl-prolyl cis-trans isomerase activity)	TIG_LACLS	1	0.99	1.01	1.06	1.09	0.014510365
Uncharacterized protein FLJ40521	16	-	YH007_HUMAN	1	0.94	0.98	1.03	1.14	0.001738786
Conjugal transfer protein TrbI	16	conjugation	gi|260655648	1	0.91	1.02	1.11	1.00	0.04124319
Dystonin, partial	16	Cell adhesion, regulation of microtubule polymerization or depolymerization (actin binding, microtubule plus-end binding)	gi|149436017	1	1.09	1.00	1.09	1.10	0.022005359
Hypothetical protein	16	-	gi|296416293	1	1.05	1.03	1.08	1.15	3.70E-04
Hypothetical protein	16	-	gi|70944686	1	1.00	1.00	1.16	1.25	0.004596717
Lipoprotein	16	-	gi|226952572	1	1.01	1.01	1.07	1.11	0.005041977
Phosphopentomutase	16	nucleotide metabolic process	gi|172057039	1	1.06	1.08	1.23	1.28	0.013457094
Protein of unknown function DUF58	16	-	gi|222528472	0	0.00	1.00	1.02	1.03	9.29E-04
Transposase, IS116/IS110/IS902 family	16	transposition, DNA-mediated (DNA binding)	gi|282879224	1	1.02	0.99	1.04	1.10	0.021498727
Unnamed protein product	16	-	gi|47213656	1	1.01	1.02	1.07	0.00	2.41E-11
Extracellular ligand-binding receptor	15	amino acid transport (receptor activity)	gi|239820623	1	1.09	1.00	1.09	1.10	0.022005359

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Glutamate-1-semialdehyde 2,1-aminomutase	15	porphyrin-containing compound biosynthetic process (glutamate-1-semialdehyde 2,1-aminomutase activity)	GSA_PROM5	1	0.91	0.95	1.08	0.00	7.22E-08
Paired box protein Pax-5	15	cell differentiation (DNA binding)	PAX5_HUMAN	1	0.94	1.04	1.10	1.15	0.01949566
Alpha adaptin-like protein, putative	15	vesicle-mediated transport (binding)	gi|86171157	1	0.99	1.04	1.11	1.10	0.009711495
Deoxyguanosinetriphosphate triphosphohydrolase	15	GTP metabolic process (dGTPase activity)	gi|188583269	1	0.98	0.97	1.16	1.15	0.032421067
Hypothetical protein BACCAC_01249	15	-	gi|153806973	1	1.09	1.02	1.06	1.23	1.45E-04
Proton-translocating NADH-quinone oxidoreductase, chain M subfamily	15	mitochondrial electron transport, NADH to ubiquinone (NADH dehydrogenase (ubiquinone) activity)	gi|254445382	1	0.99	1.04	1.11	1.10	0.009711495
Uracil-DNA glycosylase	15	DNA repair (uracil DNA N-glycosylase activity)	gi|227504428	1	0.95	0.96	1.07	1.09	0.013199708
Zinc-binding dehydrogenase	15	Oxidation-reduction process (nucleotide binding)	gi|188535420	1	0.99	1.04	1.11	1.10	0.009711495
Antibiotic biosynthesis monooxygenase	14	antibiotic biosynthetic process (monooxygenase activity)	gi|152965462	1	0.98	1.02	1.20	1.15	0.002800226
Carboxylate-amine ligase Noca_3311	14	glutathione biosynthetic process(ATP binding)	CAAL2_NOCSJ	1	0.88	0.92	1.08	0.00	3.19E-08
Chemotaxis response regulator protein-glutamate methylesterase	14	chemotaxis (Hydrolase)	CHEB_SHEFN	1	0.88	0.92	1.08	0.00	3.19E-08
DEAD/DEAH box helicase	14	ATP catabolic process (ATP binding)	gi|301062611	1	1.00	1.10	1.17	1.21	1.14E-05

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
G2/mitotic-specific cyclin-B	14	G2/M transition of mitotic cell cycle (cyclin-dependent protein kinase regulator activity)	CCNB_ASTPE	1	0.94	1.01	1.08	0.00	4.58E-12
Lipoyl synthase	14	protein lipoylation (4 iron, 4 sulfur cluster binding)	LIPA_PARDP	1	0.88	0.92	1.08	0.00	3.19E-08
Magnesium transport protein corA	14	Ion transport (cobalt ion transmembrane transporter activity)	CORA_METJA	1	0.88	0.92	1.08	0.00	3.19E-08
UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	14	lipid A biosynthetic process (N-acyltransferase activity)	LPXD_PSYCK	1	0.88	0.92	1.08	0.00	3.19E-08
Hypothetical protein HNE_2434	14	-	gi|114798242	1	1.03	0.97	1.16	1.21	0.001494441
Hypothetical protein NECHADRAFT_15869	14	-	gi|256720222	1	1.09	0.95	0.00	0.96	0.015496448
Major facilitator superfamily permease	14	Transport (transmembrane transporter activity)	gi|23308910	1	1.07	0.95	1.17	0.98	0.019548474
Phosphonate ABC transporter, Periplasmic phosphonate-binding protein	14	Transmembrane transport (ATP binding)	gi|113474247	1	0.94	0.99	1.11	1.08	0.018720636
Pyruvate carboxyltransferase	14	leucine biosynthetic process (2-isopropylmalate synthase activity)	gi|281357417	1	1.01	1.01	1.04	1.22	9.43E-05
rCG38503, isoform CRA_b	14	-	gi|149033955	1	0.98	0.97	1.16	1.15	0.032421067
Radical SAM family protein	14	Metabolic process (catalytic activity)	gi|83591239	1	0.94	0.99	1.11	1.08	0.018720636
Transglutaminase-like enzyme, putative cysteine protease	14	proteolysis (peptidase activity)	gi|15895675	1	0.89	0.91	1.03	1.15	0.004544464
ATP-dependent protease ATPase subunit HslU	13	response to heat (ATP binding)	HSLU_SILPO	1	1.09	1.13	1.29	0.00	0.003036167

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	13	Electron transport (metal ion binding)	NDUV1_BOVIN	1	1.09	1.13	1.29	0.00	0.003036167
FMN-binding negative transcriptional regulator	13	Oxidation-reduction (FMN binding)	gi|121606632	1	0.00	1.01	1.07	1.16	0.004611616
NADH-quinone oxidoreductase subunit H	13	(NADH dehydrogenase (quinone) activity)	NUOH_ROSDO	1	1.09	1.13	1.29	0.00	0.003036167
Conserved hypothetical protein	13	-	gi|115401770	1	0.94	0.97	1.06	1.12	0.042288803
Hypothetical protein ANACAC_02610	13	-	gi|167747881	1	1.01	1.05	1.10	1.14	0.004147349
Hypothetical protein Rru_A2166	13	-	gi|83593501	1	0.99	0.97	1.16	1.26	0.005768772
Nickel-dependent hydrogenase large subunit	13	Oxidation-reduction (ferredoxin hydrogenase activity)	gi|296272893	1	0.98	0.83	1.07	0.00	0.020424636
Phytochrome Agp1	13	peptidyl-histidine phosphorylation (ATP binding)	gi|51243765	1	0.92	0.99	0.97	1.24	0.017018035
tRNA and rRNA cytosine-C5-methylase	13	regulation of transcription, DNA-dependent (RNA binding)	gi|110638264	1	1.02	1.01	1.06	1.17	4.03E-05
Actin, acrosomal process isoform	12	RNA-dependent DNA replication (RNA binding)	ACTA_LIMPO	1	1.04	1.05	1.04	1.11	0.04473307
DNA mismatch repair protein MutS	12	DNA repair (ATP binding)	gi|17988084	1	1.20	1.10	0.98	1.29	0.006748606
Nucleoprotein	12	(RNA binding)	NCAP_MILVL	1	1.06	1.00	1.12	0.00	1.86E-06
Phosphoglucosamine mutase	12	UDP-N-acetylglucosamine biosynthetic process	GLMM_PROMP	1	0.95	0.99	1.10	1.14	0.036934517
Succinyl-CoA ligase [ADP-forming] subunit beta	12	tricarboxylic acid cycle (ATP binding)	SUCC_ECOBW	1	1.01	1.00	1.05	1.19	1.68E-04
Acyl-Coenzyme A oxidase 1	12	fatty acid beta-oxidation (acyl-CoA dehydrogenase activity)	gi|55741614	1	1.04	0.97	1.03	1.13	0.004848374

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Hypothetical protein	12	-	gi|225431149	1	0.93	1.02	1.07	1.11	0.001118945
Hypothetical protein Kcr_0722	12	-	gi|170290339	1	0.88	0.91	1.05	0.96	0.048885066
Hypothetical protein MPER_06122	12	-	gi|238596436	1	1.04	0.97	1.03	1.13	0.004848374
Hypothetical protein T22B7.7	12	-	gi|17569955	1	1.05	1.03	1.08	1.15	3.70E-04
Oxidoreductase/ transcriptional repressor	12	Oxidation-reduction (binding)	gi|297844608	1	0.91	0.90	0.94	1.03	0.037243493
Possible glucokinase	12	carbohydrate phosphorylation (Transferase activity)	gi|227874553	1	0.97	0.98	1.09	0.00	0.001194695
Transcription-repair coupling factor	12	regulation of transcription, DNA-dependent (ATP binding)	gi|298369031	1	1.03	1.00	1.06	1.09	0.026793158
Adaptive-response sensory-kinase sasA	11	peptidyl-histidine phosphorylation (ATP binding)	SASA_PROMA	1	0.93	1.03	1.10	1.09	0.04420454
Chromosomal replication initiator DnaA domain protein	11	DNA replication initiation (ATP binding)	gi|258406588	1	1.00	1.07	1.09	1.15	5.38E-04
L-carnitine dehydratase/bile acid-inducible protein F	11	Metabolic process (catalytic activity)	gi|254293873	1	1.00	1.03	1.18	1.17	0.001062525
Neuroplastin	11	Cell adhesion	NPTN_HUMAN	1	0.94	1.02	1.08	1.13	0.002158625
Cytochrome P450	11	Oxidation-reduction (electron carrier activity)	gi|224066581	1	1.00	1.03	1.18	1.17	0.001062525
Flavoprotein monooxygenase	11	aromatic compound catabolic process (monooxygenase activity)	gi|159469768	1	1.00	1.03	1.18	1.17	0.001062525
Integral membrane sensor signal transduction histidine kinase	11	peptidyl-histidine phosphorylation (kinase, transferase)	gi|253575400	1	1.00	1.03	1.18	1.17	0.001062525
Membrane protein M1	11	-	gi|61612084	1	1.00	1.03	1.18	1.17	0.001062525
Nuclear cap-binding protein subunit 1	11	RNA splicing (protein binding)	gi|259155144	1	1.00	1.07	1.09	1.15	5.38E-04

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Unnamed protein product	11	-	gi|47230656	1	0.95	1.01	1.03	1.13	0.034732834
Unnamed protein product	11	-	gi|170940313	1	0.94	0.94	1.00	1.06	3.12E-05
AdoMet-dependent methyltransferase involved in rRNA processing and 60S ribosomal subunit maturation	10	RNA methylation (Methyltransferase activity)	gi|6319796	1	1.05	1.03	1.08	1.15	3.70E-04
Aspartate carbamoyltransferase	10	Pyrimidine biosynthesis (protein binding)	PYRB_EHRCR	1	0.93	0.90	1.02	0.00	0.047206968
UPF0725 protein At1g02770	10	-	Y1277_ARATH	1	0.95	1.04	1.17	1.14	0.005967635
Uncharacterized oxidoreductase YKL107W	10	Oxidation-reduction (nucleotide binding)	YKK7_YEAST	1	0.95	0.96	1.07	1.21	0.004572335
Carbamate kinase	10	cellular amino acid biosynthetic process (carbamate kinase activity)	gi|146312782	1	1.02	1.01	1.06	1.17	4.03E-05
Glycine dehydrogenase	10	glycine catabolic process (decarboxylating activity)	GCSPA_CARH Z	1	1.02	1.01	1.09	1.13	0.043270014
Transporter	10	-	gi|299143613	1	1.00	1.03	1.18	1.17	0.001062525
Unknown *Zea mays*	10	-	gi|219887593	1	1.00	1.00	1.06	1.11	0.001378242
Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase	9	nucleoside biosynthetic process	COBT_GEOBB	1	0.96	1.01	1.03	1.13	9.56E-04
Peptide chain release factor 1	9	regulation of translational termination (translation release factor activity, codon specific)	RF1_HAEPS	1	0.94	1.01	1.08	0.00	4.58E-12
Sel1 domain-containing protein	9	Transport (binding)	gi|154246047	1	0.91	1.02	1.11	1.00	0.04124319

Table A1 Ovarian proteins showing significantly different expression profiles in different groups of *P. monodon* **(Cont.)**

Protein name	Score	Function	Accession no.	Relative Intensity Ratio					*P*-value
				A	B	C	D	E	
Uncharacterized protein ybdO	9	-	YBDO_BACSU	1	0.94	0.99	1.08	1.09	0.047102094
Haemoglobin type 1	9	Oxygen transport (heme binding)	gi|110319967	1	0.98	0.94	1.02	1.17	0.044588502
Elongation factor 1-alpha	9	tRNA export from nucleus (translation elongation factor activity)	gi|202071408	1	1.04	0.97	1.03	1.13	0.004848374
Nicotinate phosphoribosyltransferase	9	NAD biosynthetic process	gi|86144071	1	1.02	1.01	1.06	1.17	4.03E-05
Succinate ubiquinone oxidoreductase subunit 2	9	-	gi|11466549	1	1.08	1.02	1.16	1.21	0.027727414
Zinc finger, CCHC domain containing 8	9	RNA splicing (nucleic acid binding)	gi|221117253	1	1.02	1.01	1.06	1.17	4.03E-05
4-hydroxythreonine-4-phosphate dehydrogenase	8	pyridoxine biosynthetic process (4-hydroxythreonine-4-phosphate dehydrogenase activity)	PDXA_METPB	1	1.01	1.02	1.11	1.24	0.010371256
Cyclic AMP-responsive element-binding protein 3	8	Transcription regulation, response to unfolded protein	CREB3_BOVIN	1	0.94	1.01	1.08	0.00	4.58E-12
DNA-binding protein RHL1	8	DNA endoreduplication (DNA binding)	RHL1_ARATH	1	0.97	1.06	1.04	1.10	0.027226975
MSV199 domain-containing protein 468L	8	-	468L_IIV6	1	0.97	1.04	1.14	1.21	0.001141856
Tyrosyl-tRNA synthetase	8	tyrosyl-tRNA aminoacylation (ATP binding)	SYY_PICTO	1	0.96	0.96	1.07	1.12	0.006018259
CCA-adding enzyme	7	RNA repair (ATP binding)	CCA_STRMU	1	0.99	0.98	1.11	1.04	0.042009026
Polyprotein	7	transcription, DNA-dependent (RNA binding)	gi|609608	1	0.95	0.95	1.02	1.05	0.015917271
Integrator complex subunit 1 isoform 1	4	DNA repair (protein binding)	gi|126334195	1	0.94	0.94	1.00	1.06	3.12E-05
KIAA1440	4	-	gi|118097766	1	0.94	0.94	1.00	1.06	3.12E-05
Hypothetical protein LOC100304460	4	-	gi|259013199	1	0.94	0.94	1.00	1.06	3.12E-05
KIAA1440	4	-	gi|118097766	1	0.94	0.94	1.00	1.06	3.12E-05
Hypothetical protein *Paramecium tetraurelia strain d4-2*	3	-	gi|145349836	1	1.01	0.96	1.04	1.17	3.32E-04

