เอกสารแนบหมายเลข 4
หน้า 2/7

สัญญาเลขที่ RSA5980029

โครงการ: การพัฒนาการปฏิสนธิแบบจัดเตรียมเซลล์สืบพันธุ์โดยใช้พื้นฐานจากการศึกษาการเปลี่ยนแปลงของโปรตีน ไขมัน และเอ็นไซม์บนผิวเซลล์อสุจิและเซลล์ไข่

แบบฟอร์มรายงานความก้าวหน้าของโครงการในรอบ 42 เดือนของหัวหน้าโครงการ

ชื่อโครงการ : การพัฒนาการปฏิสนธิแบบจัดเตรียมเซลล์สืบพันธุ์โดยใช้พื้นฐานจากการศึกษาการเปลี่ยนแปลงของโปรตีน ไขมัน และเอ็นไซม์บนผิวเซลล์อสุจิและเซลล์ไข่

ระยะเวลาโครงการ : 3 ปี (16 มิถุนายน 2559 - 15 มิถุนายน 2562)
(ขอขยายเวลาต่ออีก 6 เดือน ถึง 15 ธันวาคม 2562

ชื่อหัวหน้าโครงการวิจัยผู้รับทุน : รศ.ดร.สมลักษณ์ อสุวพงษ์พัฒนา

รายงานในช่วงตั้งแต่วันที่ 16 มิถุนายน 2561 **ถึงวันที่** 15 ธันวาคม 2562

1. สำหรับหัวหน้าโครงการวิจัยผู้รับทุน รายงานความก้าวหน้าประกอบด้วย

1.1 การดำเนินงาน
☑ ได้ดำเนินงานตามแผนที่วางไว้
❑ ได้ดำเนินงานล่าช้ากว่าแผนที่วางไว้
❑ ได้เปลี่ยนแผนงานที่วางไว้ดังนี้

1.2 รายละเอียดผลการดำเนินงานของโครงการ

บทคัดย่อ

ก่อนที่จะเกิดการปฏิสนธิ (fertilization) เซลล์อสุจิ (sperm) ต้องผ่านกระบวนการหลายขั้นตอนรวมถึงการพัฒนาความสมบูรณ์ของเซลล์อสุจิ (maturation) การเพิ่มความสามารถในการปฏิสนธิ (capacitation) และเกิดการเหนี่ยวนำการแตกของถุงเอนไซม์ (acrosome reaction) กระบวนการเหล่านี้จะเกิดขึ้นที่ท่อทางเดินสืบพันธุ์เพศชาย (epididymis) และที่ท่อทางเดินสืบพันธุ์ของเพศหญิง (uterine tube) กระบวนการทั้งหมดนี้เกี่ยวข้องกับการเปลี่ยนแปลงของไขมัน (lipids) และโปรตีน (proteins) บนเยื่อหุ้มเซลล์อสุจิเพื่อเปิดเผยส่วนจับ (ligands) ที่จะไปจับกับตัวรับที่เป็นน้ำตาลต่อเชื่อมกับโปรตีน (glycoconjugate) บนผิวเซลล์ไข่ เมื่อมีการจับกันเกิดขึ้นก็จะเหนี่ยวนำให้เซลล์อสุจิเกิดปฏิกิริยาเหนี่ยวนำการแตกของถุงเอ็นไซม์บนหัวของตัวอสุจิ เพื่อย่อยผิวของเซลล์ไข่ทำให้เซลล์สืบพันธุ์ (gamete) สามารถผสมกันได้ ในการศึกษานี้ชี้ให้เห็นถึงหลักฐานบ่งชี้ว่าการปรากฏของ N-linked mannose glycoconjugate หรือ pmTSP-II ที่อยู่บนผิวเซลล์ไข่จะเป็นตัวรับที่จำเพาะในการเหนี่ยวนำให้เกิดการแตกของถุงเอนไซม์อะโครโซม การศึกษาในระดับโมเลกุลของ pmTSP-II แสดงให้เห็นว่าจุดยึดเกาะของน้ำตาลแมนโนสที่มี N-linked อยู่ในส่วนของ chitin binding และ TSP3 การปรากฏของน้ำตาลแมนโนสได้ถูกพิสูจน์โดยการใช้เลคตินชนิด ConA ที่ซึ่งใช้ประโยชน์ในการสกัดบริสุทธิ์ของ pmTSP-II (น้ำหนักโมเลกุล 250 กิโลดัลตัน) เราพบว่าหน้าที่ของน้ำตาลแมนโนสจะเกี่ยวข้องโดยตรงกับการเหนี่ยวนำการแตกของถุงเอนไซม์ซึ่งถูกรบกวนได้โดย ConA ทำให้ไม่เกิดการเหนี่ยวนำให้เกิดการแตกของถุงเอนไซม์อะโครโซม

ปฏิกริยาดังกล่าวสามารถย้อนกลับได้ด้วยการเติมน้ำตาลแมนโนส นอกจากนั้นการสังเคราะห์เปปไทด์ของ 3 โดเมนของ pmTSP-II ในแบคทีเรีย E. coli (โปรตีนสังเคราะห์ที่ได้จะไม่มีส่วนของน้ำตาล) ไม่สามารถเหนี่ยวนำให้เกิดการแตกของถุงเอนไซม์อะโครโซมได้ กล่าวโดยสรุปคือ เราได้รายงานหน้าที่สำคัญของการมีหมู่น้ำตาลแมนโนส บนสาย pmTSP-II ที่มีหน้าที่เกี่ยวข้องกับการเหนี่ยวนำให้เกิดการแตกของถุงเอนไซม์อะโครโซม (acrosome reaction) ซึ่งเป็นหน้าที่ใหม่ของโปรตีนในกลุ่ม (family) ของ TSP ที่มีอยู่ในระบบสืบพันธุ์ของกุ้ง

ทางฝั่งเซลล์อสุจิ เราได้แสดงให้เห็นถึงคุณสมบัติของเอนไซม์ Cathepsin D (CAT-D) ซึ่งเป็นเอนไซม์ที่รู้จักกันดีในประเภท serine protease ที่อยู่ในถุงไลโซโซม (lysosome) ของเซลล์ทั่วไปของร่างกาย (somatic cell) ทำหน้าที่ทำลายออร์กาแนลที่หมดอายุ การปรากฏและหน้าที่ของเอนไซม์นี้ในระบบสืบพันธุ์มีความจำเพาะอย่างมากในสัตว์แต่ละชนิด ในกุ้งก้ามกรามเอนไซม์ MrCAT-D ถูกพบทั้งในเซลล์พี่เลี้ยง (sertoli-liked cell) และเซลล์สืบพันธุ์ที่กำลังพัฒนาในอัณฑะ รวมถึงเซลล์ที่บุท่อทางเดินสืบพันธุ์ เอนไซม์ MrCAT-D ถูกพบอยู่บนผิวหุ้มเซลล์อสุจิมากกว่าอยู่ในถุงเอนไซม์อะโครโซม การวิเคราะห์ปฏิกริยาของเอนไซม์นี้จากอัณฑะและจากเซลล์อสุจิที่แยกออกมาจากอันฑะมีค่าปฏิกริยา 57.83± 2.21 นาโนโมล/ไมโครกรัม/ชม โดยวัดจากการทำปฏิกริยากับสารตั้งต้นเรืองแสงของเอนไซม์ (Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2) หน้าที่จำเพาะของ MrCAT-D คือการย่อยสลายสาย actin (น้ำหนักโมเลกุล 42 กิโลดัลตัน) ที่พบอยู่ระหว่างรอยต่อของเซลลืบพันธุ์กับเซลล์พี่เลี้ยง เรียกว่ารอยต่อนี้ว่า Ectopic Specialization (ES junction) ดังนั้นจึงสรุปว่าเอนไซม์ MrCAT-D มีหน้าที่เกี่ยวข้องกับการปลดปล่อยเซลล์อสุจิ (spermiation) ซึ่งเป็นการรายงานครั้งแรกในระบบสืบพันธุ์สัตว์น้ำ นอกจาก MrCAT-D แล้วเรายังได้ศึกษาคุณสมบัติของ Nieman Pick type 2-C (NPC2) หนึ่งในโปรตีนที่มีหน้าที่ส่งผ่านโคเลสเตอรอลในระบบสืบพันธุ์ MrNPC2 ที่พบในกุ้งก้ามกรามสามารถพบได้ที่เยื่อบุผิวของอัณฑะและท่อทางเดินสืบพันธุ์เพศผู้ ที่โดยเฉพาะเซลล์อสุจิจากท่อนำอสุจิ (vas deferens) เท่านั้นที่พบ NPC2 ในขณะที่เซลล์อสุจิจากอัณฑะไม่พบ NPC2 บนผิวเซลล์อสุจิ นอกจากนี้ยังพบการสร้าง NPC2 ในเยื่อบุผิวของท่อนำอสุจิ และหลั่งออกมาในรูป แบบของเม็ดไขมัน (vesicle) ที่ลอยอยู่ในสารน้ำภายในท่อนำอสุจิ ซึ่งเชื่อว่ามีหน้าที่ในการขนส่ง NPC2 ไปบนเยื่อหุ้มเซลล์อสุจิ จากผลการทดลองที่ผ่านมาระดับของโคเลสเตอรอลในอสุจิที่ได้มาจากท่อนำอสุจิมีค่าต่ำกว่าในเซลล์อสุจิที่มาจากอัณฑะ ในขณะที่เม็ดไขมันภายในท่อนำอสุจิมีระดับโคเลสเตอรอลสูงกว่า จึงเชื่อว่าโคเลสเตอรอลมีการขนถ่ายจากผิวของเซลล์อสุจิผ่านเม็ดไขมันเพื่อส่งต่อไปยังเซลล์บุผิวท่อนำอสุจิในระบบทางเดินสืบพันธุ์ โดยสรุปผลการศึกษาแสดงให้เห็นว่าการกระจายของ MrNPC2 ยีน และโปรตีนในทางเดินสืบพันธุ์เพศผู้ของกุ้งก้ามกราม และถูกสร้างจากเซลล์บุผิวท่อนำอสุจิเพื่อส่งต่อให้กับผิวเซลล์อสุจิโดยการเกาะเกี่ยวกับโคเลสเตอรอล ซึ่งนับเป็นข้อมูลที่สำคัญของการปรับเปลี่ยนบนชั้นผิวของเซลล์อสุจิ ที่เกี่ยวข้องกับกระบวนการทำให้เซลล์อสุจิมีความสมบูรณ์ (maturation) ในสัตว์น้ำ

Abstract

Prior to fertilization process, sperm undergo multiple steps including maturation, capacitation and acrosome reaction, which occur at the epididymis, in the female reproductive tract. These processes involve modifications of lipids and proteins on the plasma membrane to enable the exposure of sperm ligands to interact with glycoconjugates receptor on the egg extracellular matrices that initiate sperm acrosome reaction prior to gamete incorporation. Here, we evidenced the existence of *N*-linked mannose glycoconjugates on the egg's *pm*TSP-II, which is significance for sperm acrosome reaction (AR) induction. Molecular analysis of *pm*TSP-II demonstrated the anchorage sites of *N*-linked glycan at both chitin binding (CBD) and TSP3 domains. Presence of mannose residues were verified by Con A lectin histochemistry on the purified fraction of *pm*TSP-II (250 kDa with protease inhibitor). The function of mannose glycoconjugate was evident by Con A interference with *pm*-TSP-II induced AR as well as the recoverable AR induction ability by inclusion of mannoses into the treatment mixture. Additionally, recombinant proteins expressed in *E.coli* of the three signature *pm*TSP-II domains (devoid of glycosylation) showed a minimal ability to initiate the AR responses. Together, we reported here a pivotal role of mannosylated *pm*TSP-II that imparts in shrimp sperm AR modulation, a novel role of TSP family protein in shrimp reproductive biology.

On sperm site, we have characterized cathepsin D (CAT-D), a well-known serine protease that functions as a house-keeping lysosomal enzyme in all somatic cells. Its existence and functions in reproductive tissues are highly unique from species-to-species, even in the somatic derived cells. In *Macrobrachium rosenbergii*, existence of MrCAT-D and its translational product was detected in both somatic cells (Sertoli-like supporting cells) and developing spermatogenic cells of testis as well as along accessory spermatic ducts. Specifically, MrCAT-D was localized onto the sperm surface rather than within the acrosomal matrix. MrCAT-D in testicular and sperm isolates showed its active enzyme activity at 57.83±2.21 nmole/μg/hr towards its specific fluorogenic substrate (Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-D-Arg-NH_2). MrCAT-D also exerted its function towards digesting filamentous actin (42 kDa) that was proven to be localized at the junction between germ cells and supporting cells (Sertoli-liked cells) which called ES junction. Together, we have characterized and localized MrCAT-D gene and its translational product in both supporting and germ cells of testis and claimed its enzymatic function towards actin digestion, presumably at ES junction, a spermiation function that is firstly reported in marine invertebrate reproductive biology. Moreover, we also characterized Niemann Pick type 2-C (NPC2), one of the best candidate of CHO transporter among many known transporters in reproductive system. MrNPC2 existed along epithelial cells of testis and spermatic duct.

Interestingly, only the vas deferens but not testicular sperm that is reactive with anti-NPC2. This result suggests that MrNPC2, upon its biosynthesis in spermatic duct epithelium, is secreted and may be associated with the multi-lamellar vesicles within the ductal fluid to transfer NPC2 towards sperm surface. Its gauging from the lower levels of CHO in the sperm collected from spermatic duct (than testicular sperm), whilst, the spermatic vesicles inversely possess a higher level of CHO, it is presumed that CHO would be shuffled from the sperm surface via spermatic vesicles and phagocytosed by the epithelial cells. Conclusively, our results demonstrating distribution of MrNPC2 transcripts and protein in the *M. rosenbergii* male reproductive tracts as well as its presence in spermatic vesicles and binding to sperm CHO would provide insightful information in sperm surface modification which should be considered as a part of sperm maturation in marine invertebrates.

1. Executive Summary

In order to gain a full efficiency of fertilization, sperm need to acquire external factors to modify both their structures and physiological functions in addition to those occur intrinsically to the sperm cell themselves. These modifications take place in both male and female reproductive tracts, collectively known as sperm maturation and capacitation, and are well established in mammals (Yangimachi 1994). At the spawning time, female shrimp (*Penaeus monodon*) released the mature oocyte with the jelly coat known as cortical rod protein (CRP). The cortical rod components are containing an acrosome reaction inducers. The main component of cortical rod are glycoproteins, including cortical rod protein (CRP) and shrimp ovarian peritrophin (SOP). Recently, we reported that thrombospondins (TSPs) in *P. monodon* or *pmTSP*-II are found in cortical rod proteins and engage their ability to induce acrosome reaction. The sequence analysis of *pmTSP-II* composed of 1043 amino acids containing 4 domains including chitin binding, EGF-like, TSP-3 and TSP-C domains. In this study, we emphasized on the purification of *pmTSP-II* and test its function in triggering acrosome reaction, particularly, carbohydrate moieties on each domain of *pmTSP-II* was also tested using the recombinant proteins expressed in E. coli.

For purification of *pmTSP-II*, the crude water soluble cortical rod proteins were prepared in an artificial seawater (ASW) and further subjected to a size exclusion chromatography. To prove the glycosylation is an important part of acrosome induction, we synthesized the linear O-β-GlcNAc on the lipid substrate and used the CTD110.6 antibody to probe O-β-GlcNAc oligomers and on both lipid substrate as well as on purified *pmTSP*-II proteins. The crude of wsCR and purified fraction of *pmTSP-II* proteins were probed with anti-TSP against chitin binding domain antibody and further tested in

shrimp ovarian tissue. Finally, we tested the functions of O-β-GlcNAc glycoconjugates on pm-TSP-II polymerization and induction of sperm acrosome reaction.

The results showed the existence of complex O- β -GlcNAc moiety (chitin chain) in the purified *pmTSP-II* using CTD110.6 antibody at the 250-kDa immunoreactive band which also was confirmed by its recognition of CTD110.6 on the oligomeric O-β-GlcNAc linked to lipid substrate. The purified fractions of *pmTSP-II were* stained intensely with Con-A which indicate that *pmTSP-II* is enriched in the mannose residue apart from O-β-GlcNAc counterparts. On SDS-PAGE results, even pmTSP-II was exposed to a strong reducing agent and detergent, it was still-sizable at 250 kDa then we postulated the inter-molecular cross-linking of *pmTSP-II.* When we tested this hypothesis by enzymatic digestion of chitin chain on the purified *pmTSP-II,* the molecular mass of *pmTSP-II* was gradually reduced from 250 to 110 kDa. As *pmTSP-II* was enriched in mannoses, we thus used ConA as a tool for the inhibition assay and evidently verified that mannosyl glycoconjugate is an important part of *pmTSP-II* that play a role in sperm acrosome modulation.

In the sperm side, we spent our effort to investigate sperm enzymes that are significant in many sperm physiology iccluding spermiation, sperm maturation, and capacitation. Among many well-known enzymes, Cathepsin D (Cat-D), an aspartic protease which is well-characterized in the lysosomes of somatic cells and the acrosomal system of male germ cells is chosen. In male reproductive system of mammals, CAT-D has been localized in testis and epididymis of mouse, rat and human, however, its expression in the reproductive system of crustaceans is still very limited. In Decapod crustaceans, CAT-D has been reported in the gastric fluids of two species of clawed lobsters (*Homarus americanus* and *Homarus gammarus*). In the reproductive system of shrimps, CAT-D and other cathepsin family such as CAT-A, CAT-B and CAT-L are also found in the *Macrobrachium nipponense* testis cDNA library whereas CAT-D are not found in the ovary of this species which suggests that CAT-D might have a specific function in testis.