Table A2 Nuclear membrane proteins in ovaries of *P. monodon* identified by 1-DE

Protein name	Acc. No.	Function	Cellular component	Score
Lysophospholipid acyltransferase 5-like *Bombus impatiens*	gi|00015152	(transferase activity, transferring acyl groups)	integral to membrane	14.43
ABC transporter *Rhizobium leguminosarum bv. trifolii WSM2304*	gi|209546740	ATP catabolic process (ATP binding)	integral to membrane	17.05
ABC transporter-like protein *Bacillus coagulans 36D1*	gi|347752136	ATP catabolic process (ATPase activity, coupled to transmembrane movement of substances)	integral to membrane	24.63
Ankyrin repeat-containing protein	gi|00006035	(nucleotide binding)	integral to membrane	7.26
Ankyrin repeats-like	gi|00011093	(ion channel activity)	integral to membrane	12.14
Aspartyl/asparaginyl beta-hydroxylase *Candidatus Koribacter versatilis Ellin345*	gi|94969766	calcium ion transmembrane transport (oxidoreductase activity)	integral to membrane	11.5
ATP lipid-binding protein like protein	gi|00003306	ATP hydrolysis coupled proton transport (hydrogen ion transmembrane transporter activity)	integral to membrane	6.14
ATP synthase F0F1 subunit delta *Chlorobium tepidum TLS*	gi|21672859	ATP synthesis coupled proton transport (hydrogen ion transporting ATP synthase activity)	integral to membrane	11.3
ATP/ADP translocase *Pacifastacus leniusculus*	gi|00018032	(ATP binding)	integral to membrane	10.47
Bcr/CflA subfamily drug resistance transporter *Chloroflexus aggregans DSM 9485*	gi|219848827	transmembrane transport (transporter activity)	integral to membrane	17.38
Beta-amyloid precursor-like protein	gi|00014740	neuron projection morphogenesis (transition metal ion binding)	integral to membrane	9.2
Binding-protein-dependent transport systems inner membrane component *Burkholderia phymatum STM815*	gi|186471608	(transporter activity)	integral to membrane	4.3
Calcium-activated chloride channel family member 1	gi|00010434		integral to membrane	8.68

Table A2 Nuclear membrane proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
CapK; capsular polysaccharide biosynthesis protein *Sorangium cellulosum 'So ce 56'*	gi|162456127	capsule polysaccharide biosynthetic process (sugar efflux transmembrane transporter activity)	integral to membrane	6.32
Carbohydrate sulfotransferase 11	gi|00009798	carbohydrate biosynthetic process (N-acetylgalactosamine 4-O-sulfotransferase activity)	integral to membrane	13.35
Carrier protein YMC2 *Verticillium albo-atrum VaMs.102*	gi|302422118	(organic acid transmembrane transporter activity)	integral to membrane	6.73
Cation diffusion facilitator family transporter *Mycobacterium rhodesiae JS60*	gi|353193082	cellular response to zinc ion (zinc ion transmembrane transporter activity)	integral to membrane	10.19
CD63 antigen	gi|00002901	cellular protein localization	integral to membrane	16.79
Competence protein *CelB Streptococcus pneumoniae R6*	gi|15902901	establishment of competence for transformation (hydrolase activity)	integral to membrane	11.86
Cytochrome b *Penaeus monodon*	gi|00015927	respiratory electron transport chain (electron carrier activity)	integral to membrane	3.56
Cytochrome c assembly protein *Deinococcus geothermalis DSM 11300*	gi|94985348	respiratory chain complex IV assembly	integral to membrane	3.06
Cytochrome c oxidase assembly protein COX15 *Culex quinquefasciatus*	gi|00003984	heme a biosynthetic process (cytochrome-c oxidase activity)	integral to membrane	9.53
Cytochrome c oxidase subunit 1 *Spizellomyces punctatus*	gi|15147305	electron transport chain (electron carrier activity)	integral to membrane	10.37
Cytochrome c oxidase subunit II	gi|00008923	respiratory electron transport chain (copper ion binding)	integral to membrane	26.34
Cytochrome oxidase III *Nectarinia talatala*	gi|37928597	aerobic electron transport chain (cytochrome-c oxidase activity)	integral to membrane	9.07
Cytochrome P450 *Pseudonocardia sp. P1*	gi|324998845	(electron carrier activity)	integral to membrane	23.37
Cytochrome P450 CYP6BK17	gi|00009370	(electron carrier activity)	integral to membrane	15

Table A2 Nuclear membrane proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Delta12 fatty acid desaturase	gi|00013583	fatty acid biosynthetic process (oxidoreductase activity)	integral to membrane	10.93
DNA segregation ATPase FtsK/SpoIIIE and related proteins *Butyrivibrio fibrisolvens 16/4*	gi|291520655	cell cycle (ATP binding)	integral to membrane	6.64
ER lumen protein retaining receptor, putative	gi|00014384	protein transport (ER retention sequence binding)	integral to membrane	10.24
Exostosin-like 3 protein *Clonorchis sinensis*	gi|358335223	heparan sulfate proteoglycan biosynthetic process (metal ion binding)	integral to membrane	11.35
Formate dehydrogenase subunit beta *Stenotrophomonas maltophilia R551-3*	gi|194367034	cellular respiration (electron carrier activity)	integral to membrane	8.23
Gap junction Cx32.2 protein *Anoplopomafimbria*	gi|229367180	cell communication	integral to membrane	16.71
Glucose-6-phosphate translocase	gi|00008383	glucose homeostasis (glucose-6-phosphate transmembrane transporter activity)	integral to membrane	15.58
Glycoprotein gp2 *Equine herpesvirus 1*	gi|00018435	pathogenesis	integral to membrane	8.92
Heavy metal translocating P-type ATPase *Methylococcus capsulatus str. Bath*	gi|53804062	copper ion transport (ATP binding)	integral to membrane	27.35
Hypothetical transmembrane protein *Flavobacteria bacterium BBFL7*	gi|89890098	electron transport chain (protein disulfide oxidoreductase activity)	integral to membrane	8.99
Integral membrane sensor signal transduction Histidine kinase *Haliscomenobacter hydrossis DSM1100*	gi|332666709	signal transduction by phosphorylation (ATP binding)	integral to membrane	15.26
K+-transporting ATPase subunit B *Kineococcus radiotolerans SRS30216*	gi|152964985	(potassium-transporting ATPase activity)	integral to membrane	13.81
Large-conductance mechanosensitive channel *Paenibacillus larvae subsp. larvae BRL-230010*	gi|167465900	cellular water homeostasis (mechanically-gated ion channel activity)	integral to membrane	5.6
Leucine-rich receptor-like protein kinase-like *Oryza sativa Japonica Group*	gi|42408846	(ATP binding)	integral to membrane	9.06
Longevity assurance protein *Tetrahymena thermophila*	gi|118400719		integral to membrane	18.46

Table A2 Nuclear membrane proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Lysosomal-associated transmembrane protein *Aedes aegypti*	gi|00008532	transport	integral to membrane	15.23
Major facilitator transporter *Dietzia cinnamea P4*	gi|319949639	(transmembrane transporter activity)	integral to membrane	15.49
Methyl-accepting chemotaxis protein *Rhizobium etli CFN 42*	gi|86360980	chemotaxis (signal transducer activity)	integral to membrane	8.86
Methyl-accepting chemotaxis sensory transducer *Allochromatium vinosum DSM 180*	gi|288940151	(signal transducer activity)	integral to membrane	16.03
MFS alpha-glucoside transporter, putative *Talaromyces stipitatus ATCC 10500*	gi|242810901	Transport (substrate-specific transmembrane transporter activity)	integral to membrane	20.02
Multidrug ABC transporter ATPase and permease *Slackia heliotrinireducens DSM20476*	gi|257064337	ATP catabolic process (ATP binding)	integral to membrane	14.79
Na+/H+ antiporter NhaC *Shewanella pealeana ATCC 700345*	gi|157963239	(antiporter activity)	integral to membrane	7.48
Na+-driven multidrug efflux pump transmembrane protein, multi antimicrobial extrusion protein mate *Ralstonia solanacearum PSI07*	gi|300694222	(antiporter activity)	integral to membrane	11.14
Notch homolog	gi|00012008	cell differentiation (calcium ion binding)	integral to membrane	16.54
Omega-3 fatty acid desaturase transmembrane protein *Sorangium cellulosum 'So ce 56'*	gi|162457415	lipid metabolic process	integral to membrane	11.11
Outer membrane protein *Haemophilus parasuis 29755*	gi|167856673	ion transport (porin activity)	integral to membrane	9.55
PAS/PAC sensor signal transduction histidine kinase *Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293*	gi|116619022	regulation of transcription, DNA-dependent	integral to membrane	14.42
Pentatricopeptide repeat-containing protein *Medicago truncatula*	gi|357490817	(nucleic acid binding)	integral to membrane	13.04
Permease of the major facilitator superfamily protein *Alteromonas macleodii ATCC 27126*	gi|239992950	transmembrane transport	integral to membrane	9.33
gp5 *Enterobacteria phage ES18*	gi|62362218	viral attachment to host cell	integral to membrane	14.23

Table A2 Nuclear membrane proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Protein dpy-19, putative	gi|00012164		integral to membrane	9.86
Rhomboid family protein *Micromonospora aurantiaca ATCC 27029*	gi|302865368	proteolysis (serine-type endopeptidase activity)	integral to membrane	7.94
Ribophorin I	gi|00003116	protein glycosylation (dolichyl-diphosphooligosacchanide-protein glycotransferase activity)	integral to membrane	13.23
RING finger protein, putative	gi|00007656	protein ubiquitination (zinc ion binding)	integral to membrane	14.17
Ryanodine receptor *Homarus americanus*	gi|00016831	cellular calcium ion homeostasis (calcium ion binding)	integral to membrane	29.42
Semaphorin-1A	gi|00011383	negative regulation of cell size (receptor activity)	integral to membrane	11.95
Semaphorin-4G	gi|13633937	cell differentiation	integral to membrane	21.35
Sensor protein RcsC *Citrobacter sp. 30_2*	gi|237732270	intracellular signal transduction (ATP binding)	integral to membrane	17.53
Signal peptidase complex subunit 3	gi|00000755	signal peptide processing (peptidase activity)	integral to membrane	9.01
Signal peptide peptidase SppA *Sphingobium chlorophenolicum L-1*	gi|334344778	signal peptide processing (peptidase activity)	integral to membrane	9.11
Signal transduction histidine kinase, glucose-6-phosphate specific *Methylobacillus flagellatus KT*	gi|91775170	signal transduction by phosphorylation (ATP binding)	integral to membrane	5.47
Signal transduction histidine-protein kinase afsQ2 *Streptomyces sp. e14*	gi|294631082	signal transduction by phosphorylation (ATP binding)	integral to membrane	8.18
Sodium:alanine symporter *Bacillus sp. SG-1*	gi|149181621	alanine:sodium symporter activity	integral to membrane	4.62
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3 *Xenopus (Silurana) tropicalis*	gi|183986737	(amino acid transmembrane transporter activity)	integral to membrane	7.44
Voltage-dependent anion-selective channel isoform 1	gi|00001786	apoptotic process (nucleotide binding)	pore complex	8.85

Table A2 Nuclear membrane proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Sre1 cleavage activating protein Scp1 *Schizosaccharomyces pombe 972h-*	gi|19113465		integral to membrane	11.19
Tthiamine import ATP-binding protein ThiQ *Citreicella sp. SE45*	gi|260427500	ATP catabolic process (ATP binding)	integral to membrane	10.92
Transmembrane protein 102 *Camelus ferus*	gi|00018394	regulation of apoptotic process	integral to membrane	22.92
Transmembrane protein 50A	gi|00006876		integral to membrane	14.24
TRAP dicarboxylate transporter, DctM subunit *Yersinia rohdei ATCC 43380*	gi|238752902		integral to membrane	6.91
Tumor necrosis factor ligand	gi|00004193	angiogenesis	integral to membrane	16.39
Type II Secretion System PilC *alpha proteobacterium HIMB114*	gi|262277340	protein secretion	integral to membrane	13.56
Vacuolar proton ATPase	gi|00008262	ATP hydrolysis coupled proton transport	integral to membrane	10.78
VPU protein *Human immunodeficiency virus 1*	gi|00007581	apoptotic process (nucleotide binding)	integral to membrane	1.67
ANKTM1 *Drosophila melanogaster*	gi|00004630	(ion channel activity)	intergral to membrane	18.3
Poly [ADP-ribose] polymerase 1	gi|00011674	DNA damage response, detection of DNA damage (DNA binding)	nuclear envelope	22.03
Proliferating cell nuclear antigen (pcna)	gi|00012086	DNA replication (DNA binding)	nuclear lamina	12.85
Cytoplasmic dynein 1 intermediate chain, putative *Ixodes scapularis*	gi|00016124	axon cargo transport (motor activity)	nuclear membrane	7.52
Golgi phosphoprotein 3 (coat-protein GPP34)	gi|00005045	cell proliferation (lipid binding)	nuclear membrane	9.1
Ran gtpase-activating protein	gi|00004850	mitotic cell cycle (Ran GTPase activator activity)	nuclear membrane	16.82
Nucleoporin 50kDa	gi|00004023	intracellular transport	nuclear pore	15.84
Inner-membrane translocator *Thalassiobium sp. R2A62*	gi|255261884	protein transport (protein tyrosine phosphatase activity)	nuclear speck	6.36

Table A3 Nuclear proteins in ovaries of *P. monodon* identified by 1-DE

Protein name	Acc. No.	Function	Cellular component	Score
ATP-dependent RNA helicase	gi|00011591	endonucleolytic cleavage in 5'-ETS of tricistronic rRNA transcript (ATP binding)	nucleolus	23.6
DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	gi|00011274	ATP binding	nucleolus	10.34
DEAD (Asp-Glu-Ala-Asp) box polypeptide 23, partial *Strongylocentrotus purpuratus*	gi|72086508	ATP binding	nucleolus	23.64
DEAD/DEAH box helicase domain-containing protein *Desulfurispirillum indicum S5*	gi|317052319	ATP binding	nucleolus	11.24
DNA-directed RNA polymerase II, putative	gi|00012757	7-methylguanosine mRNA capping (DNA binding)	nucleolus	12.76
Exosome complex exonuclease RRP40 *Acromyrmex echinatior*	gi|00017001	nonfunctional rRNA decay (RNA binding)	nucleolus	2.67
p21-activated protein kinase-interacting protein 1-like *Caligus rogercresseyi*	gi|225710414	negative regulation of signal transduction	nucleolus	23.1
Nuclear transcription factor Y, alpha	gi|00013181	transcription, DNA-dependent (DNA binding)	nucleoplasm	18.38
26S protease regulatory subunit 6A-B *Uncinocarpus reesii 1704*	gi|258563624	protein catabolic process (ATP binding)	nucleus	15.09
26S protease regulatory subunit 8	gi|00012887	protein catabolic process (ATP binding)	nucleus	11.33
26S proteasome non-ATPase regulatory subunit 2 *Ascaris suum*	gi|324502812	hyperosmotic response (endopeptidase activity)	nucleus	17.09
60S acidic ribosomal protein P0	gi|00010441	SRP-dependent cotranslational protein targeting to membrane (RNA binding)	nucleus	8.01
Apoptosis regulator BAX *Lepeophtheirus salmonis*	gi|000038354	apoptotic DNA fragmentation (channel activity)	nucleus	12.79
Bromodomain-containing protein, putative *Ixodes scapularis*	gi|000016060	chromatin modification (chromatin binding)	nucleus	12.62
Cell division control protein Cdc6 *Aspergillus fumigatus Af293*	gi|70987197	cell division (ATP binding)	nucleus	6.59
Cell division cycle 2 protein	gi|00007188	cell division (cyclin-dependent protein kinase activity)	nucleus	11.63
Cell division cycle 6 protein-like *Saccoglossus kowalevskii*	gi|000055840	cell division (cyclin-dependent protein kinase activity)	nucleus	14.55