In this study, we characterized the full length of CAT-D gene and protein product and elucidated its function in releasing sperm from the supporting cells into testicular lumen, a process refer to as “spermiation”. The full-length of MrCAT-D was consisted of 1,361 nucleotides and 385 amino acid residues including 15 amino acid residues of signal peptide at N-terminus. Upon the proteolytic processing, the mature form was consisted of two chains which are light and heavy chain. The predicted molecular weight of the cleaved CAT-D was 42 kDa and an isoelectric point was 6.35. A phylogenetic tree analysis revealed that MrCAT-D was closely related to CAT-D of *M. japonicas* and diverged from a common ancestor. RT-PCR amplification was performed in various tissues of M.

rosenbergii using CAT-D primers designed and constructed from *M. rosenbergii* CAT-D nucleotides sequences to give a deduced RT-PCR product length of 323 bp. The results revealed a single, intense PCR amplifying band at 323 bp in testis, vas deferens, spermatophores as well as other tissues. Enzymatic assay of tissues extracts from testis (TE) and sperm (Tsp) was performed using fluorogenic substrate (Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-D-Arg-NH_2). The results clearly showed that TE and Tsp engaged high cathepsin enzyme activity 57.83±2.21 nmole/μg/hr). This enzyme activity is proven to be localized on the sperm surface, as the staining of anti-CAT-D were similar between live and aldehyde fixed isolated sperm. This result prompted us to propose MrCAT-D function in cleaving actin meshwork at the junction between supporting cells and sperm, since one of the well-known natural substrate of CAT-D is filamentous actin. Active hydrolysis of filamentous actin (isolated from neuronal stem cells, SH-5y5y) was evident. These results suggested that there are modifications of sperm by the surrounding fluid proteins during sperm transit from testis into vas deferens and spermatophores in male reproductive tract of *M. rosenbergii.* Moreover, the modification processes are extensively reported at sperm plasma membrane especially, membrane lipid modifications when sperm residing in the vas deferens, the lipid pattern of which is markedly different from that of testicular sperm. One of the best candidate of lipid binding proteins in reproductive system is a cholesterol transporter Niemann Pick type 2-C (NPC2). The transcripts of NPC2 and the expressing protein are distributed along spermatic tracts of male shrimp. The shrimp sperm cholesterol is depleted during their transit in spermatic duct which makes it reasonable to postulate that the NPC2 homolog that is present in *M. rosenbergii* male reproductive tract serves a function to regulate the level of cholesterol in reproductive tracts and/or membrane lipid modification in sperm cells in this shrimp species.

2. ผลงานวิจัย

(1) วัตถุประสงค์ของโครงการ

1. To search for the key modulators, both proteins and lipids that are involved in fertilizing processes – capacitation, acrosome reaction.
2. To characterization, distribution and role of pmTSP-II in modulating shrimp sperm acrosome reaction
3. To investigate the distribution of CAT-D in male reproductive tissues (testis, vas deferens, spermatophore) and the enzymatic activity of this protein during sperm maturation process in the freshwater prawn (*Macrobrachium rosenbergii*).

4. To determine the distribution of Niemann Pick type 2C (NPC2) in shrimp reproductive tracts and its binding ability to sperm cholesterol.

(2) การดำเนินงานวิจัยตามวัตถุประสงค์

2.2.1 Molecular analysis, cortical rod protein and pmTSPII purification

- Molecular analysis of pmTSP-II and its carbohydrate anchoring sites

The deduced molecular weight, glycoconjugate sites and putative disulfide bonding of *pmTSP-II* were predicted by an Expasy software (http://web.expasy.org/cgi-bin/compute_pi/pi_tool). The secondary structure was predicted using a Sopma program (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_sopma.pl). The protein structure modeling was performed using a Swiss Model according to the method described previously (Kiefer et al., 2009, Guex et al., 2009). A phylogenetic analysis of TSP sequences was performed using the neighbor-joining method, which was based on the distances between the TSP amino acids analyzed by a Molecular Evolutionary Genetics Analysis version 5 (MEGA 5) software (Tamaru et al., 2011). A phylogenetic tree was tested for reliability using 500 bootstrap replications. Percentages of the bootstrap confidence value were shown at the branch nodes.

- Cortical rod (CR) isolation and pmTSP-II purification

Mature female shrimp were obtained from Gulf of Thailand, Chonburi province, Thailand and from Shrimp Genetic Improvement Center, Surat Thani province, Thailand. The animal handling methods were followed the guideline of the Aquatic Animal Research Committee, Mahidol University, Thailand. They were acclimatized in the 500-L plastic tanks at 28°C with seawater salinity of 20 ppt for at least one day prior to use for experiments. Mature stage IV ovaries were identified using a transmitted light method through shrimp cephalathorax and the ovaries were collected and processed for CR collection as described previously (Kreuvaisayawan et al., 2007). Briefly, the ovaries were homogenized using a Dounce's homogenizer and the suspension were loaded on a discontinuous sucrose gradient and centrifuged (8,000 ×g, 4°C, 60 min). CRs were collected as an opaque greenish pellet at the bottom of 40% sucrose. The yolk contaminants were washed away from CRs by a single step centrifugation (1,000 ×g, 4°C, 5 min), through a 30% sucrose and re-suspended in an artificial seawater.

Crude water soluble CR (wsCR) was prepared by re-suspending isolated CRs in an artificial seawater (ASW: 423 mM NaCl, 9 mM KCl, 9.3 mM $CaCl_2$, 23 mM $MgCl_2$, 9.3 mM $MgSO_4$, 2.1 mM $NaHCO_3$, pH 7.8) and left overnight (4°C) with gentle agitation. For *pmTSP-II* purification, crude CRs were further subjected to a size exclusion chromatography using a 30-ml sepharose column. One-milliliter

fractions were collected and measured for its concentration using Bradford protein assay kit (Sigma, St. Louis, MO). The protein profile was then checked by gel electrophoresis as mentioned below.

2.2.2 Antibody production and testing its recognition and localization on *O-ß-GlcNAc* of *pm*TSP-II

- Synthesis of linear O- ß-GlcNAc on the lipid substrate

Three forms of linear O-β-GlcNAc oligomers including *N*-acetylchitotriose, *N*-acetylchitotetraose and *N*-acetyl-chitopentaose were synthesized (Funakoshi, Japan) and chemically linked to phosphatidylethanolamine (PE). In brief, glycosyl residuals were dissolved in warmed distilled water at the concentration of 50 mg/ml (2 h, 60ºC). Sodium cyanoborohydride ($NaBH_3CN$) was added and incubated overnight at 60ºC. The PE glycolipid cores were resuspended at the concentration of 5 mg/ml in 1:2 chloroform:methanol (v/v). The successful linkage of carbohydrate-PE lipid was proved by thin layer chromatography (TLC) in ethylacetate/pyridine/acetic acid/DW at the ratio of 5:5:1:3 (v/v/v/v) solvent separation system. Resolved lipids were sprayed with 1% orcinol solution and dried with heat to detect the purple spots of glycolipids.

- Recognition of O-ß-GlcNAc oligomers by a CTD110.6 antibody

It has been reported that CTD110.6 antibody is used to detect O-β-GlcNAc monomer on its substrate. We thus used this CTD110.6 antibody to verify its recognition towards the oligomeric (tri-, tetra- and penta-) O-β-GlcNAc linked PE lipid substrate using an ELISA assay. Approximately 5 μg/ml of either the PE-linked chitotriose or chitotetraose or chitopentaose mixture was added into 96-well plate and incubated (37ºC, 2 h). After extensive washed with PBS, the carbohydrates were blocked with 1% BSA in PBS, washed and incubated with 1:500 dilution of CTD110.6 antibody (37ºC, 2 h), followed by HRP conjugated goat anti-mouse IgG at a dilution 1:1,000. Binding of antibody was detected by O-phenylenediamine substrate in 0.1 M Tris-HCl pH 6.8 containing 3% H_2O_2. The reaction was stopped by adding 2 N H_2SO_4 and the developing color was quantified at 490 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

- Detection of naturally anchored O- ß -GlcNAc and mannoses on pmTSP-II

Approximately 10 μg purified *pm*-TSP-II protein were loaded onto12.5% SDS-PAGE and subjected to a silver staining. Proteins in the duplicated gel were transferred to PVDF membrane and blocked (room temperature, 1 h) with 1% BSA in PBS containing 0.2% Tween 20 (PBST). CTD110.6 antibody was used to detect O-β-GlcNAc chain on the purified *pmTSP-II* protein at a dilution of 1:3,000 (4ºC, overnight). After an extensive wash with PBST, the membrane was further incubated with

1:10,000 horse radish peroxidase (HRP) conjugated goat anti-mouse IgG (room temperature, 1 h). The immunoreactive band was visualized by an enhanced chemiluminescence kit (Amersham Pharmacia, Buckinghamshire, UK). The detection of mannoses and other sugar residues on purified *pmTSP-II* was carried out by probing with biotinylated lectins (Concanavalin A (Con A, recognizing mannoses and to a lesser extent glucoses); Wheat germ agglutinin (WGA for neuraminic acid) under a similar condition described for CTD110.6 antibody staining except the secondary antibody used was replaced by HRP-streptavidin.

- Production of polyclonal antibody and Western immunoblotting

Polyclonal antibody directed against chitin binding domain (CBD) of *pmTSP-II* was prepared in Slc:ddY strain mice (Japan SLC). The animal handling and care were performed at University of Nagoya in accordance with the NIH guidelines. The immunogenic peptide designed from CBD domain was NH2-DDCNGFVYNTTSRTCNGEMQC-COOH and synthesized by Thermo Fisher Scientific. The obtaining peptides were further purified through Sephadex G-25 column. The mice were intraperitonealy injected with 200 μg of peptide emulsified with a complete Freund's adjuvant (1:1, v/v) and further boosted with an incomplete adjuvant 2 and 4 weeks after the first injection. The blood was collected and allowed to clot (37ºC, 1 h) and the sera were obtained after centrifugation (3,000 ×g, 10 min). The sera were further purified by CNBr sepharose column and eluted with 100 mM glycine-HCl, pH 2.5 and neutralized with 1 M Tris-HCl, pH 9. Eluted fractions were pooled and dialyzed then the affinity purified IgG antibody were kept in -20ºC until used.

For Western blotting, approximately 4-10 μg of crude wsCR and purified fraction of *pmTSP-II* proteins were resolved by SDS-PAGE and transferred onto PVDF membrane. Non-specific antibody binding was blocked with 5% skimmed milk and probed with anti-TSP antibody under similar conditions described for CTD110.6 antibody above.

- Immunolocalization of anti-TSP in shrimp ovarian tissues

The shrimp ovaries were cut into small pieces, fixed in Bouin (for light microscopy) or Karnovsky fixative (4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M PBS, for electron microscopy). The tissues were dehydrated and processed for embedding in paraffin or LR-White hydrophilic resin (London Resin, Berkshire, UK) polymerized at 58°C for overnight. Thin (5 μm thick) or ultrathin sections (50-70 nm thick) were blocked the non-specific antibody staining with 5% normal goat serum (2h, room temperature) and washed with 0.05% Tween-20 in PBS (PBST). The sections were incubated with anti-TSP IgG (2 h, room temperature) and further incubated with goat anti-rabbit IgG coupled either with HRP or with 10 nm gold particle (2 h, room temperature). They were viewed

under an Eclipse Olympus light microscope. For TEM, the sections were counterstained with 1% uranyl acetate and viewed under a FEI Tecnai 200 transmission electron microscope operated at 80 kv. Control sections were those incubated with a non-immune rabbit serum in place of primary antibody.

2.2.3 Identification of pmTSP-II function on inducing sperm acrosome reaction

- Testing of glycoconjugates on pmTSP-II polymerization and induction of sperm AR

The function of naturally conjugated O-β-GlcNAc in molecular polymerization of purified *pmTSP-II* was tested by these following experiments. The pooled HPLC fractions of purified *pm*TSPII was subjected to 0.1-0.2 μg/ml chitinase digestion at various time points (1, 6, and 12 hr, respectively). The samples at various enzyme concentrations and digestion timing were then resolved by SDS-PAGE and silver staining. Alternatively, the digested *pmTSP-II* was transferred onto PVDF membrane and probed with CTD110.6 antibody to prove the remaining of O-β-GlcNAc residues on the purified *pmTSP-II* under the conditions described above.

Induction of *P. monodon* sperm AR was followed the protocol described by Kreuvaisayawan et al., (2007). Briefly, sperm taken from mature female thelycum (aged > 12 month-old) were washed free from thelycal contents by centrifugation (500 ×*g*, 10 min) and resuspened in calcium free artificial seawater (CFASW: 423 mM NaCl, 9 mM KCl, 23 mM $MgCl_2$, 9.3 mM $MgSO_4$, 2.1 mM $NaHCO_3$, pH 7.8) to prevent spontaneous AR. Sperm were resuspended at a final concentration of 10^6 sperm/ml. After pelleting by centrifugation, *pmTSP-II* at concentrations of 2-16 μg/ml in ASW was added to re-suspend the sperm and allowed to stand in ambient temperature for 30 min. AR induction was stopped by addition of an equal volume of 8% paraformaldehyde in ASW. The parallel sperm treatments included addition of 1) 20 μg/ml crude wsCR (positive control); 2) 8 μg/ml *pmTSP-II* pretreated with 100 μg/ml Con A; 3) 100 μg/ml Con A pre-incubated with 1 mg/ml mannose (6 h) and subsequently treated with 8 μg/ml *pmTSP-II* (1 h) prior to incubation with sperm; 4) 100 μg/ml Con A (lectin control) and 5) 16 μg/ml recombinant *pmTSP-II* (both CBD and EGF-like portions). Aldehyde fixed sperm were then pipetted onto slide, covered with cover glass, and viewed under a light microscope. Approximately, 200 sperm from >8 randomized microscopic fields were counted and the percentages of AR sperm or intact sperm (AI) were analyzed and expressed as mean ± S.D. in the triplicate experiments.

2.2.4 Characterize and localize the expression of CAT-D in male reproductive tissues

- Tissue preparation

Mature male giant freshwater prawns were anesthetized with ice for 2-3 min. The testis, vas deferens and spermatophores were dissected out and immediately immersed into 4%

paraformaldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.4 at 4°C for 24 h and then placed in 70% ethanol. Subsequently, the specimens were dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, and 100%), cleared in toluene and infiltrated in paraffin by using automate tissue processor. Paraffin embedded tissues were cut at 4-6 µm thick and the tissues were picked up onto glass slides coated with silane.

- RNA extraction

Total RNA were extracted from testis, vas deferens, spermatophore, muscle, hepatopancreas, stomach, heart, and gill of the freshwater prawns by Trizol reagent (1 ml of Trizol reagent per 50-100 mg of tissue). The tissue samples were homogenized by homogenizers and then incubated at room temperature (RT) for 5 min. The homogenated samples were added with chloroform (0.2 ml of chloroform per 1 ml of Trizol reagent), gently mixed for 15 sec and then incubated at RT for 5 min. The mixture solution centrifuged at 12,000 x g for 15 min at 4°C. After centrifugation, the supernatant in upper phase was transferred to the new tube and then added 0.5 ml of isopropyl alcohol for RNA precipitation which was separated by centrifugation at 12,000 x g for 20 min at 4°C. The RNA pellet was washed twice with 75% ethanol with RNase-free water and centrifuged at 7,500 x g for 5 min at 4°C. The ethanol was removed and RNA pellet was dried at RT for 10 min. Finally, the RNA pellet was resuspended in 30 µl of diethylpyrocarbonate (DEPC)-treated H_2O. The total extracted RNA concentrations from these tissues were determined by measuring the absorbance at 260 nm of Nanodrop (Thermo Scientific NanoDrop 2000). The ratio of reading values at 260 nm and 280 nm (A_{260}/A_{280}) is about 1.8-2 that indicates the purity of nucleic acid according to the recommendation of the manufacturer (Thermo Scientific, Waltham, Massachusetts). The total extracted RNA was stored at -80 ^{0}C.

- Reverse transcriptase polymerase chain reaction (RT-PCR)

The 2 µg of RNA extracted samples from the tissues of mature male giant prawns were mixed with 20 µl of the reaction mixture (random primers, RT buffer, dNTPmix and reverse transcriptase) as provide in the commercial kit. The cDNA products from reverse transcription were stored at -20 °C before analysis by PCR. To amplify target DNA sequence, 2 µl of reaction mixture from reverse transcription containing the first-strand cDNA was used as the template for PCR. The Oligo nucleotide primers used in this study were showed in Table 1.β-actin of *Macrobrachium rosenbergii* was used as reference gene. The primers of CAT-D are CatD-F: 5'-CCT GTT TTC TAC AAT ATG GTT A-3' and CatD-R: 5'- GGC TTA GCA CCA ATC TTC TTG TTG-3' (Pacific Science, Thailand). These primers sequence was designed and selected by using NCBI/primer blast and Clustal Omega/multiple sequence alignment.

The PCR reaction was performed in PCR Thermal Cycler (Bio-Rad). The reaction was initiated a cycle of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec and finally end with further extension at 72°C for 10 min. To analyze the RT-PCR reaction, PCR products (expected product length = 323 bp) were checked by 1.2% agarose electrophoresis and then stained with 0.5 µg/ml ethidium bromide for 5 min followed by visualization under an ultraviolet lamp (UV Transilluminator, Gel Doc 1000; BioRad).

Table 1. Oligonucleotide primers

Primer name	Nucleotide sequence (5'→ 3')	Purpose
CatD-F	CCTGTTTTCTACAATATGGTTA	RT-PCR expression analysis and *In situ* hybridization
CatD-R	GGCTTAGCACCAATCTTCTTGTTG	
β-actin-F	ATTGGACTTCGAGCAGGAGA	Reference genes
β-actin-R	ACAGGTCCTTACGGATGTCG	

- *In situ* hybridization

The sections will be dewaxed in fresh xylene twice for 5 min each and rehydrated in graded series ethanol (100%-70%) and distilled water for 5 min each. Then, the sections will be incubated with TNE buffer (50 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, pH 7.4) containing 10 µg/ml RNase-free proteinase K at 37°C for 10-15 min. The sections will be post-fixed with DEPC-PBS containing 4% paraformaldehyde for 5 min at 4°C. Before hybridization, the sections will be incubated with prehybridization buffer (4x SSC (600 mM Nacl, 60 mM Na-Citrate in DEPC-H_2O), 50% deionized formamide) at 37°C for 2 h. After prehybridization buffer was drained, each slide was treated in 100 µl of hybridization buffer (50% deionized formamide, 50% dextran sulfate, 50x Denhardt's soltution, 20x SSC, 10 mg/ml denatured sheared salmon sperm DNA) containing 100 ng of DIG-labeled oligonucleotide probes at 42°C overnight in humid chamber. The probes for *Macrobrachium rosenbergii* CAT-D are probe I: 5'-CCT GTT TTC TAC AAT ATG GTT A -3' and probe II : 5'- GGC TTA GCA CCA ATC TTC TTG TTG-3' (Pacific science co.th.,Thailand). The negative controls will be done with non-cDNA probe hybridization in hybridization buffer. The slides will be washed in a shaking water bath as follows: 2xSSC for 30 min at 37°C, 1x SSC for 30 min at 42°C and 0.5xSCC for 30 min at 42°C.

The sections will be washed with buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 5 min and then will be incubated for 2 h at RT in humid chamber with buffer I (blocking solution (10% NGS) in buffer I). The slides will be decanted buffer II and incubated with the anti-DIG-alkaline phosphatase antibody (1:500) for 2 h at RT in humid chamber. Subsequently, the slides will be washed with buffer I twice for 10 min each and then will be incubated with buffer III for 5 min. To develop the color with NBT/BCIP (Promega, Madison, Winconsin) and stop the reaction with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.1), mounted by 60% glycerol, observe and photograph under a light microscope.