Table A3 Nuclear proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Centromere protein A isoform 2 *Canis lupus familiaris*	gi|70568888	nucleosome assembly (DNA binding)	nucleus	25.37
Cold shock protein *Bradyrhizobium japonicum USDA 110*	gi|27379706	DNA duplex unwinding (RNA binding)	nucleus	16.54
Conserved repeat domain protein *Methanosaeta harundinacea 6Ac*	gi|357209873	carboxypeptidase activity	nucleus	13.18
CRE-RNP-7 protein *Caenorhabditis remanei*	gi|308501349	(nucleic acid binding)	nucleus	21.52
CRISPR-associated protein	gi|194336719	ATP bindin	nucleus	7.51
Cyclin dependent kinase 2	gi|00001880	meiosis (cyclin-dependent protein kinase activity)	nucleus	3.5
Cyclin T2 *Mus musculus*	gi|56550069	regulation of cyclin-dependent protein kinase activity	nucleus	7.26
Cytidylate kinase	gi|00002310		nucleus	12.56
Deformed *Physopelta quadriguttata*	gi|285206865	sequence-specific DNA binding	nucleus	14.77
DNA topoisomerase (ATP-hydrolyzing) subunit B *Chryseobacterium gleum ATCC 35910*	gi|300777682	DNA topological change (ATP binding)	nucleus	15.08
DNA topoisomerase *Edwardsiella tarda ATCC 23685*	gi|294635998	DNA topological change (ATP binding)	nucleus	7.67
DNA topoisomerase 2-like *Nasonia vitripennis*	gi|345490822	DNA topological change (ATP binding)	nucleus	21.99
DNA topoisomerase I *Caldicellulosiruptor saccharolyticus DSM 8903*	gi|146296272	DNA topological change (ATP binding)	nucleus	7.21
DNA-3-methyladenine glycosylase I *Rhodopseudomonas palustris CGA009*	gi|39934142	DNA dealkylation involved in DNA repair (DNA binding)	nucleus	8.7
DNA-directed RNA polymerase subunit beta *Methylomonas methanica MC09*	gi|333985467	transcription, DNA-dependent (DNA binding)	nucleus	20.57
Dual specificity tyrosine-phosphorylation-regulated kinase 2 *Danio rerio*	gi|113677529	positive regulation of transcription, DNA-dependent (ATP binding)	nucleus	10.82
E3 ubiquitin ligase, putative *Ixodes scapularis*	gi|000004273	apoptotic process (ribonucleoprotein complex binding	nucleus	8.88
Ets protein	gi|00005038	(sequence-specific DNA binding)	nucleus	9.86

Table A3 Nuclear proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
FACT complex subunit spt16 isoform 1 *Acyrthosiphion pisum*	gi|00008052	DNA repair (chromatin binding)	nucleus	12.08
Forkhead box transcription factor subgroup N2	gi|00007511	transcription, DNA-dependent (sequence-specific DNA binding)	nucleus	19.98
Forkhead protein/ forkhead protein domain, putative *Pediculus humanus corporis*	gi|242024936	transcription, DNA-dependent (sequence-specific DNA binding)	nucleus	15.03
Formamidopyrimidine-DNA glycosylase *Haliangium ochraceum DSM 14365*	gi|262200032	base-excision repair (damaged DNA binding)	nucleus	22.38
GATA-binding protein 4	gi|00005771	positive regulation of transcription, DNA-dependent (sequence-specific DNA binding)	nucleus	11.41
Glucose-6-phosphate 1-dehydrogenase *Rhodopirellula baltica SH 1*	gi|32472128	cellular response to oxidative stress (NADP binding)	nucleus	15.89
Glutamyl-tRNA amidotransferase subunit C *Mycoplasmapenetrans HF-2*	gi|26553662	regulation of translational fidelity (ATP binding)	nucleus	23.28
Glutamyl-tRNA(Gln) amidotransferase subunit B *Peptoniphilus indolicus ATCC 29427*	gi|350566306	regulation of translational fidelity (ATP binding)	nucleus	8.27
Glycine rich protein	gi|00005291	regulation of transcription, DNA-dependent (DNA binding)	nucleus	5.9
Glycogen phosphorylase isoform 2 *Apis mellifera*	gi|00017177	glycogen catabolic process (nucleotide binding)	nucleus	6.92
Groucho/TLE N-terminal Q-rich domain-containing protein *Loa loa*	gi|312072370	regulation of transcription, DNA-dependent	nucleus	13.98
Group II intron reverse transcriptase/maturase *Bacillus cereus G9241*	gi|47567760	RNA-dependent DNA replication (RNA binding)	nucleus	14.38
Guanine-specific ribonuclease N1 and T1 *Pirellula staleyi DSM 6068*	gi|283781994	(RNA binding)	nucleus	25.88
Histone acetyltransferase MYST2 *Harpegnathos saltator*	gi|307213696	(sequence-specific DNA binding transcription factor activity)	nucleus	12.71
Histone H1	gi|00011348	nucleosome assembly (DNA binding)	nucleus	13.27
Histone H3 3 type 2-like *Callithrix jacchus*	gi|296194875	nucleosome assembly (DNA binding)	nucleus	3.68
Importin alpha *Schizosaccharomyces pombe 972h*	gi|19113424	protein import into nucleus (protein transporter activity)	nucleus	10.5

Table A3 Nuclear proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Histone acetyltransferase MYST2 *Harpegnathos saltator*	gi|307213696	(sequence-specific DNA binding transcription factor activity)	nucleus	12.71
Histone H1	gi|00011348	nucleosome assembly (DNA binding)	nucleus	13.27
Histone H3.3 type 2-like *Callithrixjacchus*	gi|296194875	nucleosome assembly (DNA binding)	nucleus	3.68
Importin alpha *Schizosaccharomyces pombe 972h*	gi|19113424	protein import into nucleus (protein transporter activity)	nucleus	10.5
Importin subunit alpha-2	gi|00011302	protein import into nucleus (protein transporter activity)	nucleus	26.38
Integrase family protein *Burkholderia phymatum STM815*	gi|186473761	DNA integration (DNA binding)	nucleus	15.3
IRA1 protein *Acyrthosiphon pisum*	gi|000015848		nucleus	9.68
Kynureninase *Stackebrandtia nassauensis DSM 44728*	gi|291302088	L-kynurenine catabolic process (kynureninase activity)	nucleus	14.23
Late gene regulator	gi|00000581	RNA biosynthetic process (DNA-directed RNA polymerase activity)	nucleus	9.05
LReO_3	gi|00000566	DNA integration (RNA binding)	nucleus	20.48
Males absent on the first *Drosophila immigrans*	gi|316927902	regulation of transcription, DNA-dependent (transferase activity)	nucleus	16.43
Mariner transposase	gi|00000418	DNA integration (DNA binding)	nucleus	18.41
Myeloid/lymphoid or mixed-lineage leukemia 3	gi|00012891	intracellular signal transduction (DNA binding)	nucleus	10.42
Myosin heavy chain, nonmuscle or smooth muscle *Aedes aegypti*	gi|00016198	(ATP binding)	nucleus	5.63
Myosin light chain *Marsupenaeus japonicus*	gi|00016905	(calcium ion binding)	nucleus	10.68
Myosin xv *Acyrthosiphon pisum*	gi|000002631	(ATP binding)	nucleus	13.92
Novel KRAB box and zinc finger	gi|00013337	regulation of transcription, DNA-dependent (nucleic acid binding)	nucleus	7.04
Nuclear autoantigenic sperm protein	gi|00014057	histone exchange (histone binding)	nucleus	2.93
Nuclear DNA-binding protein	gi|00005720	adipose tissue development	nucleus	5.65

Table A3 Nuclear proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
p53 *Marsupenaeus japonicus*	gi|00000714	transcription, DNA-dependent (sequence-specific DNA binding transcription factor activity)	nucleus	15.13
ParB family chromosome partitioning protein *Arthrospira platensis str. Paraca*	gi|284054522	chromosome segregation (DNA binding)	nucleus	5.98
Peroxiredoxin	gi|00006627	cell proliferation (thioredoxin peroxidase activity)	nucleus	8.38
phd finger protein	gi|00002509	regulation of transcription, DNA-dependent (zinc ion binding)	nucleus	10.5
PHP domain-containing protein *Clostridium saccharolyticum WM1*	gi|302387539	DNA replication (DNA binding)	nucleus	20.64
pim-3 oncogene	gi|00010637	negative regulation of apoptotic process (ATP binding)	nucleus	1.83
Polycomb protein Suz12 (Suppressor of zeste 12 protein homolog)	gi|00003969	histone methylation (chromatin binding)	nucleus	20.61
Polyubiquitin 2	gi|00001032	DNA repair (ATP-dependent protein binding)	nucleus	3.3
PRP39 pre-mRNA processing factor 39 homolog (yeast) *Tribolium castaneum*	gi|00002849	mRNA splicing, via spliceosome	nucleus	7.08
RING finger protein *Eriocheir sinensis*	gi|00012347	cell differentiation (ubiquitin-protein ligase activity)	nucleus	10.72
RNA polymerase factor sigma-54 *Geobacillus thermodenitrificans NG80-2*	gi|138896647	DNA-dependent transcription, initiation (DNA binding)	nucleus	6.86
RNA-binding protein 39 *Harpegnathos saltator*	gi|00004914	mRNA processing (RNA binding)	nucleus	10.82
RUNX1 protein *Homo sapiens*	gi|00016006	signal transduction (3',5'-cyclic-nucleotide phosphodiesterase activity)	nucleus	6.63
Structural maintenance of chromosomes protein *Medicago truncatula*	gi|357486255	DNA recombination (ATP binding)	nucleus	24.24
TBC domain containing protein	gi|00014287	regulation of protein localization (Rab GTPase activator activity)	nucleus	15.29
TetR family transcriptional regulator *Pseudonocardia dioxanivorans CB1190*	gi|331698342	regulation of transcription, DNA-dependent (DNA binding)	nucleus	11.49

Table A3 Nuclear proteins in ovaries of *P. monodon* identified by 1-DE (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Topoisomerase IV subunit B *gamma proteobacterium IMCC1989*	gi|331006356	DNA topological change (ATP binding)	nucleus	29.22
Transcription accessory protein *Streptomyces ghanaensis ATCC 14672*	gi|291435829	DNA repair (DNA binding)	nucleus	12.69
Transcription factor Cys6 *Botryotinia fuckeliana*	gi|347830951	regulation of transcription from RNA polymerase II promoter (zinc ion binding)	nucleus	8.46
Transcription initiation factor TFIID subunit 11 *Harpegnathos saltator*	gi|307204822	regulation of transcription, DNA-dependent (translation initiation factor activity)	nucleus	15.05
Transcriptional repressor CTCF-like *Nasonia vitripennis*	gi|345481141	DNA methylation involved in gamete generation (histone binding)	nucleus	16.82
Twinkle protein, putative *Ixodes scapularis*	gi|000027526	DNA duplex unwinding (5'-3'DNA helicase activity)	nucleus	11.74
Uracil phosphoribosyltransferase homolog *Bombus terrestris*	gi|00018362	UMP biosynthetic process	nucleus	6.15
Vasa *Botryllus primigenus*	gi|00015422	(ATP binding)	nucleus	8.88
Y-box binding protein isoform 2	gi|00011460	mRNA stabilization (DNA binding)	nucleus	15.42
Zinc finger protein	gi|00012510	(DNA binding)	nucleus	8.65
Zinc finger protein 160	gi|00001223	hemopoiesis (DNA binding)	nucleus	5.39
Zinc finger protein 235 (Zinc finger protein 93 homolog) (Zfp-93) (Zinc finger protein 6) (HZF6)	gi|00011564	regulation of transcription, DNA-dependent (DNA binding)	nucleus	5.47
Zinc finger protein ZIC 5	gi|00003421	cell differentiation (DNA binding)	nucleus	22.91
TATA box-binding protein	gi|00011790	cell death (transcription regulatory region DNA binding)	pronucleus	11.02

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS

Protein name	Acc. No.	Function	Cellular component	Score
26S proteasome non-ATPase regulatory subunit 14-like isoform 1 *Strongylocentrotus purpuratus*	gi|00001362	-	Not known	16.59
LReO_3-like *Saccoglossus kowalevskii*	gi|00000243	-	Not known	12.66
UBA-like domain-containing protein 1-like isoform X1 *Bombyx mori*	gi|00002491	-	Not known	21.57
Ubiquitin-conjugating enzyme E2 variant 2-like *Bombyx mori*	gi|00000699	(acid-amino acid ligase activity)	Not known	13.26
AraC family transcriptional regulator *Paenibacillus sp. JDR-2*	gi|251795765	transcription, DNA-dependent (sequence-specific DNA binding)	Intracellular	19.32
Disks large-like protein 5 *Acromyrmex echinatior*	gi|332022248	regulation of apoptosis process	Intracellular	17.19
Aldehyde dehydrogenase type III, isoform J *Drosophila melanogaster*	gi|00005808	cellular aldehyde metabolic process (aldehyde dehydrogenase [NAD(P)+] activity)	lipid particle	17.94
14.5 kDa translational inhibitor protein, p14.5 *Gallus gallus*	gi|118087216	-	Not known	11.26
235 kDa rhoptry protein, putative Wolbachia endosymbiont of *Drosophila ananassae*	gi|58699286	-	Not known	21.72
2-alkenal reductase *Desulfomicrobium baculatum DSM 4028*	gi|256828238	(ATP binding)	Not known	17.27
2-amino-4-hydroxy-6-Hydroxymethyldihydropteridine pyrophosphokinase, partial *Rhodanobacter sp. 2APBS1*	gi|352090293	folic acid biosynthetic process (kinase activity)	Not known	8.32
2-hydroxychromene-2-carboxylate isomerase *Aspergillus fumigatus Af293*	gi|70994518	aromatic compound catabolic process (isomerase activity)	Not known	10.37
2-keto-3-deoxygalactonate kinase *Bradyrhizobium sp. BTAi1*	gi|148253576	(kinase activity)	Not known	23.1
2-oxoglutarate dehydrogenase, E2 component *Candidatus Sulcia muelleri GWSS*	gi|161833786	tricarboxylic acid cycle (dihydrolipoyllysine-residue succinyltransferase activity)	Not known	26.7
3'-5' exonuclease *Stackebrandtia nassauensis DSM 44728*	gi|291299881	(3'-5' exonuclease activity)	Not known	12.97

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
6-aminohexanoate-dimer hydrolase *Anaeromyxobacter sp. Fw109-5*	gi|153006138	nylon catabolic process (6-aminohexanoate-dimer hydrolase activity)	Not known	5.25
ABC transporter ATPase *Methanobacterium sp. AL-21*	gi|325959572	-	Not known	25.2
ABC transporter transmembrane protein *Streptomyces zinciresistens K42*	gi|345853108	-	Not known	13.84
ABC transporter, ATP-binding protein *Bryantella formatexigens DSM 14469*	gi|255280196	-	Not known	22.47
ABC1 family protein *Arabidopsis thaliana*	gi|18420844	(protein kinase activity) isoleucine biosynthetic process	Not known	15.8
Acetolactate synthase small subunit *Sulfitobacter sp. EE-36*	gi|83942256	(acetolactate synthase activity)	Not known	12.94
Acetyltransferase	gi|00009643	(N-acetyltransferase activity) tricarboxylic acid cycle (citrate hydro-lyase)	Not known	15.24
Aconitate hydratase *Acidithiobacillus ferrooxidans ATCC 53993*	gi|198282725	(cis-aconitate-forming activity)	Not known	21.92
ActA *Listeria monocytogenes*	gi|9050037	-	Not known	10.58
Actin	gi|00005721	(ATP binding)	Not known	17.6
Actin Patches Distal protein 1, putative *Ogataea parapolymorpha DL-1*	gi|320580619	-	Not known	10.71
Activator of basal transcription, putative *Pediculus humanus corporis*	gi|242025622	(nucleic acid binding)	Not known	20.08
Acyl-CoA dehydrogenase *Shewanella woodyi ATCC 51908*	gi|170726888	(acyl-CoA dehydrogenase activity)	Not known	11.86
Acyl-CoA dehydrogenase domain-containing protein	gi|00013965	(acyl-CoA dehydrogenase activity)	Not known	5.71
Acyl-CoA dehydrogenase, short/branched chain *Xenopus (Silurana) tropicalis*	gi|54020956	(acyl-CoA dehydrogenase activity)	Not known	5.44
Acyltransferase *Ruegeria pomeroyi DSS-3*	gi|56695955	-	Not known	6.65
ADP-ribosylation factor GTPase-activating protein, putative	gi|00010816	regulation of ARF GTPase activity (ARF GTPase activator activity)	Not known	19.32
ADR316Wp *Ashbya gossypii ATCC 10895*	gi|45188189	-	Not known	13.13
Afadin, putative *Pediculus humanus corporis*	gi|000035688	signal transduction	Not known	13.26