- Immunohistochemistry with anti-CAT-D

The paraffin sections of reproductive tissue of mature male giant freshwater prawns were deparaffinized with series of xylene and rehydrated in a decreasing graded ethanol (100% to 70%). The sections were treated with 1% hydrogen peroxide (H_2O_2) to quench endogenous peroxidase. To block free aldehyde groups, sections were immersed in with 0.1 M glycine PBS. The tissues were retrieved antigen by 0.1 M citrate buffer, pH 6.0 at 60°C. The sections were treated with 0.1%Triton-X 100 in PBS for 15 min for cell permeability. To detect CAT-D in the tissues, 4% bovine serum albumin (BSA) was used to block non-specific antibody binding sites before incubating the sections with rabbit anti-CAT-D antibody (EMD Merck, Darmstadt, Germany) at dilution 1:200 at 4°C overnight followed by exposing the sections with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (EMD Merck, Darmstadt, Germany) at dilution of 1:400 for 1 h. Enzymatic reaction was developed using 3,3'-Diaminobenzidine (DAB) until a reddish brown color on the section appeared. Reaction was stopped by submersing the sections in the distilled water followed by extensively rinsed with distilled water. The sections were dehydrated by passing the slide through increasing concentrations of ethanol (50% to 100%) and then immersed in xylene. The sections were observed under Olympus light microscope.

2.2.5 Determine CAT-D is secreted protein existing in the fluid of male reproductive tract

- Sperm and its spermatic duct fluid preparation

Mature male giant freshwater prawns were anesthetized with ice as described in 4.1.2. The testis (Tsp), vas deferens (Vsp) and spermatophores (Ssp) were dissected out. The tissues were cut into small pieces in phosphate buffered saline (0.1M PBS, pH 7.4). The tissues were homogenized and filtered through a 45 µm metal sieve (Endercotts, London, UK) to remove the cell debris. Sperm isolated from all tissues were washed by centrifugation (500 x g, 4°C, 10 min) and the sperm pellets were collected. Whereas, spermatic duct fluid from testis, vas deferens, and spermatophores were collected from each part of male reproductive tract by cutting tissues into small pieces. The tissue pieces were gently agitated to release their fluid and sperm into PBS then filtered through a 45 µm

metal sieve. The sperm were washed out by centrifugation (500 x g, 4°C, 10 min). Fluids were washed at least twice with PBS buffer by centrifugation (12,000 x g, 4°C, 15 min) to remove the debris and residual sperm. The fluids were stored at -20°C for further study.

- Sperm proteins and their fluid profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sperm proteins extracted from testis (Tsp), vas deferens (Vsp), spermatophore (Ssp) and their fluid samples, testicular fluid (TF), vas deferens fluid (VF) and spermatophore fluid (SF) were separated on 12.5% SDS-PAGE under a reducing condition. Electrophoresis used 100-125 volts for 1.30 h. The running gel was contained 12.5% polyacrylamide, Tris-HCl pH 8.8, 10% SDS, distilled H_2O, 10% Ammonium persulphate and TEMED whereas the stacking gel were contained 4% polyacrylamide, Tris-HCl pH 6.8, 10% SDS, distilled H_2O, 10% Ammonium persulphate and TEMED. After electrophoresis, the running gel were stained with Coomassie staining (0.025% Coomassie Brilliant Blue R-250, 40%methaol, 7% acetic acid) for overnight and de-stained with de-stained solution I (40% methanol, 7% acetic acid) and de-stained solution II (5%methanol,7% acetic acid) for 30 min, respectively.

- Western blot analysis

Sperm proteins and their fluid from Testis, Vas deferens and Spermatophores were separated by 12.5% SDS-PAGE and transferred to PVDF membranes (Amersham BioSceinces, Little Chalfont, UK). The membrane was incubated with 3% skim milk and 2% BSA in PBS containing 0.05% Tween 20 (PBST) to block non-specific binding for overnight at 4°C. Thereafter, the membrane was incubated with rabbit anti-CAT-D antibody (EMD Merck, Darmstadt, Germany) diluted in the blocking solution at 1:1000 dilution for 2 h at RT. Subsequently, the membrane was washed with 0.2% PBST for 3 times and then incubated with HRP conjugated goat anti-rabbit IgG (EMD Merck, Darmstadt, Germany) diluted in the blocking solution at 1:5000 for 1 h at RT. Visualization of the antigen-antibody complexes was performed by enhanced chemiluminescent method using an ECL kit.

2.2.6 Activity of CAT-D in male reproductive tract of the freshwater prawn

- Function analysis of MrCAT-D

- Zymography

Sperm proteins and their fluid samples extracted from testis (TF), vas deferens (VF) and spermatophore (SF) will be separated on 12.5% SDS-PAGE containing 2% gelatin under a non-reducing condition. Electrophoresis will be used 100-125 volts, 10-15 mA for 1.30 h. These samples will be mixed with a 2X loading dye without dithiothreitol (DTT) or mercaptoehanol. After electrophoresis, the gels will be treated with renaturing buffer (2.5% Triton X-100 in PBS) at RT for 1 h and then incubated

in the incubation buffer (0.5 M sodium acetate buffer (pH 5), 0.05% NaN_3 pH 5.0) at 37ºC for 18 h. The gels will be stained with the staining solution (0.025% Coomassie Brilliant Blue R-250, 40% methaol, 7% acetic acid) for overnight and destained with destained solution I (40% methanol,7% acetic acid) and destained solution II (5%methanol, 7% acetic acid) for 30 min, respectively. Visualization of the proteolytic active band will be performed as a clear band against dark background.

- Actin digestion

F- actin from rat skeletal muscle were prepared by methods of Tonomura et al. Actin was digested by CAT-D at 25 C using a 1:10 (w/w) ratio of protease/actin in the presence of 2 mM MgCl2 or 2 mM EDTA. G-actin was also digested by CAT-D over G-actin in a 2mM tris buffer (pH 8.0) containing 0.2 mM ATP, 0.2 mM CaCl2, and 0.1 mM dithiothreitol. The samples were then denatured and examined on SDS- PAGE and stained with Coomassie blue.

2.2.7 Characterize Niemann Pick type 2C (NPC2) in shrimp reproductive tracts

- Animal and Tissue preparation

Fresh water prawn, *Macrobrachium Rosenbergii*, was purchased from commercialized livestock at Prannok market, Bangkok, Thailand. The prawns were acclimatized with aeration before tissue collection. The various tissues including brain, thoracic ganglion, hepatopancreas, stomach, gill, testis, vas deferens, and spermatophore were collected and immediately put into Trizol reagent (Invitrogen, CA) then stored at -80°C for RNA extraction. Male reproductive tracts were divided into three parts which are testis, vas deferens and spermatophore.

- RNA Extraction and gene identification

Tissues samples including testis, vas deferens, hepatopancreas, muscle, heart, gill, stomach, brain and thoracic ganglion were collected and immediately put into Trizol reagent (Invitrogen, CA), following the manufacturer's instruction. The samples were homogenized in Trizol reagent and then centrifuged at 12,000 rpm at 4ºC for 15 min. The supernatant which contained RNA was transferred to a new tube. Thereafter, 200 µl of chloroform was added into the solution and then centrifuged at 12,000 rpm at 4ºC for 15 min for phase separation. The aqueous phase was transferred into a new tube. The isopropanol was added in the solution for RNA precipitation and incubated at 4ºC for 10 min. The mixtures suspension were centrifuged at 12,000 rpm at 4ºC for 30 min. The RNA pellets was collected and then washed with 75% Ethanol for 2 times. The RNA pellet was kept at -80ºC for further study. The purity and quantity of RNA were measured by Nanodrop-2000C spectrophotometer (Thermo Scientific, Massachusetts).

- cDNA Synthesis & amplication

Total RNA was extracted and converted to first-strand cDNA by a kit from GeneAll (Korea) following the manufacturer's protocol. Twenty microliters of reaction contained 2 μg of total RNA, 25× dNTP mixture, 10× random primer and Multi Serobe^{TM} reverse transcriptase (200 U/μl). The mixtures were amplified at 25ºC for 10 min, 37ºC for 120 min, 85ºC for 5 min and 4ºC, respectively. The cDNA was kept at -80ºC for further studies. To investigate the expression of NPC2 gene in the male prawns, specific primers were designed from the *M. rosenbergii* NPC2 (MrNPC2) gene. The specific primer sequences (MrNPC2-F1 and MrNPC2-R1) were shown in Table 1. Since *M. rosenbergii* transcriptomes have been previously investigated and are available in the NCBI database (SRA accession number: SRP049917), the search of MrNPC2 gene was performed by using NCBI tBLASTn program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in which the known crustacean NPC2 proteins were used as queries. The parameter was set as follows: matrix, BLOSUM62; e-value, 100. The hit nucleotide sequence with lowest e-value was collected and translated into amino acid sequences using ExPASy translate tool (http://web.expasy.org/translate). Subsequently, the deduced protein was characterized based on their sequence similarity and homology to previously identified NPC2, using Clustal Omega alignment and SignalP 4.1 online tools (http://www.ebi.ac.uk/Tools/msa/clustalo/ and http://www.cbs.dtu.dk/services/SignalP/). In addition, the hit NPC2 protein was annotated against the NCBI database using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) program for further confirmation. The second set of primers (MrNPC2-F2 and MrNPC2-R2) were additionally designed based on nucleotide sequence of MrNPC2 derived from transcriptomes before custom synthesized (Pacific Science, Thailand).