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
AGC/MAST/MAST protein kinase *Salpingoeca sp. ATCC 50818*	gi|326427941	(ATP binding)	Not known	21.1
Aggrecan *Oncorhynchus keta*	gi|319918507	cartilage condensation (calcium ion binding)	Not known	8.76
Alanine aminotransferase, putative	gi|00011611	L-alanine catabolic process, by transamination (ATP binding)	Not known	4.97
Alanyl-tRNA synthetase, mitochondrial precursor *Phytophthora infestans T30-4*	gi|301102849	-	Not known	14.21
ALB protein *Bos taurus*	gi|154425704	-	Not known	55.68
Alginate biosynthesis protein Alg44 *Pseudomonas syringae pv. tomato str. DC3000*	gi|28868452	alginic acid biosynthetic process (cyclic-di-GMP binding)	Not known	23.43
Alginate biosynthesis protein AlgX *Pseudomonas syringae pv. oryzae str. 1_6*	gi|237799839	-	Not known	5.76
Alpha 2 macroglobulin	gi|00007423	(endopeptidase inhibitor activity)	Not known	12.05
Alpha glucosidase *Litopenaeus vannamei*	gi|00018399	cellular polysaccharide catabolic process (alpha-glucosidase activity)	Not known	13.82
Alpha-2-macroglobulin-like *Cricetulus griseus*	gi|354487225	negative regulation of endopeptidase activity (endopeptidase inhibitor activity)	Not known	14.09
Alpha-galactosidase 1 *Saccharomyces Cerevisiae*	gi|113501	carbohydrate metabolic process (cation binding)	Not known	21.46
Alpha-isopropylmalate/homocitrate synthase transferase *Streptomyces griseus subsp. griseus NBRC 13350*	gi|182435772	leucine biosynthetic process (2-isopropylmalate synthase activity)	Not known	11.93
Alpha-methylacyl-CoA racemase *Xenopus laevis*	gi|291290961	fatty acid metabolic process (alpha-methylacyl-CoA racemase activity)	Not known	12.68
Amidinotransferase *Chelativorans sp. BNC1*	gi|110634457	arginine catabolic process (arginine deiminase activity)	Not known	9.38
amino acid adenylation domain protein *Methylomicrobium album BG8*	gi|334112224	biosynthetic process (hydrolase activity, acting on ester bonds)	Not known	7.94
Aminotransferase *Candidatus Solibacter usitatus Ellin6076*	gi|116620793	-	Not known	12.64
AMP-binding domain protein	gi|00003091	(acyl-CoA dehydrogenase activit)	Not known	7.68

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Anaerobic nitric oxide reductase transcription regulator *Vibrio angustum S14*	gi|90578316	regulation of transcription, DNA-dependent (ATP binding)	Not known	11.3
Anaphase promoting complex subunit 10-like *Saccoglossus kowalevskii*	gi|291239008	-	Not known	2.79
Ankyrin repeat domain-containing protein 13D *Pan troglodytes*	gi|343960170	-	Not known	6.63
Ankyrin repeat protein	gi|00004187	-	Not known	9.11
Anter-specific proline-rich protein APG, putative *Ricinus communis*	gi|255568004	lipid metabolic process (hydrolase activity, acting on ester bonds)	Not known	21.77
Antibiotic biosynthesis monooxygenase *Ralstonia pickettii 12D*	gi|241662733	(monooxygenase activity)	Not known	16.34
Anti-codon nuclease masking agent *Campylobacter concisus 13826*	gi|157164495	DNA modification (DNA binding)	Not known	9.92
Arc CG6741-PB	gi|00012238	-	Not known	23.79
Archaeal/vacuolar-type H+-ATPase subunit A *Alistipes shahii WAL 8301*	gi|291515525	ATP hydrolysis coupled proton transport (hydrogen ion transmembrane transporter activity)	Not known	16.67
Arginine kinase [Fenneropenaeus chinensis	gi|00015647	(arginine kinase activity)	Not known	23.33
arginyl-tRNA synthetase *Corynebacterium striatum ATCC 6940*	gi|227503835	-	Not known	15.41
Argonaute-2	gi|00001233	(nucleic acid binding)	Not known	11.95
arrestin domain-containing protein, putative	gi|00004452	signal transduction	Not known	9.95
arthrodial cuticle protein AMP16.3	gi|00000482	(structural constituent of cuticle)	Not known	15.04
Asf2p *Saccharomyces cerevisiae Vin13*	gi|323338386	-	Not known	4.33
aspartyl aminopeptidase *Ajellomyces capsulatus G186AR*	gi|225562419	-	Not known	14.12
ATP synthase subunit gamma, mitochondrial precursor *Lepeophtheirus salmonis*	gi|000006159	ATP synthesis coupled proton transport (hydrogen ion transporting ATP synthase activity)	Not known	13.28
ATPase (AAA+ superfamily)-like protein *Desulfovibrio vulgaris str. Miyazaki F*	gi|218887442	(ATP binding)	Not known	17.49

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
ATPase, class I, type 8B, member 2	gi|00003257	(metal ion binding)	Not known	13.76
ATP-binding ABC transporter protein *Aromatoleum aromaticum EbN1*	gi|56476145	ATP catabolic process (ATP binding)	Not known	19.61
ATP-binding Cassette (ABC) Superfamily *Phytophthora infestans T30-4*	gi|301098081	-	Not known	11.53
ATP-dependent Clp protease, ATP-binding subunit	gi|00002634	proteolysis (ATP binding)	Not known	12.92
ATP-dependent DNA helicase HFM1-like *Glycine max*	gi|356560587	(ATP binding)	Not known	6.67
ATP-dependent exoDNAse beta subunit *Methylophaga aminisulfidivorans MP*	gi|335044085	DNA repair (ATP binding)	Not known	9.88
ATP-dependent nuclease, subunit A *Listeria monocytogenes str. 4b H7858*	gi|47094504	double-strand break repair (ATP binding)	Not known	12.32
ATP-dependent protease ATP-binding subunit HslU *Lactobacillus sakei subsp. sakei 23K*	gi|81428595	ATP catabolic process (ATP binding)	Not known	9.23
BAI1-associated protein 2-like 1 *Xenopus (Silurana) tropicalis*	gi|154147654	signal transduction	Not known	10.59
Bardet-Biedl syndrome 10 protein homolog *Ailuropoda melanoleuca*	gi|301770545	Golgi to plasma membrane protein transport	Not known	13.64
Basic proline-rich protein precursor	gi|00018391	-	Not known	3.03
BCS-1 *Baianus amphitrite*	gi|00016357	(structural constituent of cuticle)	Not known	17.72
BCS-2	gi|00005653	- lactose catabolic process (alkali metal ion binding)	Not known	7.56
Beta-galactosidase	gi|00011054		Not known	12.75
Beta-ketoacyl synthase *Streptomyces sp. MP8E7-PKS1-rr4*	gi|207560107	(catalytic activity)	Not known	10.63
Bifunctional short chain isoprenyl diphosphate Synthase IdsA *Methanobrevibacter ruminantium M1*	gi|288560181	isoprenoid biosynthetic process (dimethylallyltranstransferase activity)	Not known	16.46
Biuret hydrolase *Thermomicrobium roseum DSM 5159*	gi|221632338	atrazine catabolic process (biuret amidohydrolase activity)	Not known	8.65
BRD group protein	gi|00005196	-	Not known	5.11

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
BSD domain-containing protein 1 isoform 1 *Danio rerio*	gi|000054796	-	Not known	15.95
BVpp12b protein *Chelonus inanitus*	gi|297372578	-	Not known	11.15
CAIB/BAIF family enzyme *Magnaporthe oryzae 70-15*	gi|351645466	(catalytic activity)	Not known	13.06
Calcified cuticle protein CP14.1	gi|00004659	(structural constituent of cuticle)	Not known	13.36
Calcitonin I precursor SCT-Gly (synthetic construct)	gi|220946	-	Not known	15.02
Calponin homology (CH) domain-containing, Chdc/Irch *Ixodes scapularis*	gi|241998146	(phosphoprotein phosphatase activity)	Not known	8.02
CAP CG18408-PC, isoform C *Apis mellifera*	gi|00016368	-	Not known	15.35
CBXX/CFQX family protein *Mycobacterium sp. JDM601*	gi|333988687	(ATP binding)	Not known	15.52
CCDC46 protein *Ciona intestinalis*	gi|198438205	-	Not known	24.64
CDC68 like aminopeptidase family chromatinic Protein (possible inactive enzyme) *Cryptosporidium parvum Iowa II*	gi|66362808	cellular process (aminopeptidase activity)	Not known	13.5
Cell wall-associated hydrolase *Vibrio vulnificus CMCP6*	gi|00017625	(hydrolase activity)	Not known	10.62
cement precursor protein 3B variant 2	gi|00007466	-	Not known	8.18
CG14534-PA *Apis mellifera*	gi|00016612	-	Not known	13.39
CG1523 CG1523-PA *Acyrthosiphon pisum*	gi|000002090	-	Not known	11.62
CG16711, isoform A *Drosophila melanogaster*	gi|24661837	-	Not known	8.97
CG16973-PE, partial *Nasonia vitripennis*	gi|000035548	-	Not known	5.61
CG17680-PA *Tribolium castaneum*	gi|00017667	-	Not known	10.47
CG1850-PA *Drosophila melanogaster*	gi|00017910	-	Not known	12.52
CG2691 *Drosophila melanogaster*	gi|24641825	-	Not known	1.37
CG2691 *Drosophila melanogaster*	gi|24641825	-	Not known	1.37
CG2918, isoform A *Drosophila melanogaster*	gi|20128923	-	Not known	16.41
CG32556, isoform A *Drosophila melanogaster*	gi|24642834	-	Not known	17.5
CG8144-PK *Nasonia vitripennis*	gi|000035833	-	Not known	8.19
CG8486 CG8486-PC *Tribolium castaneum*	gi|189237536	-	Not known	5.12

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Chain A, Crystal Structure Of Homo Sapien Glycerol-3-Phosphate Dehydrogenase 1	gi|9031624	-	Not known	9.99
Chain A, Crystal Structure Of M111, Bcl-2 Homolog From Myxoma Virus	gi|134105043	-	Not known	9.57
Chain A, Crystal Structure Of The Rna Binding Domain Of Puf4 From *Saccharomyces Cerevisiae*	gi|169791859	-	Not known	11.04
Chain A, Phd2-R127 With Jnj41536014	gi|00015865	-	Not known	27.13
Chain A, Ranasmurfin	gi|161761123	-	Not known	5.94
Chain B, Ran-Rcc1-So4 Complex	gi|000035584	-	Not known	11.24
Strongylocentrotus purpuratus Chitin binding PM protein	gi|00006355	carbohydrate metabolic process (hydrolase activity,)	Not known	10.87
Chitinase 1 precursor	gi|00005887	-	Not known	12.17
CHK1 checkpoint-like protein	gi|00004433	-	Not known	10.46
CHL4 family chromosome segregation protein *Aspergillus kawachii IFO 4308*	gi|358365957	-	Not known	13.97
Chloramphenicol 3-O phosphotransferase *Streptomyces ambofaciens ATCC 23877*	gi|117164606	response to antibiotic (ATP binding)	Not known	9.81
Chloride ion current inducer protein, putative	gi|00011657	-	Not known	5.4
Chordin-like protein *Hydra magnipapillata*	gi|00015545	BMP signaling pathway	Not known	9.68
CitG protein *Clostridium perfringens str. 13*	gi|18310127	phosphorylation (ATP binding)	Not known	8.64
Clp domain serine proteinase 1	gi|00002178	proteolysis (serine-type endopeptidase activity)	Not known	3.72
Coatomer subunit delta-like *Megachile rotundata*	gi|00001837	vesicle-mediated transport	Not known	9.08
Cobalt transport protein *Nocardioidaceae bacterium Broad-1*	gi|326330680	-	Not known	7.88
CobN/Mg-chelatase family protein *Methanopyrus kandleri AV19*	gi|20093947	biosynthetic process (magnesium chelatase activity)	Not known	7.1
cof family hydrolase *Sebaldella termitidis ATCC 33386*	gi|269118962	(hydrolase activity)	Not known	13.24
complement component	gi|00006956	induction of apoptosis	Not known	14.18

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Conjugal transfer protein TraL *Desulfovibrio salexigens DSM 2638*	gi|242279183	-	Not known	19.55
Conjugative transfer protein TraA *Sphingobium japonicum UT26S*	gi|294057979	-	Not known	10.95
Conserved oligomeric Golgi complex subunit 4-like *Bombus impatiens*	gi|00015206	-	Not known	7.57
Cop9 complex subunit	gi|00005716	-	Not known	6.56
COP9 signalosome complex subunit, putative	gi|00014910	-	Not known	5.13
Copine family protein *Brugia malayi*	gi|170592234	-	Not known	14.24
Copper homeostasis protein *Streptomyces lividans TK24*	gi|289768241	copper ion homeostasis (copper ion binding)	Not known	11.35
CRE-NASP-1 protein *Caenorhabditis remanei*	gi|308510080	-	Not known	16.12
CRS1/YhbY domain containing protein *Pseudogulbenkiania sp. NH8B*	gi|347541234	RNA binding	Not known	11
Crustacean calcium-binding protein 23 (CCBP-23 protein)	gi|00018368	(calcium ion binding)	Not known	15.52
C-terminal domain containing protein	gi|00011998	-	Not known	8.98
C-type lectin	gi|00012640	-	Not known	10.34
Cuticle protein CP1158 (CPCP1158).	gi|00017838	-	Not known	12.1
Cyclase family protein *Desulfotomaculum ruminis DSM 2154*	gi|334090181	-	Not known	11.75
Cyclase/dehydrase *Rhodopseudomonas palustris CGA009*	gi|39935654	-	Not known	25.54
Cyclic AMP-regulated protein like protein	gi|00006901	-	Not known	13.3
Cysteine-rich C-terminal protein 1 *Homo sapiens*	gi|9506923	-	Not known	13.63
Cysteine-rich protein 1 (Cysteine-rich intestinal protein) (CRIP) *Apis mellifera*	gi|00017776	-	Not known	12.92
Cytochrome b5, putative *Pediculus humanus corporis*	gi|00013894	lipid metabolic process (heme binding)	Not known	7.96
Cytosine deaminase or related metal-dependent hydrolase *Ralstonia eutropha H16*	gi|116694384	(hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds)	Not known	14.22