The PCR products were obtained using Vivantis DNA polymerase (Vivantis Technologies, Subang Jaya, Malaysia), following the manufacturer's instruction. A PCR reaction was contained 10 μl of 2-Taq DNA polymerase, 0.5 μl of forward primer, 0.5 μl of reverse primer, 0.5 μl of cDNA template and 8.5 μl of DEPC treated water. Thermocycling condition was: 1 cycle of initial denaturation at 95ºC for 3 min, followed by 34 cycles of denaturation at 95ºC for 30 sec, annealing temperature at 60ºC for MrNPC2 and at 55ºC for actin for 30 sec, extension at 72ºC for 1 min and the final extension cycle at 72ºC for 5 min. All PCR products were analyzed using 1.5% agarose gel/Tris-Borate-Ethyllenediaminetetra acetic acid (TBE) buffer which contained 0.5 μg/ml of ethidium bromide. The PCR bands were visualized using UVP EpiChemi III *Darkroom* (UVP Bioimaging System, USA). The predicted size were cut, purified using GeneAll gel extraction kit (GeneAll, Geneall biotechnology. CO., LTD, Korea) and cloned into a pGEM cloning vector (pGEM cloning vector T eazy, Promega, USA).

Plasmids with inserted sequences were purified using High-Speed Plasmid Mini Kit (Geneaid) and sequenced by Macrogen (Korea).

- Bioinformatic analyses

The full-length of NPC2 nucleotide sequence was translated into a deduced amino acid sequence by the Expasy bioinformatics resource portal-translate tool (http://web.expasy.org/translate). The similar proteins sequence of other species were retrieved from National Center of Biotechnology International (NCBI). The sequence alignment to other species was performed using the Clustal omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). The protein identification was performed by BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cg). A phylogenetic tree was performed by used MAGA7 program. Proteins prediction was conducted by Signal P-4.1 program (http://www.cbs.dtu.dk/services/SignalP/).

Table 2 Summary of the primers of *NPC2*

Primer	Direction	Nucleotide sequence	Reaction
MrNPC2-F1	Forward	5'-GCTGAGGTTCGAGAGGACAC-3'	F1 fragment
MrNPC2-R1	Reverse	5'-GCGAATGTGAATCTGAACGA-3'	F1 fragment
MrNPC2expF2	Forward	5'-CATATGAACACCAACGCTCTTATTATCCTCC-3'	Cloning expression in *E.coli*
MrNPC2expR2	Reverse	5'CTCGAGTCAATGATGATGATGATGATGCTTCAG CTGGACTGGGAATTTGATGCAGAC-3'	Cloning expression in *E.coli*
MrNPC2-F1	Forward	5'-GCTGAGGTTCGAGAGGACAC-3'	In Situ Hybridization
MrNPC2-R1	Reverse	5'-GCGAATGTGAATCTGAACGA-3'	In Situ Hybridization
β-Actin	Forward	5'-ATTGGACTTCGAGCAGGAGA-3'	Control of tissue distribution
β-Actin	Reverse	5'-ACAGGTCCTTACGGATGTCG-3'	Control of tissue distribution

3

(3) ผลงานวิจัยที่ได้รับ

3.1 ***Molecular analysis, recombinant protein pmTSPII production & localization and identification its function***

- Molecular characteristics of pmTSP-II and its glycoconjugate analysis

We first analyzed carbohydrate anchoring domains on *pmTSP-II* which are known to play many significant roles in biological processes. As shown in the sequence comparison among crustacean TSPs,

carbohydrate anchoring motifs both *N*-linked glycosylation at the site of NX(S/T) (Carlson et al., 2008) and *O*-linked glycosylation of O-β-GlcNAc at CXXG(Y/F)(T/S)GZ2-5C motif (Brain et al., 2012) could be found at the amino acids positioned XXX and YYY, respectively, along the full sequence of *pmTSP-II*. There were at least 2 sites of *N*-linked glycosylation found at the CBD and calcium binding (TSP3) domains, which were presumably mannosyl anchoring sites, known to be the major sugar moiety of cortical proteins in *P. monodon* (Kraeuvaisayawan et al., 2007). The anchorage of O-β-GlcNAc on *pmTSP-II* was deemed to be on the EGF-like motifs which were located in the middle of the sequence. In addition, we could also detect 6 putative cysteine residuals that may be involved in formation of three disulfide bridging (both intramolecular and intermolecular) to subsequently form a higher order structure as reported earlier for other TSPs. However, as summarized in Figure 8 below, the intermolecular bundling of *pmTSP-II* did not depend on disulfide bridging but it was rather based on chitin-CBD interaction to form the supramolecular architecture. Therefore, we believe that these putative cysteine residues would be important more for the intramolecular folding of the protein structure. In the protein structure point of view, we also demonstrated the linear arrangement of multi-domains in *pmTSP-II* and the important positions where O-β-GlcNAc (on EGF-like domain, blue) and *N*-linked mannoses (on CBD and TSP3, pink and purple) were associated with the peptide chain in the deduced three dimensional structure of *pmTSP-II* (Fig. 1).

Phylogenic tree analysis in Figure 2 clearly revealed sequence comparison of TSPs that were closely similar among marine shrimp species, particularly those in penaeus and marsupenaeus species. The sequence similarity (score >84) spanned throughout the evolutionary scale towards mammalian species including humans. However, a relatively low correlation that was seen at the twig of *F. chinensis*'s TSP (score = 69) may be due to the differences in the number of CBD and EGF-like domains and some variations found in non-conserved domain at the C-terminal end in the protein sequence of *F. chinensis*'s TSP.

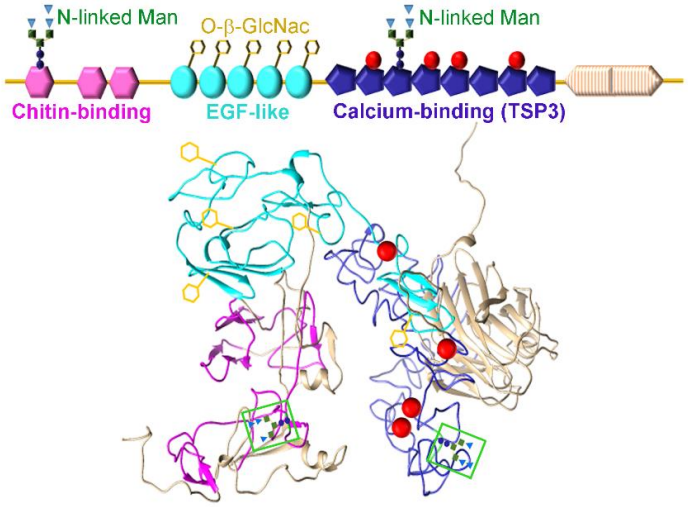

Figure 1. The linear arrangement of multi-domains in *pmTSP-II* and the important positions where O-β-GlcNAc (on EGF-like domain, blue) and *N*-linked mannoses (on CBD and TSP3, pink and purple) were associated with the peptide chain in the deduced three dimensional structure of *pmTSP-II.*

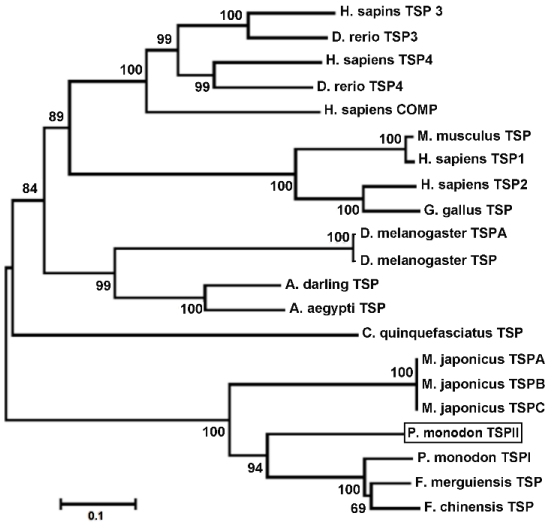

Figure 2. The Phylogenic tree analysis revealed sequence comparison of TSPs that were closely similar among marine shrimp species.

- Detection of O-β-GlcNAc residues on lipids substrate and purified pmTSP-II

As the deduced amino acid sequence of *pmTSP-II* has indicated the anchorage of O-β-GlcNAc residues, we thus aimed to investigate the existence of complex O-β-GlcNAc moiety (chitin chain) in the purified *pmTSP-II* using CTD110.6 antibody. Since the antibody has been proved to recognize O-β-

GlcNAc monosaccharide, its recognition towards the oligomeric O-β-GlcNAc linked to lipid substrate was then tested. The TLC results in Figure 3A clearly demonstrated a successful linkage of *N*-acetyl-triose, -tetraose and -pentaose (as a purplish blue enzyme precipitates resulting from charring reaction of carbohydrates with an orcinol) to the PE lipid substrate. The more sugars attached to the PE lipids, the slower the band mobility observed. Through ELISA analysis, these oligomeric sugar-lipid linkages were well recognized by a CTD110.6 antibody with the dilution factor ranging from 1:12.5 to 1:50 and the optical density (O.D.) from 0.05-0.3 (Fig. 3B). This result extrapolated the recognition of this antibody from monomeric sugar towards linear chained sugars.