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Daughterless	gi|00008078	-	Not known	8.91
DbpA RNA-binding domain protein				
Spirochaeta sp. Buddy	gi|325970724	(ATP binding)	Not known	12.76
DD9B *Marsupenaeus japonicus*	gi|00017079	(structural constituent of cuticle)	Not known	8.51
Dehydrogenase E1 and transketolase domain-containing protein 1		tricarboxylic acid cycle (oxoglutarate dehydrogenase (succinyl-transferring)		
Coprinopsis cinerea okayama7#130	gi|299742536	activity)	Not known	12.31
Deoxyribodipyrimidine photolyase				
Roseovarius sp. TM1035	gi|149202510	DNA repair (DNA binding)	Not known	26.08
		actin filament polymerization		
Diaphanous	gi|00010323	(Rho GTPase binding)	Not known	5.15
Diguanylate cyclase and metal dependent Phospholydrolase		cyclic nucleotide biosynthetic process		
Thermanaerovibrio acidaminovorans DSM6589	gi|269791787	(metal ion binding)	Not known	16.93
Diguanylate phosphodiesterase				
Verminephrobacter eiseniae EF01-2	gi|121608685	-	Not known	11.92
Dihydroxyacetone kinase		anaerobic glycerol catabolic process		
Burkholderia pseudomallei B7210	gi|167845538	(ATP binding)	Not known	6
Dinucleotide-utilizing enzyme involved in Molybdopterin and thiamine biosynthesis family 1				
Pseudovibrio sp. JE062	gi|254471277	metabolic process (nucleotide binding)	Not known	2.26
Dipeptidyl aminopeptidase/acylaminoacyl peptidase *Gluconacetobacter oboediens 174Bp2*	gi|349687895	proteolysis (aminopeptidase activity)	Not known	8.6
Dipeptidyl peptidase 9-like *Ailuropoda melanoleuca*	gi|301787997	-	Not known	3.24
Disease resistance protein				
Brassica rapa subsp. pekinensis	gi|227438125	-	Not known	16.25
Disease resistance-like protein *Glycine max*	gi|7263119	-	Not known	4.55
Di-trans-poly-cis-decaprenylcistransferase				
Janibacter sp. HTCC2649	gi|84496822	-	Not known	9.2
DNA polymerase iota *Xenopus (Silurana) tropicalis*	gi|70955242	-	Not known	13.66
DNA primase *Clostridium sp. DL-VIII*	gi|357169920	replisome (DNA binding)	Not known	2.36

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
DNA primase small subunit *Paracoccidioides brasiliensis Pb01*	gi|295668429	DNA replication initiation	Not known	9.32
DNA topoisomerase TopA *Selenomonas noxia ATCC 43541*	gi|292670828	-	Not known	9.23
DNA topoisomerase/gyrase *Aedes aegypti*	gi|000002822	DNA topoisomerase/gyrase	Not known	23.14
DNA2-like helicase-like *Oreochromis niloticus*	gi|348534058	-	Not known	9.75
DNA-binding domain-containing protein *Prosthecochloris aestuarii DSM 271*	gi|194334891	-	Not known	7.79
DNA-binding domain-containing protein, AraC-type *Brevibacterium linens BL2*	gi|260904381	-	Not known	16.53
DNA-directed RNA polymerase, sigma subunit (sigma70/sigma32) *Actinobacillus pleuropneumoniae serovar 1 str. 4074*	gi|32034495	-	Not known	6.89
DnaJ (Hsp40) homolog, subfamily B, member 9	gi|00013709	-	Not known	9.54
DNAJ heat shock N-terminal domain-containing protein *Arabidopsis thaliana*	gi|334182674	response to stress (nucleic acid binding)	Not known	9.12
double WAP domain-containing protein	gi|00003636	negative regulation of peptidase activity (peptidase inhibitor activity)	Not known	7.94
DSBA oxidoreductase *Xylella fastidiosa Dixon*	gi|71274788	-	Not known	9.15
Dual 3',5'-cyclic-AMP and -GMP phosphodiesterase 11 *Harpegnathos saltator*	gi|00004011	-	Not known	13.38
E3 50K (CR1b) *Human adenovirus 36*	gi|261875916	-	Not known	4.09
e3 ubiquitin-protein ligase HECTD1-like *Amphimedon queenslandica*	gi|340381960	protein ubiquitination (ubiquitin-protein ligase activity)	Not known	20.4
EAL domain-containing protein *Butyrivibrio proteoclasticus B316*	gi|302671195	-	Not known	17.68
early cuticle protein 5 *Calinectes sapidus*	gi|00001732	-	Not known	27.27
early gene regulator	gi|00008035	-	Not known	10.87
ebna2 binding protein P100	gi|00002987	-	Not known	14.65
ECF subfamily RNA polymerase sigma-24 factor *Candidatus Koribacter versatilis Ellin345*	gi|94967618	DNA-dependent transcription, initiation (sequence-specific DNA binding)	Not known	33.31

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Eco57I restriction endonuclease *Marinithermus hydrothermalis DSM 14884*	gi|328949574	DNA modification (DNA binding)	Not known	6.48
EDTP(egg derived tyrosine phosphatase) *Sarcophaga peregrina*	gi|4586367	peptidyl-tyrosine dephosphorylation (protein tyrosine phosphatase activity)	Not known	13.4
EGF-like domain containing protein	gi|00014977	-	Not known	5.17
Electron transfer flavoprotein subunit beta *Roseiflexus sp. RS-1*	gi|148656423	-	Not known	13.32
Electron-transfer-flavoprotein beta polypeptide	gi|00006170	-	Not known	7.71
Enoyl-ACP reductase *Alcanivorax borkumensis SK2*	gi|110834076	-	Not known	17.95
ENSANGP00000021035-like	gi|00001106	-	Not known	8.36
ENSANGP00000030076 *Anopheles gambiae str. PEST*	gi|00015814	-	Not known	5.48
Est1p-like protein B	gi|00014598	-	Not known	7.89
EvpL *Edwardsiella tarda*	gi|158512119	-	Not known	14.82
excinuclease ABC subunit C *Helicobacter bilis ATCC 43879*	gi|237751708	-	Not known	13.9
Expressed hypothetical protein	gi|00008366	-	Not known	9.35
Expressed protein *Chlorella variabilis*	gi|307108252	-	Not known	15.01
Extensin-like protein	gi|00013108	-	Not known	7.84
F0F1 ATP synthase subunit epsilon *Saccharopolyspora spinosa NRRL 18395*	gi|348169093	-	Not known	6.55
F20B17.20 *Arabidopsis thaliana*	gi|7715593	-	Not known	12.47
F28C1.1 *Caenorhabditis elegans*	gi|00016908	-	Not known	12.2
FAD linked oxidase domain-containing protein *Mycobacterium thermoresistibile ATCC 19527*	gi|357022269	(UDP-N-acetylmuramate dehydrogenase activity)	Not known	36.05
FAD linked oxidase-like protein *Pseudoalteromonas atlantica T6c*	gi|109898066	(UDP-N-acetylmuramate dehydrogenase activity)	Not known	6.85
FAD-dependent pyridine nucleotide-disulfide Oxidoreductase *Jannaschia sp. CCS1*	gi|89052931	(flavin adenine dinucleotide binding)	Not known	20.83
family 1 extracellular solute-binding protein *Lactobacillus buchneri NRRL B-30929*	gi|331702325	(transporter activity)	Not known	7.81

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Family with sequence similarity 76, member A	gi|00013434	-	Not known	18.52
Farnesoic acid o-methyltransferase-like isoform 1 protein	gi|00008380	(methyltransferase activity)	Not known	12.79
Fast myosin heavy chain	gi|00006946	(ATP binding)	Not known	8.38
Fatty acid hydroxylase	gi|332667208	fatty acid biosynthetic process	Not known	7.68
Haliscomenobacter hydrossis DSM 1100		(electron carrier activity)		
FBOX domain-containing protein *Naegleria gruberi*	gi|290995883	-	Not known	6.98
FdxD *Mycobacterium rhodesiae JS60*	gi|353192332	-	Not known	9.77
Fe/S oxidoreductase *Thermus thermophilus HB27*	gi|46199246	cofactor biosynthetic process (4 iron, 4 sulfur cluster binding)	Not known	10.1
Fibronectin type III domain-containing protein *Escherichia coli E482*	gi|322942216	-	Not known	14.68
Fis family PAS modulated sigma-54 specific Transcriptional regulator *Desulfomicrobium baculatum DSM 4028*	gi|256829149	-	Not known	9.46
Flavocytochrome c *Shewanella sp. W3-18-1*	gi|120598393	FMN binding	Not known	6.95
Flavonol reductase/cinnamoyl-CoA reductase, putative *Ixodes scapularis*	gi|241703753	cellular metabolic process (coenzyme binding)	Not known	7.39
FLJ16542 protein *Homo sapiens*	gi|00016347	-	Not known	8.66
Formylmethanofuran dehydrogenase subunit C *Anaerobaculum hydrogeniformans ATCC BAA-1850*	gi|289524236	methanogenesis (transition metal ion binding)	Not known	20.78
GA17051 *Drosophila pseudoobscura pseudoobscura*	gi|198468151	-	Not known	12.22
GD13716 *Drosophila simulans*	gi|195587318	-	Not known	42.08
GE18379 *Drosophila yakuba*	gi|195471577	-	Not known	12.09
Gef26 CG9491-PA	gi|00007089	-	Not known	6.7
Geranylgeranyl pyrophosphate synthetase *gamma proteobacterium NOR51-B*	gi|254283313	isoprenoid biosynthetic process (farnesyltranstransferase activity)	Not known	17.74
Gluconate utilization system Gnt-I transcriptional repressor *Vibrio splendidus 12B01*	gi|84394258	transcription, DNA-dependent (DNA binding)	Not known	28.6
Glucosamine-6-phosphate deaminase 1	gi|00013678	N-acetylglucosamine catabolic process (hydrolase activity)	Not known	9.09

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Glucose dehydrogenase *Streptomyces scabiei 87.22*	gi|290963206	alcohol metabolic process (choline dehydrogenase activity)	Not known	21.27
Glutamate cysteine ligase *Aedes aegypti*	gi|00017814	cellular response to arsenic-containing substance (ATP binding)	Not known	11.88
Glutamine--scyllo-inositol transaminase *azollae' 0708*	*Nostoc* gi|298490475	metabolic process (catalytic activity)	Not known	14.16
Glutaminyl-tma synthetase	gi|00002755	-	Not known	7.25
Glutamyl-tRNA synthetase *Onion yellows phytoplasma OY-M*	gi|39938626	-	Not known	5.89
Glycosyl transferase, WecB/TagA/CpsF family protein *Asticcacaulis biprosthecum C19*	gi|329847658	biosynthetic process (transferase activity, transferring glycosyl groups)	Not known	15.95
Glycosyltransferase *Ciona intestinalis*	gi|56550356	biosynthetic process (transferase activity, transferring glycosyl groups)	Not known	8.12
Glyoxalase/bleomycin resistance Protein/dioxygenase *Rhizobium etli CIAT 894*	gi|218677517	(oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen)	Not known	20.54
gp47 *Burkholderia phage Bcep176*	gi|77864672	hydrolase activity, acting on ester bonds	Not known	19.05
Group 1 glycosyl transferase *Acidimicrobium ferrooxidans DSM 10331*	gi|256372003	biosynthetic process (transferase activity)	Not known	19.74
Guanine aminohydrolase *Azorhizobium caulinodans ORS 571*	gi|158426249	guanine catabolic process (guanine deaminase activity)	Not known	15.3
GYD family protein *Streptomyces pristinaespiralis ATCC 25486*	gi|297192072	-	Not known	20.39
GyrA *Brucella abortus*	gi|133853699	DNA topological change	Not known	10.13
H+ transporting ATP synthase gamma subunit	gi|00003141	-	Not known	13.28
Haloacid dehalogenase *Marinomonas mediterranea MMB-1*	gi|326795411	(hydrolase activity, acting on acid halide bonds, in C-halide compounds)	Not known	15.65
Halogenase *Streptomyces sp. L133(2010)*	gi|288816949	(oxidoreductase activity)	Not known	10.2

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
HECT domain containing 1 *Apis mellifera*	gi|00016985	(metal ion binding)	Not known	6.32
Helicase domain-containing protein *Thiorhodococcus drewsii AZ1*	gi|345873267	(ATP binding)	Not known	10.94
Helix-turn-helix domain-containing protein *Thermoanaerobacter wiegelii Rt8.B1*	gi|345018435	(sequence-specific DNA binding)	Not known	10.62
Heme oxygenase *Streptomyces viridochromogenes DSM 40736*	gi|302550972	pyridoxal phosphate biosynthetic process (FMN binding)	Not known	5.82
Hemocyte kazal-type proteinase inhibitor	gi|00008251	-	Not known	11.65
Hemocyte protein-glutamine gamma-glutamyltransferase, putative	gi|00012148	peptide cross-linking (metal ion binding)	Not known	5.58
Hemolymph clottable protein	gi|00013564	hemolymph coagulation (lipid transporter activity)	Not known	8.2
Hemolysin activation/secretion protein *alpha proteobacterium BAL199*	gi|163795779	protein transport	Not known	11.04
heterogeneous nuclear ribonucleoprotein L, putative	gi|00013176	ribonucleoprotein complex (nucleic acid binding)	Not known	6.56
HipA domain-containing protein *Sphingomonas wittichii RW1*	gi|148550653	-	Not known	10.16
Histidine kinase *Oscillatoria sp. PCC 6506*	gi|300865287	intracellular signal transduction (ATP binding)	Not known	15.25
Histone-lysine N-methyltransferase MLL3-like *Xenopus (Silurana) tropicalis*	gi|301605820	(methyltransferase activity)	Not known	10.42
Holliday junction DNA helicase RuvB *Thermanaerovibrio acidaminovorans DSM 6589*	gi|269792532	DNA recombination (ATP binding)	Not known	11.23
Hydrogenase assembly chaperone HypC/HupF *Selenomonas sputigena ATCC 35185*	gi|260887614	-	Not known	5.92
Hydrolase *Capsaspora owczarzaki ATCC 30864*	gi|00007589	-	Not known	17.08
Hypothetical UPF0327 protein isoform 2 *Bos taurus*	gi|00016916	-	Not known	13.22
Indolepyruvate oxidoreductase subunit alpha *Methanothermobacter thermautotrophicus str. Delta H*	gi|15679840	(electron carrier activity)	Not known	10.96

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Integrator complex subunit 12	gi|00011156	-	Not known	10.49
Intermediate filament tail domain-containing Protein, partial *Wuchereria bancrofti*	gi|00012168	-	Not known	13.96
Intersectin 2-like	gi|00014719	endocytosis	Not known	3.4
Intracellular fatty acid binding protein	gi|00002085	Transport (lipid binding)	Not known	9.49
Ipk2 CG13688-PA *Apis mellifera*	gi|00018388	-	Not known	9.12
Iron(3+)-hydroxamate import system permease	gi|282882408	-	Not known	6.31
Protein FhuB *Peptoniphilus lacrimalis 315-B*				
Iron(III) dicitrate-binding periplasmic protein	gi|28210666	-	Not known	14.07
hmuT *Clostridium tetani E88*				
Isoquinoline 1-oxidoreductase	gi|332665712	(electron carrier activity)	Not known	25.81
Haliscomenobacter hydrossis DSM 1100				
Jumonji/Zn finger-class transcription factor ELF6	gi|79507158	-	Not known	3.32
Arabidopsis thaliana				
Kelch repeat and BTB domain-containing protein 13-like *Danio rerio*	gi|326669200	-	Not known	14.84
Lactate utilization protein B/C	gi|350573001	-	Not known	16.74
Thiorhodovibrio sp. 970				
lamin Dm0-like isoform 2 *Nasonia vitripennis*	gi|00000668	-	Not known	2.82
LD31582p	gi|00002927	-	Not known	5.52
Lethal (2) 01424 CG3845-PB, isoform B	gi|00000929	-	Not known	7.33
Leucine Rich Repeat family protein	gi|00015236	-	Not known	13.13
L-glutaminase *Alistipes shahii WAL 8301*	gi|291513775	-	Not known	12.99
LigA	gi|00007452	DNA repair (DNA binding)	Not known	2.31
lingerer, putative	gi|00008314	-	Not known	12.43
Lipase family protein	gi|00012131	lipid metabolic process (triglyceride lipase activity)	Not known	17.68
Lipoprotein	gi|00000497	-	Not known	21.01
LOW QUALITY PROTEIN: BTB/POZ and MATH domain-containing protein 2-like *Brachypodium distachyon*	gi|357152766	-	Not known	11.15