We further investigated the recognition of CTD110.6 antibody towards a more complex sugar chain, namely, a natural *N*-linked O-β-GlcNAc (or chitin) chain on the crude CR extracts without protease inhibitors. It was revealed that the antibody recognized a multiple intense immunoreactive bands at the molecular mass of 250, 180, 150 kDa and a moderate intensity band at 110 kDa protein bands (Fig. 3C, arrowheads). A single immunoreactive band of 250 kDa was observed when the purified fraction of *pmTSP-II* was probed with CTD1110.6 antibody (See Figure 6). Together, our results indicated that CTD110.6 antibody could recognize the synthetic linear oligomeric O-β-GlcNAc chain on the lipid substrate and could also selectively interact with a naturally linked O-β-GlcNAc (chitin) that was anchored on the purified *pmTSP-II* molecules.

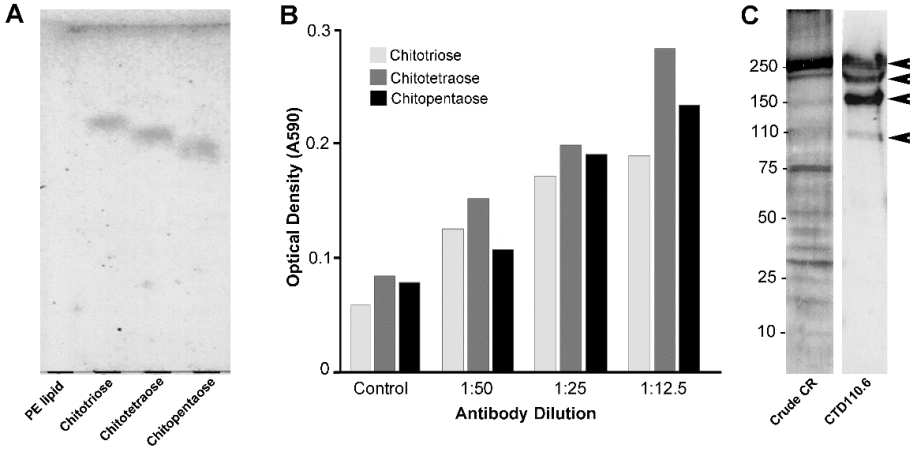

Figure 3. The detection of O-β-GlcNAc with CTD110.6 antibody in the thin layer chromatography of synthesize oligomeric (A) with the dilution of antibody (B) and on the cortical rod protein (C).

- ***Presence of mannose residues on pmTSP-II***

We have previously reported the extensive presence of mannose residues over many other sugars in the water soluble fractions of the cortical rods (wsCR) using many probing lectins (Kruevaisayawan et al., 2007). Here, we thus selected only Con A lectin (recognizing mainly ∝-D-mannose for CR proteins) to test with the purified fraction of *pmTSP-II* and WGA lectin (for sialic acid) as negative control. We could purify *pmTSP-II* from the crude sample of water soluble CR using a single-step, size-exclusion FPLC yielding a single protein band of 250 kDa in the fractions F44-F48 (Fig. 4A). Either the purified protein or freshly extracted CR was specifically reactive with anti-TSP (raised against synthetic CBD peptide designed from the protein sequence of *pmTSP-II*) (Fig. 4B), which partly supported that the purified protein was of TSP origin. Lectin histochemical results revealed that the representative purified fractions (F44) were stained intensely by Con A lectin but not WGA (Fig. 4C, arrowhead). The results thus indicate that *pmTSP-II*, as a part of wsCR, is also enriched in the mannose residues apart from O-β-GlcNAc counterparts.

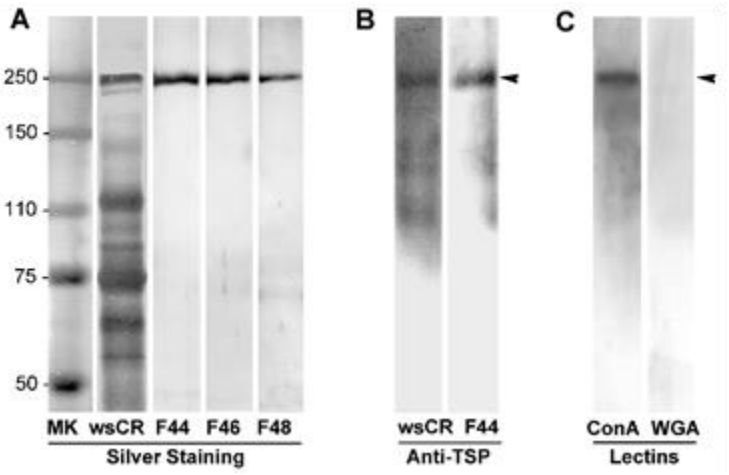

Figure 4. Siver staining of of wsCR and pmTSP-II purification fractions (A) and probe with anti-TSP (B) and ConA (C).

- Localization of pmTSP-II in the extracellular cortical rods

We performed immunolocalization at both light and electron microscopic levels to search for the localization of *pmTSP-II*, particularly in the cortical rods situated within the extracellular crypts of the mature oocytes. Using anti-TSP as a probe, the intense enzymatic products were detected specifically at the periphery of cortical rods in the stage IV oocytes (Fig. 5A, CR). A moderate staining could also be seen in the cytoplasmic granules of stage II and III oocytes, presumably endoplasmic reticulum around accumulating yolk pellets and cortical granules, which is known to be the site for

synthesis and secretion of CR proteins. At the EM level, precipitation of gold particles were dispersed throughout the matrix of CR in particular in the area of bottle-brush structure (Fig. 5C) which was resembled to a glycoproteoglycan-based structure. Negative control showed a minimal antibody staining in the periphery or matrix of CR (Fig. 5B and D). The results thus supported the fact that *pmTSP-II* was localized mainly at the periphery of cortical rods and readily to be dispersed into seawater to make up of egg water, a well-known natural AR inducer in *P. monodon* and many other shrimp species.

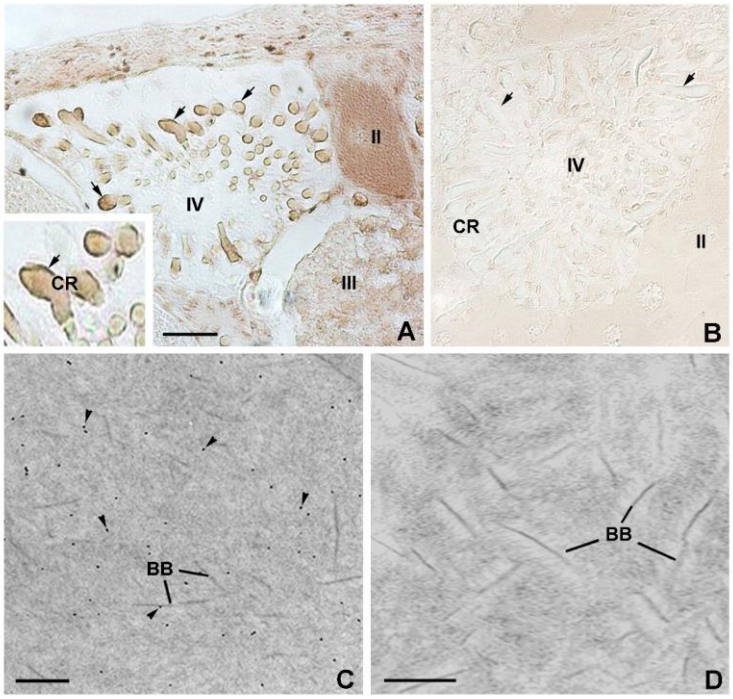

Figure 5. Immunolocalization of pmTSP-II in cortical rods (A), negative control (B). Immunogold revealed the gold precipitation on the extracellular matrix protein of cortical rods (C), negative control (D).

- O-ß-GlcNAc chain in pmTSP-II is involved in inter-molecular polymerization

Despite many reports indicating the presence of cysteine-based disulfide bridging that is involved in a supra-molecular structure of mammalian TSP proteins (whose deduced molecular mass is about 110-120 kDa) our SDS-PAGE results demonstrated a still-sizable 250 kDa *pmTSP-II* even they were exposed to a strong reducing agent and detergent in the PAGE resolving buffer (Fig. 6, lanes 1-2). We thus aimed to search for the inter-molecular cross-linkage of *pmTSP-II* strands, one of which could be a carbohydrate binding with its complimentary peptide domain. Through our bioinformatics

analysis on *pmTSP-II* sequence, it contained both O-β-GlcNAc (chitin chain) anchorage site on the *N*-terminus EGF-like domain as well as several CBD in the middle part of the molecule (See Fig. 1B), a unique feature of crustacean TSP. Cross-interaction between chitin and CBD could thus hypothetically generate a multimeric-bundling structure of *pmTSP-II*.

We tested this hypothesis by enzymatic digestion of chitin chain on the purified *pmTSP-II* by chitinase enzyme, which should dissolve its bundling structure resulting in a singlet chain of a 110 kDa protein. The results in Figure 6A clearly indicated that the molecular mass of *pmTSP-II* was gradually reduced to be a mixture of 250 to 110 kDa bands upon digested with 0.1 µg/ml chitinase for 1-6 h (Lanes 4-8). Either longer exposure to 0.1 µg/ml chitinase (12-24 h) or higher the amount of the enzyme (0.1 µg/ml) led to an apparent reduction of the upper bands (250 and 180 kDa) while increasing the intensity of the 150 and 110 kDa bands (lanes 14-18). Moreover, when these purified *pmTSP-II* proteins were probed with CTD110.6 antibody, the 250 kDa band in the non-digested proteins was intensely reactive with the antibody (Fig. 6B, lanes 1-2). Reactivity of CTD110.6 antibody was gradually shifted towards the lower molecular weighted bands, particularly the 110 kDa protein, in the moderately digested proteins (lanes 8-12). The prolonged or intensely digested samples (lanes 14-18) showed merely or completely absence of antibody reactivity, suggesting a complete removal of chitin chain from *pmTSP-II* peptide core. The results thus indicated the significance of chitin chain at O-β-GlcNAc anchorage site on *pmTSP-II* molecular polymerization.