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
LOW QUALITY PROTEIN: PWWP domain-containing protein 2A-like *Callithrix jacchus*	gi|296192645	-	Not known	9.51
LPXTG-motif cell wall anchor domain protein *Bacillus cereus E33L*	gi|00016304	-	Not known	21.66
LuxR family transcriptional regulator *Burkholderia mallei ATCC 23344*	gi|53717271	transcription, DNA-dependent (sequence-specific DNA binding)	Not known	14.64
Lysine 2,3-aminomutase YodO family protein *Marinobacter aquaeolei VT8*	gi|120555694	metabolic process (iron-sulfur cluster binding)	Not known	10.01
Lysosomal acid lipase, putative	gi|00001313	-	Not known	5.44
LysR family transcripitonal regulator *Erwinia billingiae Eb661*	gi|300716920	transcription, DNA-dependent (DNA binding)	Not known	17.29
mab-3-related transcription factor 3-like protein *Anopheles merus*	gi|240268764	-	Not known	17.3
Malonyl CoA-acyl carrier protein transacylase *Rhodotorula glutinis ATCC 204091*	gi|342321338	(transferase activity)	Not known	11.24
Mannose-binding protein *Pacifastacus leniusculus*	gi|00015783	(carbohydrate binding)	Not known	12.62
Membrane coat complex retromer, subunit VPS29/PEP11, putative	gi|00014720	(hydrolase activity, acting on ester bonds)	Not known	11.96
Membrane nuclease *Mycoplasma arthritidis 158L3-1*	gi|193217053	-	Not known	8.86
Membrane protein *Sphingomonas sp. S17*	gi|332186963	-	Not known	41.16
Membrane-associated guanylate kinase WW and PDZ domain-containing protein 3 *Clonorchis sinensis*	gi|358334242	(kinase activity)	Not known	16.21
MerR family transcriptional regulator *Streptomyces violaceusniger Tu 4113*	gi|345010390	(DNA binding)	Not known	9.46
Metallothionein [Penaeus monodon]	gi|000056117	-	Not known	8.9
Methyl coenzyme M reductase alpha subunit *uncultured archaeon*	gi|345449509	methanogenesis (coenzyme-B sulfoethylthiotransferase activity)	Not known	12.28
Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha) *Nasonia vitripennis*	gi|000026401	(ATP binding)	Not known	3.5

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Methyltransferase type 11 *Chelativorans sp. BNC1*	gi|110636356	(ATP binding)	Not known	11.3
Microcompartments protein *Mahella australiensis 50-1 BON*	gi|332981409	(DNA binding)	Not known	7.8
MORN repeat protein *Ichthyophthirius multifiliis*	gi|340500584	-	Not known	16.43
Mov34/MPN/PAD-1 family protein *Tetrahymena thermophila*	gi|118365100	mRNA splicing, via spliceosome	Not known	5.1
MPXV-WRAIR169 *Monkeypox virus*	gi|00017298	-	Not known	11.72
Multiple pdz domain protein, putative	gi|00006364	-	Not known	9.42
MutS family DNA mismatch repair protein *Zunongwangia profunda SM-A87*	gi|295134547	mismatch repair(ATP binding)	Not known	8.23
Myosin heavy chain, non-muscle *Harpegnathos saltator*	gi|00007858	(ATP binding)	Not known	23.22
Myosin-1	gi|190359856	actomyosin contractile ring contraction (ATP binding)	Not known	7.78
Myosin-like protein *Medicago truncatula*	gi|357463145	(ATP binding)	Not known	16.66
Myosin-VIIa *Tribolium castaneum*	gi|00017538	actin filament organization (ATP binding)	Not known	17.86
N-6 DNA methylase *Nostoc punctiforme PCC 73102*	gi|186685409	DNA methylation on adenine (DNA binding)	Not known	21.68
N-acetyl sugar amidotransferase family protein *Subdoligranulum variabile DSM 15176*	gi|261368058	-	Not known	15.65
N-acyl-L-amino acid amidohydrolase *Xanthomonas campestris pv. campestris str. ATCC 33913*	gi|21233214	(aminoacylase activity)	Not known	12.68
NADH kinase, putative	gi|00013095	NADP biosynthetic process (NAD+ kinase activity)	Not known	8.99
NADPH:quinone reductase *Mycobacterium rhodesiae JS60*	gi|353185023	(zinc ion binding)	Not known	14.28
NADPH-dependent FMN/FAD containing oxidoreductase *Trypanosoma vivax Y486*	gi|340053222	(iron ion binding)	Not known	16.47
NBS-LRR resistance-like protein RGC662 *Helianthus paradoxus*	gi|148286930	(ADP binding)	Not known	7.8

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Neuramidase *Beutenbergia cavernae DSM 12333*	gi|229819930	(exo-alpha-sialidase activity)	Not known	33.78
Neutral ceramidase Heterocephalus glaber	gi|351715569	-	Not known	3.61
Nickel-dependent hydrogenase large subunit *Desulfurivibrio alkaliphilus AHT2*	gi|297568874	(ferredoxin hydrogenase activity)	Not known	12.95
Nicotinamide riboside kinase 1	gi|00004048	NAD biosynthetic process (metal ion binding)	Not known	10.76
NiFe hydrogenase maturation protein HypF *Azospirillum amazonense Y2*	gi|347739749	protein carbamoylation (double-stranded RNA binding)	Not known	3.21
Nin protein *Enterobacteria phage lambda*	gi|00018182	centrosome-templated microtubule nucleation (calcium ion binding)	Not known	17.07
Nipsnap	gi|00012675	-	Not known	12.42
Nitrate ABC superfamily ATP binding cassette transporter	gi|148657988	ATP catabolic process (ATP binding)	Not known	7.9
Nitrate transport ATP-binding subunits C and D *Rhodopseudomonas palustris HaA2*	gi|86748881	ATP catabolic process (ATP binding)	Not known	9.91
Nitrilase family, member 2	gi|00000765	nitrogen compound metabolic process (hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds)	Not known	3.34
Nitrilotriacetate monooxygenase component A *Sinorhizobium meliloti 1021*	gi|16263599	(monooxygenase activity)	Not known	14.13
NLI interacting factor-like phosphatase family protein	gi|00008934	dephosphorylation (phosphatase activity)	Not known	5.82
N-myc downstream regulated	gi|00014451	-	Not known	11.43
No on or off transient A CG4211-PB, isoform B	gi|00003355	-	Not known	17.28
Non-ribosomal peptide synthetase *Pseudomonas syringae* pv. tabaci ATCC 11528	gi|257485057	oxidation-reduction process (oxidoreductase activity)	Not known	4.68
Non-ribosomal peptide synthetase MbtF *Mycobacterium kansasii ATCC 12478*	gi|240169922	(ligase activity)	Not known	15.09
Nucleolar complex protein 14 *Arthroderma otae CBS 113480*	gi|296806513	-	Not known	8.75
Nucleolar protein c7b	gi|00002393	-	Not known	7.55

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Nudel CG10129-PA, partial *Apis mellifera*	gi|000035297	-	Not known	18.82
Odd-skipped 2 protein	gi|00006907	(nucleic acid binding)	Not known	9.41
Olfactory enriched transcipt 14.35	gi|00001993	-	Not known	19.62
Olfactory enriched transcipt 15.22	gi|00003598	-	Not known	16.29
O-methyltransferase	gi|00000838	(O-methyltransferase activity)	Not known	22.08
Ornithine decarboxylase, putative	gi|00000967	polyamine biosynthetic process (catalytic activity)	Not known	2.41
Orotate phosphoribosyltransferase *Thermus aquaticus Y51MC23*	gi|218294655	de novo' UMP biosynthetic process (magnesium ion binding)	Not known	12.3
Ovarian cancer-associated gene 2 protein homolog *Xenopus (Silurana) tropicalis*	gi|00017976	-	Not known	28.35
Ovarian peritrophin 1 precursor	gi|00002808	-	Not known	10.47
Oxidoreductase	gi|291435583	oxidoreductase activity	Not known	21.44
Streptomyces ghanaensis ATCC 14672 Oxidoreductase, short chain dehydrogenase/reductase family *Clostridium botulinum D str. 1873*	gi|253681649	oxidation-reduction process (oxidoreductase activity)	Not known	7.99
PA14 domain-containing protein *Roseiflexus sp. RS-1*	gi|148655418	-	Not known	7.41
Papilin, putative	gi|00006263	(serine-type endopeptidase inhibitor activity)	Not known	14.77
par domain protein *Aedes aegypti*	gi|00006815	-	Not known	12
Pc13g05420 *Penicillium chrysogenum Wisconsin 54-1255*	gi|255935899	DNA integration (nucleic acid binding)	Not known	9.44
Pc22g19160 *Penicillium chrysogenum Wisconsin 54-1255*	gi|255950104	RNA metabolic process (DNA binding)	Not known	18.43
Pcoln3 protein *Strongylocentrotus purpuratus*	gi|00016382	-	Not known	11.61
Pentaphosphate guanosine-3'-Pyrophosphohydrolase *Xanthomonas oryzae pv. oryzae KACC10331*	gi|58580772	guanosine tetraphosphate metabolic process (hydrolase activity)	Not known	13.77
Pentatricopeptide, putative, expressed *Oryza sativa Japonica Group*	gi|108712143	-	Not known	13.43

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
PE-PGRS family protein *Azospirillum amazonense Y2*	gi|347736506	-	Not known	12.71
Peptidoglycan-binding LysM *Streptomyces sp. Tu6071*	gi|333025155	-	Not known	17.68
PH domain containing protein *Entamoeba histolytica HM-1:IMSS*	gi|67482706	phospholipid binding	Not known	8.01
Phosphate acetyltransferase *Neisseria bacilliformis ATCC BAA-1200*	gi|329120612	(phosphate acetyltransferase activity)	Not known	10.63
Phosphatidylserine synthase *Vibrio scophthalmi LMG 19158*	gi|343509456	phospholipid biosynthetic process (CDP-diacylglycerol-serine O-phosphatidyltransferase activity)	Not known	12.85
Phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthase-like protein *Geobacter sp. FRC-32*	gi|222053811	(catalytic activity)	Not known	17.06
phospholipase, patatin-like family protein *Sulfitobacter sp. EE-36*	gi|83942531	(lipid metabolic process)	Not known	42.77
PII uridylyl-transferase *Pseudomonas aeruginosa PAO1*	gi|15598854	cellular amino acid metabolic process (amino acid binding)	Not known	17.92
PilO, putative	gi|00002577	-	Not known	9.65
Pinn	gi|00013112	-	Not known	6.69
Polehole-like protein *Penaeus monodon*	gi|000069312	-	Not known	29.73
Pol-like protein	gi|00004531	(zinc ion binding)	Not known	6.17
Poly(3-hydroxyalkanoate) synthetase-like protein *Sphingomonas sp. KC8*	gi|357975589	-	Not known	16.88
Poly(A) polymerase, putative	gi|00013372	-	Not known	11.42
Poly-beta-hydroxybutyrate polymerase domain protein *Hyphomicrobium denitrificans 1NES1*	gi|353210572	poly-hydroxybutyrate biosynthetic process (transferase activity, transferring acyl groups)	Not known	6.95
Polyferredoxin *Clostridium botulinum C str. Eklund*	gi|168186747	(4 iron, 4 sulfur cluster binding)	Not known	2.35
Polypeptide of 976 aa, putative	gi|00007651	-	Not known	12.87
Polyphosphate kinase *Prevotella bergensis DSM 17361*	gi|261879163	polyphosphate biosynthetic process (ATP binding)	Not known	11.5
Portal vertex protein *Pseudomonas mendocina NK-01*	gi|330503114	-	Not known	10

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Possible transcriptional regulator *Actinomyces urogenitalis DSM 15434*	gi|227495778	transcription, DNA-dependent (DNA binding)	Not known	15.77
Potassium voltage-gated channel subfamily H member 1 isoform 2 *Homo sapiens*	gi|4504831	protein heterooligomerization (inward rectifier potassium channel activity)	Not known	8.73
Pre-mRNA splicing factor ATP-dependent RNA helicase PRP43 *Leptosphaeria maculans JN3*	gi|312212832	(ATP binding)	Not known	24.17
Preprotein translocase subunit secY *Ochrobactrum intermedium LMG 3301*	gi|239834933	protein transport	Not known	8.27
Prevents mitotic catastrophe 2 homolog	gi|00008367	(exonuclease activity)	Not known	10.59
Probable phosphoserine aminotransferase *Harpegnathos saltator*	gi|00007969	L-serine biosynthetic process (O-phospho-L-serine:2-oxoglutarate aminotransferase activity)	Not known	18.68
Prophage Lp1 protein 52, endolysin *Fructobacillus fructosus KCTC 3544*	gi|339624806	-	Not known	30.59
protein alphaB, Ca binding.	gi|00017947	-	Not known	6.03
Protein CLP1-like protein *Acromyrmex echinatior*	gi|00002825	(ATP binding)	Not known	14.16
Protein daughter of sevenless *Camponotus floridanus*	gi|307172991	(phospholipid binding)	Not known	13.11
Protein Mo25 (dMo25)	gi|00003380	embryonic development via the syncytial blastoderm	Not known	7.57
Protein singed, putative	gi|00000945	-	Not known	6.55
Protein translation factor SUI1 homolog	gi|00010528	regulation of translation (translation initiation factor activity)	Not known	10.05
Protein tyrosine phosphatase receptor type B *Clonorchis sinensis*	gi|00009341	peptidyl-tyrosine dephosphorylation (protein tyrosine phosphatase activity)	Not known	14.78
Proteophosphoglycan 5 *Leishmania major strain Friedlin*	gi|00018165	-	Not known	17.49
PRP38 pre-mRNA processing factor 38 domain containing B	gi|00014972	-	Not known	11.22
PT repeat/fibro-slime domain-containing protein *Teredinibacter turnerae T7901*	gi|254785927	-	Not known	18.13

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Purple acid phosphatase, putative	gi|00009271	(acid phosphatase activity)	Not known	19.48
Putative eukaryotic translation initiation factor 3, Theta subunit *Danaus plexippus*	gi|00005693	-	Not known	14.76
Putative GTP-binding protein 5 *Aedes aegypti*	gi|00013923	GTP catabolic process (GTP binding)	Not known	5.89
PVR3-like protein *Zea mays*	gi|226498322	-	Not known	5.73
Pyoverdine chromophore synthetase *Streptomyces hygroscopicus ATCC 53653*	gi|302540441	(catalytic activity)	Not known	28.07
Receptor for activated protein kinase c1	gi|00008161	-	Not known	12.15
Recombinase *Nitrosomonas sp. AL212*	gi|325983030	-	Not known	8.02
Reductase-related protein	gi|00010391	-	Not known	17.81
Regulator of de-novo NAD biosynthesis NadR *Bacillus licheniformis ATCC 14580*	gi|52081269	(small molecule binding)	Not known	17.05
Rep B partitioning protein/ParB-like protein *Roseovarius nubinhibens ISM*	gi|83952923	(DNA binding)	Not known	11.01
Replicative DNA helicase *Tsukamurella paurometabola DSM 20162*	gi|296141938	DNA duplex unwinding (ATP binding)	Not known	6.44
Resolvase *Erwinia pyrifoliae*	gi|27228698	DNA integration (DNA binding)	Not known	10.7
Resolvase domain-containing protein *Halothermothrix orenii H 168*	gi|220931984	(DNA binding)	Not known	11.2
Respiratory-chain NADH dehydrogenase subunit 1 *Candidatus Puniceispirillum marinum IMCC1322*	gi|294083718	(4 iron, 4 sulfur cluster binding)	Not known	9.62
Response regulator receiver protein *Archaeoglobus profundus DSM 5631*	gi|284162475	intracellular signal transduction (phosphorelay response regulator activity)	Not known	6.95
Reticulocalbin-2 *Harpegnathos saltator*	gi|307202124	(calcium ion binding)	Not known	16.07
Reverse transcriptase	gi|00003665	DNA integration (RNA binding)	Not known	15.56
RNA polymerase I large subunit *Ciona intestinalis*	gi|000037371	-	Not known	15.83
RNA polymerase II largest subunit *Candida sequanensis*	gi|169247220	-	Not known	14.35
RNA polymerase sigma factor *Cyanothece sp. PCC 7424*	gi|218440234	DNA-dependent transcription, initiation (DNA binding)	Not known	12.13
RNA-binding protein 12 *Camponotus floridanus*	gi|307179523	(nucleic acid binding)	Not known	13.8

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
RNA-metabolising metallo-beta-lactamase domain-containing protein, putative	gi|00009298	(hydrolase activity)	Not known	5.33
ROK family protein *Gordonia neofelifaecis NRRL B-59395*	gi|326382534	-	Not known	13.01
ROK family transcriptional regulator *Rhizobium leguminosarum bv. viciae 3841*	gi|116251421	-	Not known	21.01
rRNA (guanine-N(1)-)-methyltransferase Marinobacter aquaeolei VT8	gi|120554258	-	Not known	9.37
rRNA methylase *Methylomicrobium album BG8*	gi|334110009	-	Not known	21.31
Salivary proline-rich protein	gi|00008671	-	Not known	12.1
Saposin isoform 1	gi|00009172	-	Not known	19.05
Secreted protein with cysteine rich repeats and a Mucin like threonine rich repeat, signal peptide	gi|00005545	-	Not known	9.8
Secreted salivary gland peptide, putative	gi|00013384	-	Not known	12.81
Selenoprotein S *Xenopus (Silurana) tropicalis*	gi|144922660	-	Not known	9.03
Semaphorin 2a	gi|00002749	-	Not known	11.32
Sentrin-specific protease 8-like isoform 1 *Nasonia vitripennis*	gi|00009303	-	Not known	14.83
Serine/threonine-protein kinase B-raf-like *Amphimedon queenslandica*	gi|340374266	-	Not known	15.73
Serine/threonine-protein kinase CTR1 *Ectocarpus siliculosus*	gi|299117098	-	Not known	20.61
Serine/threonine-protein kinase pim-3-like *Ciona intestinalis*	gi|00008992	(ATP binding)	Not known	19.72
Seven transmembrane helix receptor	gi|00011883	-	Not known	9.58
SHC transforming protein, putative	gi|00012740	-	Not known	21.72
Shematrin-1	gi|00015174	-	Not known	12.26
Short wing CG18000-PK, isoform K	gi|00011263	-	Not known	9.7
Signal transduction protein *Vibrio angustum S14*	gi|90579529	cyclic nucleotide biosynthetic process (phosphorus-oxygen lyase activity)	Not known	23.08
Site-specific recombinase, phage integrase family domain protein *Vibrio splendidus 12B01*	gi|84387354	-	Not known	13.03

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
SJCHGC01957 protein	gi|00009815	-	Not known	20.31
Skuld CG9936-PD, isoform D	gi|00013240	-	Not known	8.09
S-layer domain-containing protein *Paenibacillus elgii B69*	gi|357009137	-	Not known	4.14
Slit-robo rho GTPase activating protein 1,3	gi|00003191	signal transduction	Not known	8.07
Slow muscle myosin S1 heavy chain *Homarus americanus*	gi|00016769	(motor activity)	Not known	15.02
Small nuclear ribonucleoprotein polypeptide G	gi|00004760	-	Not known	4.49
SMC domain-containing protein *Thermoanaerobacter italicus Ab9*	gi|289577486	-	Not known	16.83
SNF2-like protein *Haliscomenobacter hydrossis DSM 1100*	gi|332662947	(ATP binding)	Not known	12.4
SNF7 family protein *Tribolium castaneum*	gi|00011013	protein transport	Not known	10.15
Spastic paraplegia 21 (H. sapiens)	gi|00007403	-	Not known	24.78
Splicing factor 3A subunit 3 (Spliceosome Associated protein 61) (SAP 61) (SF3a60)	gi|00016550	RNA splicing (nucleic acid binding)	Not known	17.75
Splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)	gi|00008002	(nucleic acid binding)	Not known	11.17
Splicing factor yt521-b	gi|00006902	-	Not known	7.69
SpoVT / AbrB like domain protein *Synechococcus sp. PCC 7335*	gi|254426198	-	Not known	3.32
SR Protein (splicing factor) family member (rsp-7) *Caenorhabditis elegans*	gi|00016478	-	Not known	23.31
SRPK1a protein kinase	gi|00011688	(ATP binding)	Not known	15.47
Stylicine 2	gi|00009392	-	Not known	14.44
Sulfate adenylyltransferase *Synechocystis sp. PCC 6803*	gi|16330927	hydrogen sulfide biosynthetic process (ATP binding)	Not known	3.85
Super cysteine rich protein	gi|00002021	-	Not known	5.2
Tail assembly protein I from prophage *Escherichia coli O104:H4 str. 01-09591*	gi|340734555	-	Not known	6.03

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
TAR RNA-binding protein 2	gi|00010345	(double-stranded RNA binding)	Not known	10.06
Thioredoxin reductase *Blastopirellula marina DSM 3645*	gi|87310515	(Oxidoreductase activity)	Not known	18.86
Thioredoxin-related *Raphidiopsis brookii D9*	gi|282897452	cell redox homeostasis (electron carrier activity)	Not known	11.26
Thymidylate synthase thyX *Treponema succinifaciens DSM 2489*	gi|328949144	dTMP biosynthetic process (flavin adenine dinucleotide binding)	Not known	14.18
TIGR00268 family protein *Desulfosporosinus orientis DSM 765*	gi|357214132	-	Not known	10.47
TM helix repeat-containing protein *Acidovorax citrulli AAC00-1*	gi|120610701	-	Not known	5.39
TniB *Marinomonas sp. MED121*	gi|87120757	-	Not known	18.95
Transcription regulation repressor hexr protein *Ralstonia solanacearum Po82*	gi|334198994	carbohydrate metabolic process (sequence-specific DNA binding transcription factor activity)	Not known	16.48
Transcription termination factor *Enterobacteria phage HK022*	gi|19343387	DNA-dependent transcription, termination	Not known	8.8
Transcriptional regulator protein *Rhizobium etli 8C-3*	gi|218512383	-	Not known	20.39
Transcriptional regulator, HxlR family *Actinomyces sp. oral taxon 170 str. F0386*	gi|329945218	-	Not known	18.36
Transcriptional regulator, LacI family protein *Vibrio scophthalmi LMG 19158*	gi|343511170	transcription, DNA-dependent (DNA binding)	Not known	13.74
Transcriptional regulator, LysR family protein *Roseobacter sp. AzwK-3b*	gi|149916438	transcription, DNA-dependent (DNA binding)	Not known	8.36
transcriptional regulator, PadR family *Blautia hansenii DSM 20583*	gi|260589309	-	Not known	6.97
Transcriptional regulatory protein TctD *Roseibium sp. TrichSKD4*	gi|307943941	intracellular signal transduction (DNA binding)	Not known	11.6
Transglutaminase	gi|00010890	-	Not known	9.95
Transglutaminase domain protein *Frankia sp. EUN1f*	gi|288918868	-	Not known	12.91

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Transglutaminase y *Taeniopygia guttata*	gi|224078077	-	Not known	15.69
Transparent testa 8 *Arabidopsis thaliana*	gi|11121434	-	Not known	14.46
Transposable element MDG1 ORF111	gi|227732	-	Not known	4.07
Transposase *Corynebacterium casei UCMA 3821*	gi|356607650	transposition, DNA-mediated (DNA binding)	Not known	16.02
Transposase family protein A *Lactobacillus johnsonii ATCC 33200*	gi|227888988	transposition, DNA-mediated (DNA binding)	Not known	17.72
Transposase, IS4 family *Escherichia coli MS 182-1*	gi|300923979	transposition, DNA-mediated (DNA binding)	Not known	9.31
Transposon Ty3-I Gag-Pol polyprotein *Camponotus floridanus*	gi|00004434	DNA integration (nucleic acid binding)	Not known	9.66
Trehalase inhibitor	gi|124987	-	Not known	31.64
tRNA (uracil-5-)-methyltransferase *Chlamydomonas reinhardtii*	gi|159462564	-	Not known	9.11
tRNA pseudouridine synthase-like 1 *Camponotus floridanus*	gi|00002799	tRNA processing (RNA binding)	Not known	5.14
Troponin T isoform 3	gi|00004198	-	Not known	13.14
Twitching motility protein *Acidovorax delafieldii 2AN*	gi|241764782	transport (ATP binding)	Not known	18.07
Two-component sensor histidine kinase protein *Rhizobium etli IE4771*	gi|218659984	-	Not known	7.66
Two-component system response regulator *Streptomyces sviceus ATCC 29083*	gi|297199299	-	Not known	17.86
Two-component system sensor histidine kinase/response regulator, hybrid *Salinibacter ruber DSM 13855*	gi|83816000	-	Not known	5.6
Type 12 methyltransferase *Frankia symbiont of Datisca glomerata*	gi|336177322	(methyltransferase activity)	Not known	7.28
Type III effector protein *Ralstonia solanacearum CFBP2957*	gi|300705555	(ATP binding)	Not known	11.92

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
U2 small nuclear riboprotein auxiliary factor 50 CG9998-PA	gi|00009867	-	Not known	11.72
U88	gi|00013452	-	Not known	12.54
Ubiquinone binding protein *Papilio xuthus*	gi|00009587	(ubiquinol-cytochrome-c reductase activity)	Not known	6.08
Ubiquitin C, isoform CRA_a *Homo sapiens*	gi|00015584	-	Not known	13.82
Ubiquitin carboxyl-terminal hydrolase, putative *Pediculus humanus corporis*	gi|242020942	ubiquitin-dependent protein catabolic process (ubiquitin thiolesterase activity)	Not known	13.27
Ubiquitin conjugating enzyme 7 interacting protein	gi|00006522	(zinc ion binding)	Not known	12.23
Ubiquitin specific protease 14 isoform 2	gi|00002551	-	Not known	14.32
Ubiquitin-activating enzyme E1 *Tribolium castaneum*	gi|00016936	cellular protein modification process (ATP binding)	Not known	13.41
Ubiquitin-conjugating enzyme E2-17 kDa *Harpegnathos saltator*	gi|00016442	(acid-amino acid ligase activity)	Not known	5.11
Ubiquitin-like 1-activating enzyme E1B (SUMO-1-activating enzyme subunit 2) (Anthracycline-associated resistance ARX) *Rattus norvegicus*	gi|00016462	cellular protein modification process (ATP binding)	Not known	8.47
UBX domain-containing protein 2 *Caligus rogercresseyi*	gi|225711798	-	Not known	15.44
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5-like *Callithrix jacchus*	gi|296224671	-	Not known	10.29
UDP-glucose 4-epimerase *Ictalurus punctatus*	gi|318056070	cellular metabolic process (UDP-glucose 4-epimerase activity)	Not known	11.39
UDP-glucose 6-dehydrogenase *Fusobacterium mortiferum ATCC 9817*	gi|340750046	(NAD binding)	Not known	6.71
UDP-glucose/GDP-mannose dehydrogenase *Geobacter metallireducens GS-15*	gi|78222642	(NAD binding)	Not known	3.25
Umps-prov protein	gi|00010145	de novo' UMP biosynthetic process (orotate phosphoribosyltransferase activity)	Not known	13.32
Uncharacterized component of phosphonate metabolism *uncultured Rhodospirillales bacterium HF0500_23A22*	gi|297171689	-	Not known	5.23

Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

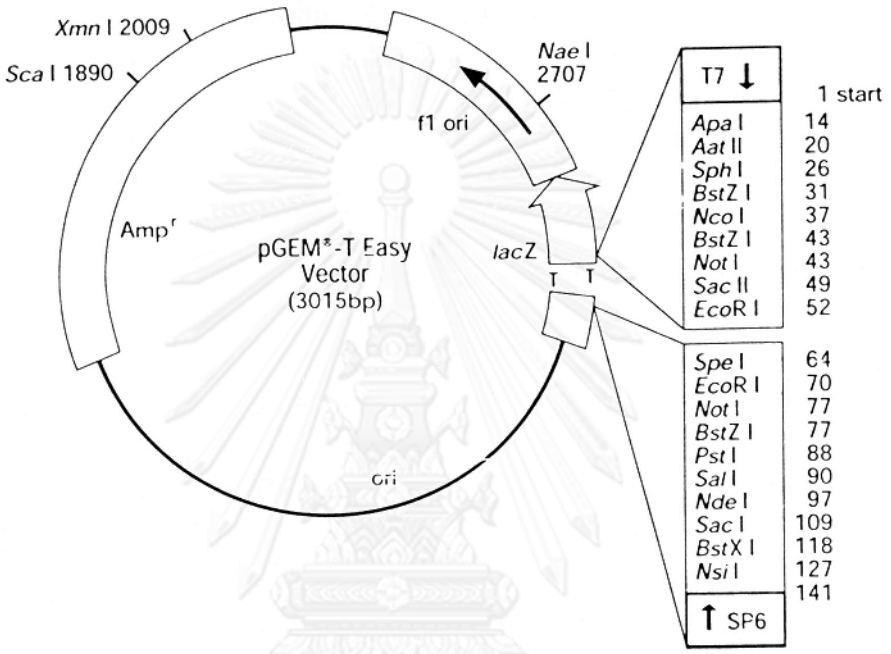
Protein name	Acc. No.	Function	Cellular component	Score
UPF0585 protein C16orf13 homolog *Anolis carolinensis*	gi|327291306	-	Not known	20.68
UV radiation resistance-associated gene protein *Pyrenophora tritici-repentis Pt-1C-BFP*	gi|189208161	positive regulation of autophagy	Not known	15.62
Vacuolar ATPase subunit C	gi|00004496	-	Not known	13.97
Vacuolar protein sorting 8 homolog	gi|00005365	(zinc ion binding)	Not known	13.87
Vacuolar protein-sorting-associated protein 36 *Cordyceps militaris CM01*	gi|346321194	(phosphatidylinositol-3-phosphate binding)	Not known	20.75
VenA *Streptomyces venezuelae*	gi|2555096	(transferase activity)	Not known	6.98
Vezatin, adherens junctions transmembrane protein, isoform CRA_f *Mus musculus*	gi|148689625	-	Not known	19.62
VgrG like protein *Paracoccus denitrificans SD1*	gi|338849989	-	Not known	16.18
VH region of G7 Ab 2.9 *Mus musculus*	gi|1333986	-	Not known	7.66
Virion protein US2 *Bovine herpesvirus 1*	gi|9629883	-	Not known	6.19
Vomeronasal type-1 receptor 2-like *Cricetulus griseus*	gi|354502641	-	Not known	26.23
WD-repeat protein, putative *Ixodes scapularis*	gi|00011412	-	Not known	17.58
xanthine dehydrogenase *Pereskia portulacifolia*	gi|160690150	(UDP-N-acetylmuramate dehydrogenase activity)	Not known	20.47
X-linked deafness dystonia protein	gi|00014176	-	Not known	6.62
YALI0F21681p *Yarrowia lipolytica*	gi|50556618	-	Not known	5.69
YD repeat protein *Pantoea sp. aB*	gi|304399325	-	Not known	9.75
YHR077Cp-like protein	gi|00010353	RNA metabolic process (DNA binding)	Not known	5.85
YqjA *Bacillus subtilis*	gi|1303952	-	Not known	7.8
Zgc:85671 protein *Danio rerio*	gi|00016984	-	Not known	5.24
Zinc finger and SCAN domain-containing protein 21 *Clonorchis sinensis*	gi|358338670	(nucleic acid binding)	Not known	18.51
Zinc finger CCCH domain-containing protein 4 *Ascaris suum*	gi|324503865	(nucleic acid binding)	Not known	11.75
Zinc finger protein 273-like *Pongo abelii*	gi|297680333	-	Not known	3.22

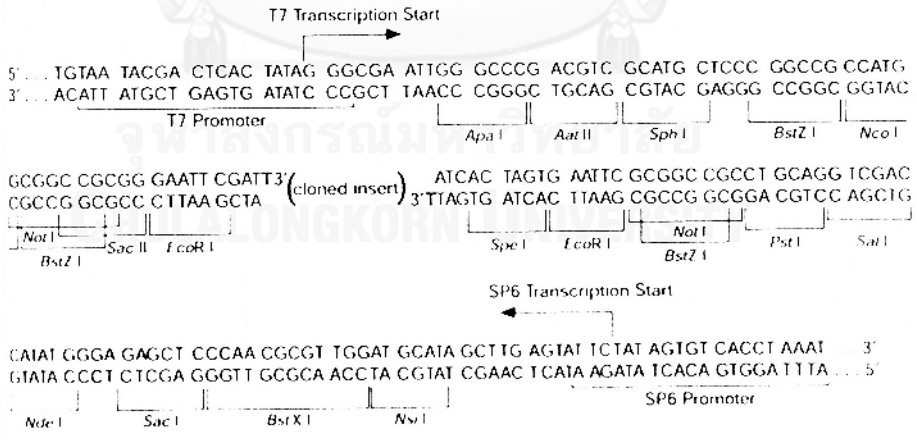
Table A4 Proteins from ovaries with unknown cellular component identified by GeLC-MS/MS (Cont.)

Protein name	Acc. No.	Function	Cellular component	Score
Zinc finger protein 273-like *Pongo abelii*	gi|297680333	-	Not known	3.22
Zinc finger protein 593 homolog *Megachile rotundata*	gi|00010671	(zinc ion binding)	Not known	11.35
Zinc finger protein 804A *Canis lupus familiaris*	gi|345797077	(zinc ion binding)	Not known	7.53
Zinc knuckle containing protein-like *Oryza sativa Japonica* Group	gi|55297470	-	Not known	12.47
Zn binding domain-containing protein *Ectocarpus siliculosus*	gi|298708171	(ATP binding)	Not known	25.31
Zn-dependent hydrolase, glyoxylase *Desulfosporosinus orientis DSM 765*	gi|357212018	(hydrolase activity)	Not known	15.62
3-hydroxy-3-methylglutaryl coenzyme A synthase *Dendroctonus jeffreyi*	gi|9621905	isoprenoid biosynthetic process (hydroxymethylglutaryl-CoA synthase activity)	Not known	6.48
3-isopropylmalate dehydrogenase *Saccharopolyspora erythraea NRRL 2338*	gi|134099735	cellular response to amino acid starvation (3-isopropylmalate dehydrogenase activity)	Not known	18.56
3-methyl-2-oxobutanoate hydroxymethyltransferase *Paracoccus denitrificans SD1*	gi|338849003	pantothenate biosynthetic process from valine	Not known	18.35
3-oxoacyl-ACP reductase *Desulfotomaculum acetoxidans DSM 771*	gi|258514466	-	Not known	12.72
4-coumarate:CoA ligase	gi|00004142	phenylpropanoid metabolic process (4-coumarate-CoA ligase activity) (iron-sulfur cluster binding)	Not known	17.19
4Fe-4S ferredoxin	gi|00012889		Not known	18.48
4-hydroxyphenylacetate 3-monooxygenase, oxygenase subunit *Deinococcus maricopensis DSM 21211*	gi|320334483	phenylacetate catabolic process (4-hydroxyphenylacetate 3-monooxygenase activity)	Not known	4.63

Appendix B

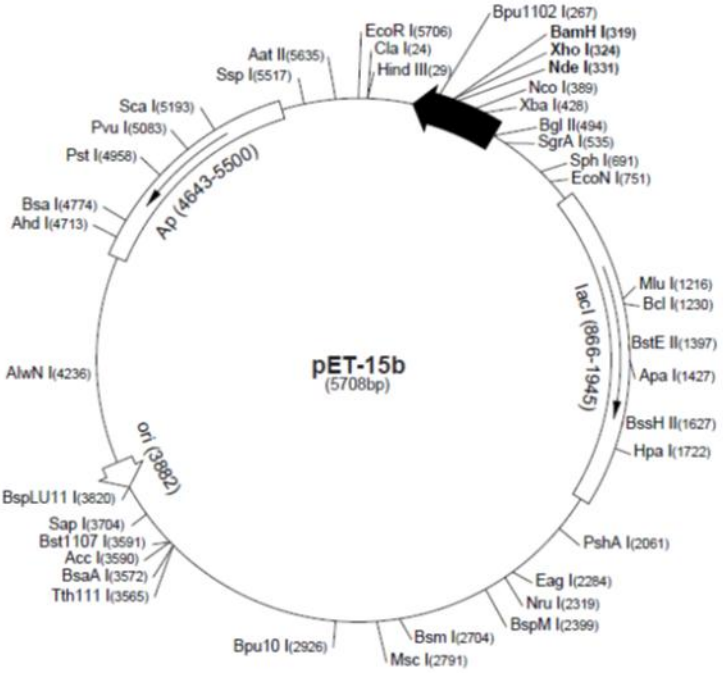
Restriction mapping of pGEM® T-easy Vector

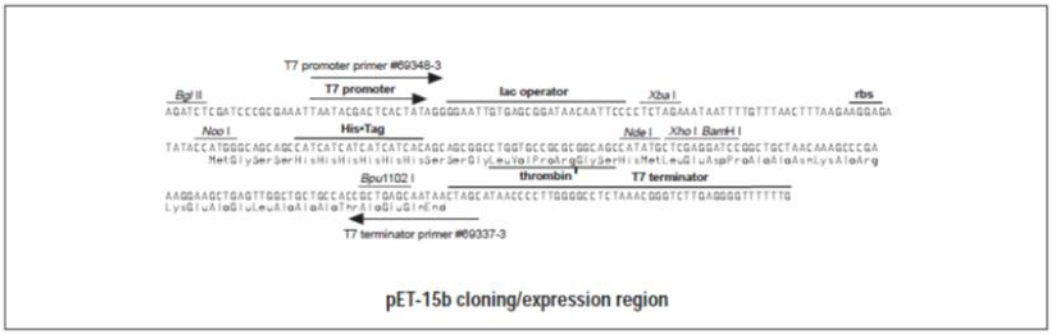




Restriction mapping of pET15b expression Vector

pET-15b sequence landmarks	
T7 promoter	453-469
T7 transcription start	452
His•Tag coding sequence	362-380
Multiple cloning sites	
(*Nde* I - *Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	(866-1945)
pBR322 origin	3882
bla coding sequence	4643-5500

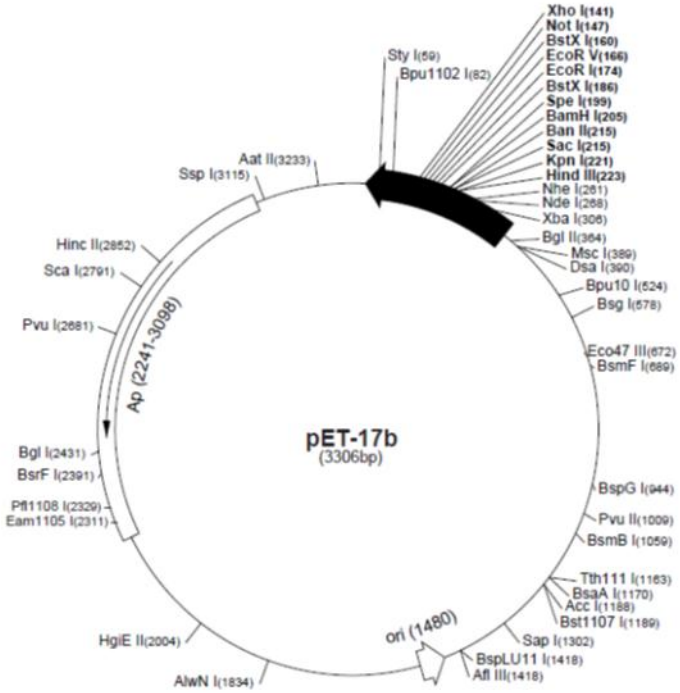


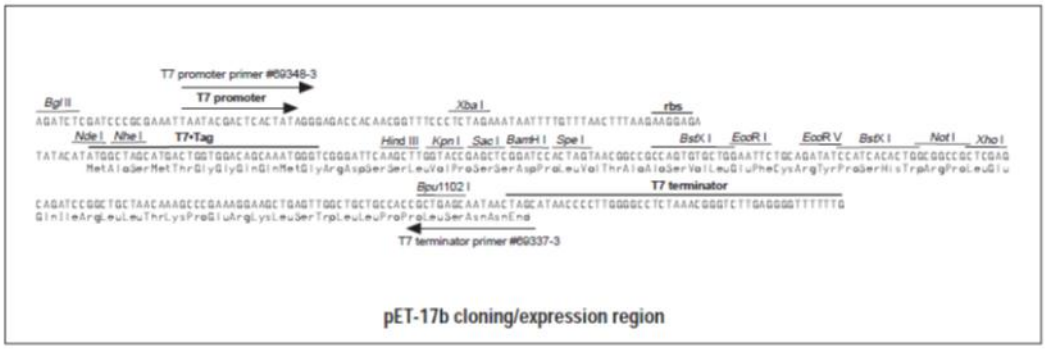


pET-15b cloning/expression region

Restriction mapping of pET17b expression Vector

pET-17b sequence landmarks	
T7 promoter	333-349
T7 transcription start	332
T7•Tag coding sequence	237-269
Multiple cloning sites	
(*Hin*d III - *Xho* I)	141-228
T7 terminator	28-74
pBR322 origin	1480
bla coding sequence	2241-3098

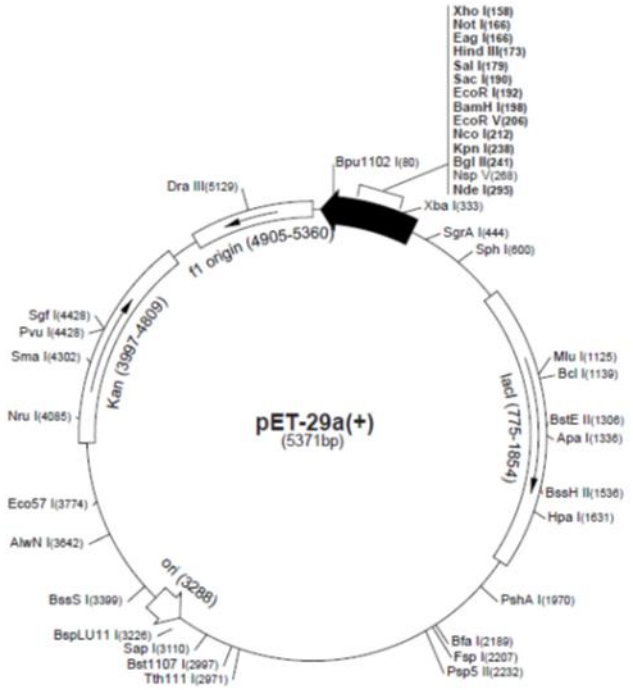




Restriction mapping of pET29a expression Vector

pET-29a(+) sequence landmarks	
T7 promoter	368-384
T7 transcription start	367
S•Tag coding sequence	249-293
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-217
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	775-1854
pBR322 origin	3288
Kan coding sequence	3997-4809
f1 origin	4905-5360

The maps for pET-29b(+) and pET-29c(+) are the same as pET-29a(+) (shown) with the following exceptions: pET-29b(+) is a 5370bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-29c(+) is a 5372bp plasmid; add 1bp to each site beyond *Bam*H I at 198.



VITA

Miss. Witchulada Talakhun was born on September 10, 1983 in Sakonnakhon. She graduated with the degree of Bachelor of Science from the Department of Science (Biotechnology), Ramkhumhang University in 2005. She graduated a Master degree at the Program in Biotechnology, Chulalongkorn University in 2008. She has enrolled a Doctor's degree program at the Program in Biotechnology, Chulalongkorn University since 2009.

Publications

International publications

1. Talakhun, W., Roytrakul, S., Phaonakrop, N., Kittisenachai, S., Khamnamtong, B., Klinbunga, S., Menasveta, P. 2012. Identification of reproduction-related proteins and characterization of the protein disulfide isomerase A6 cDNA in ovaries of the giant tiger shrimp Penaeus monodon. Comp. Biochem. Physiol. D 7, 180–190.

2. Talakhun,W., Khamnamtong, B., Nounurai, P., Klinbunga, S., Menasveta, P. 2014 Characterization, expression and localization of valosin-containing protein in ovaries of the giant tiger shrimp Penaeus monodon, Gene 533, 188-198.

International conferences

1.Talakhun, W., Kittisenachai, S., Roytrakul, S., Klinbunga, S. and Menasveta, P. (2008). Identification and characterization of proteins related to ovarian development of the giant tiger shrimp Penaeus monodon. The 20th Annual Meeting and International conference of the Thai Society for Biotechnology, 14–17 October 2008, Maha Sarakham, Thailand (Poster presentation).

2.Talakhun, W., Phaonakrop, N., Roytrakul, S., Klinbunga, S., Menasveta, P., Khamnamtong, B. (2013). Identification of reproduction-related proteins and characterization of thymosin-β in ovaries of the giant tiger shrimp Penaeus monodon. 10th Asian Fisheries And Aquaculture Forum. April 30- May 4 2013. The Ocean Resort Hotel, Yeosu, Korea (Oral presentation).

National conferences

1. Talakhun, W., Kittisenachai, S., Roytrakul, S., Klinbunga, S. and Menasveta, P. (2008). Identification of proteins related to ovarian development of the giant tiger shrimp Penaeus monodon using two dimensional gel electrophoresis. 34th Congress

on Science and Technology of Thailand, 31 October-2 November 2008, Queen Sirikit National Convention Hall, Bangkok, Thailand (Oral presentation).

2. Talakhun, W., Phaonakrop, N., Roytrakul, S., Khamnamtong, B., Klinbunga, S. and Menasveta, P. (2012). Identification of reproduction-related protein in ovaries of the giant tiger shrimp Penaeus monodon by the proteomic approch. 38th Congress on Science and Technology of Thailand, 17-19 October 2012, The empress hotel, Chaingmai, Thailand (Oral presentation).

3. Thamniemdee, N., Talakhun, W. and Klinbunga, S. (2009). Identification of genes involved in ovarian development of the giant tiger shrimp Penaeus monodon analyzed by cDNA-AFLP. 35th Congress on Science and Technology of Thailand, 15-17 October 2009, The Tide Resort, Burapha University, Chonburi, Thailand (Oral presentation).

Book chapter

Klinbunga, S., Khamnamtong, B., Preechaphol, R., Leelatanawit, R., Talakhun, W., Karoonuthaisiri, N., Roytrakul, S., Penman, D.J., McAndrew, B.J. and Menasveta, P. (Genetics and its applications for increasing management and culture efficiency of the giant tiger shrimp (Penaeus monodon). In: Alday-Sanz, V, editor. The shrimp book. 2010. P.149-192.