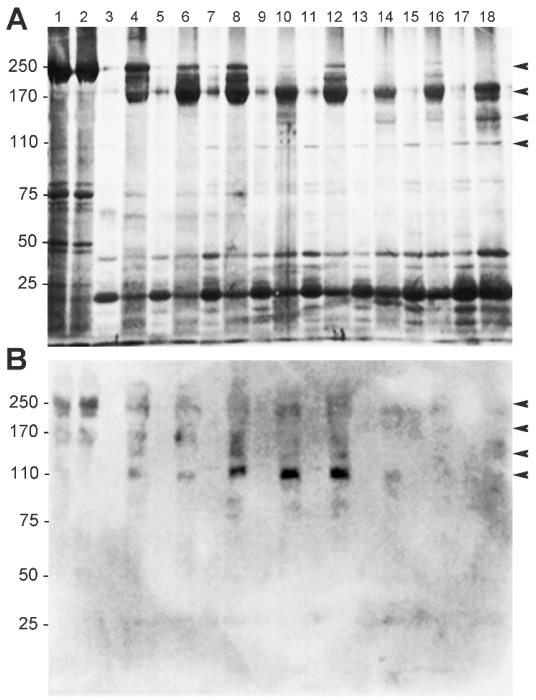

Figure 6. Analysis of physicochemical of *pm*TSP protein using chitinase activity. (A) The silver stained gel of the various concentration of chitinase. Chitinase were added and incubated with CRP for 3 h and overnight. Interestingly, chitinase digested the high MW of CRP to be smaller MW. (B) CTD110.6 blotted membrane shown other reacted band at 110 kDa after digested by chitinase.

- Role of mannose glycoconjugates in an induction of acrosome reaction

We have shown previously that mannoses are the major sugar in wsCR glycoproteins which are significant for sperm AR induction in this shrimp species (Kruevaisayawan et al., 2007). As we also detected here a considerable amount of Con-A reactive mannoses in the purified *pmTSP-II*, we thus tested their possible involvement in the sperm AR induction. It was shown that purified *pmTSP-II* was able to induce sperm AR in a concentration-dependent manner (Fig. 7A). The highest AR inducing ability could be generated by 16 µg/ml purified *pmTSP-II*, a concentration that was in the same ballpark with that of the natural inducer, egg water or wsCR reported earlier (Vanichviriyakit et al., 2004; Kruevaisayawan et al., 2007). At lower concentrations of *pmTSP-II* (2-8 µg/ml), the percentages of AR induction were in the range of 40-65% (Fig. 7A). We chose *pmTSP-II* at 8 µg/ml for further Con A inhibitory assay, in order to avoid a plateau saturation of sperm AR induction at 16 µg/ml *pmTSP-II*. The results showed that pretreatment of *pmTSP-II* with100 µg/ml Con A drastically inhibited the ability of sperm to undergo AR – only 15% AR was observed which was accountable for 90% AR inhibition

(Fig. 7B). Sperm treated with Con A alone (lectin control) revealed about 18% AR which was closely resembled to the spontaneous AR percentage (15%) shown in Figure 7A. Interestingly, pretreatment of Con A with 1 mg/ml mannoses prior to incubation with *pmTSP-II* and subsequent sperm treatment could effectively rescue sperm AR induction back to about 50% (compared to 65% AR induced by 8 µg/ml *pmTSP-II* itself). This was accountable for about 70% recovery of AR induction, confirming the role of mannosyl glycoconjugates in sperm AR modulation.

Role of *pmTSP-II*'s carbohydrates in AR induction was also confirmed by the use of three different recombinant proteins that were expressed in *E. coli* (devoid of glycosylation on the nascent proteins). It is apparent that the peptides that were designed from the three signature domains of *pmTSP-II* lacked their abilities to trigger sperm AR response. The AR percentages ranged between 14-18 percent (Fig. 7C), which was resembled to that of spontaneous AR where the sperm were exposed to ASW alone. Together, the results strongly suggest the significance of carbohydrate extension, particularly N-linked mannoses on pmTSP-II on sperm AR modulation.

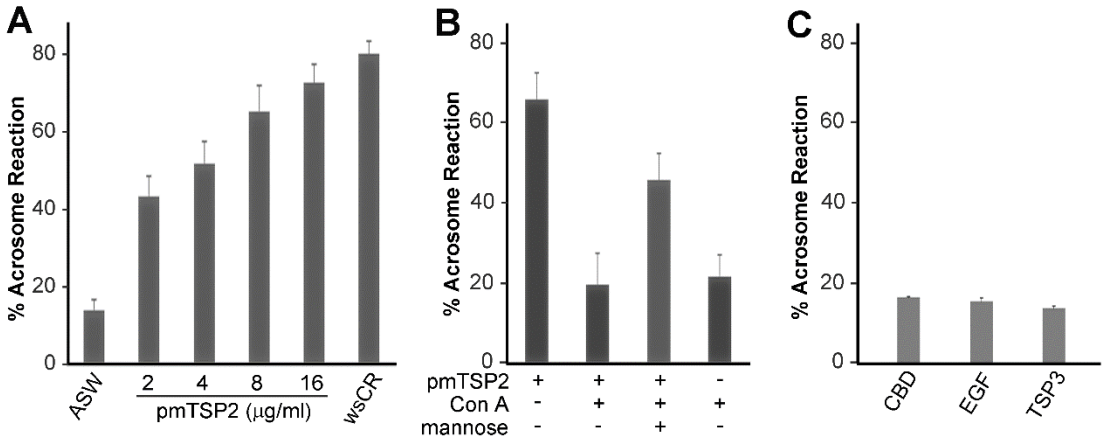

Figure 7. Inhibition of the sperm acrosome reaction by anti-TSP antibody. Water soluble CRs (wsCRs) at 16 µg/ml were pre-incubated with various concentrations of anti-TSP and used for AR induction in thelycal sperm. Controls were sperm treated with wsCR pre-incubated with pre-immune serum or with anti-TSP without wsCR. Data were calculated from triplicate experiments and expressed as mean ± S.D of the percentages of the AR responses (A), as percentages of the AR inhibition (B) and recombinant of domain proteins of pmTSP-II (C).

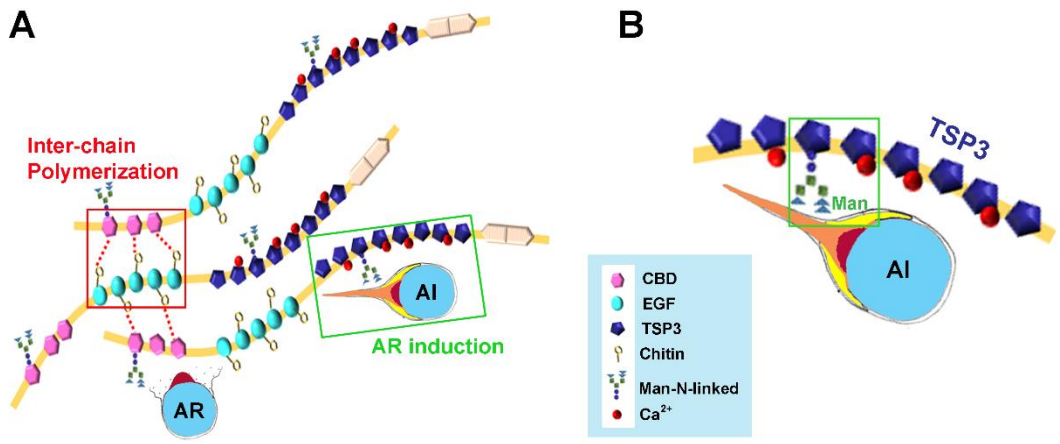

Figure 8. The diagram of pmTSP-II polymerization (A) and induction of acrosome reaction with the mannosyl glycoconjugation (B).

3.2 Existence of Cathepsin-D in male shrimp reproductive tissue

- Characterization of M. rosenbergii CAT-D genes expression (MrCAT-D)

To identify CAT-D gene, we determined the fraction cDNA sequences from transcriptomes analysis of *M. rosenbergii* that retrieved from NCBI database. The full-length of MrCAT-D was consisted of 1,361 nucleotides and 385 amino acid residues (Figure 9) including 15 amino acid residues of signal peptide at N-terminus was shown in gray color (Figure 9), 3 kDa of the cleavage of pro-peptide and the single chain of pro-cathepsin-D (pCAT-D) which is an intermediate form. Further of the proteolytic processing, the mature form was consisted of two chains which are light and heavy chain. The predicted molecular weight of MrpCAT-D was 42 kDa (http://web.expasy.org/compute_pi/) and an isoelectric point was 6.35. The light chain of MrCAT-D was 8.9 kDa whereas the heavy chain was 28.4 kDa. It was indicated that the sequence at 16-385 amino acid residues were predicted to be pCAT-D. The amino acid at Trp46-Tyr116 was predicted to be light chain and Asp118-Ala385 was predicted to be heavy chain. The cleavage of propeptide was shown in red color (Figure 9). An inhibitor binding domain was Asp81- Iso172 and conserved inhibitor binding site was Asp81, Gly83 and Ser85. The catalytic motif was Asp81, Thr82 and gly83 as show in box of figure 3. The active flap was Ala123-Gly133. It was six cysteine residues to form three disulfide bonds which are Cys94-Cys102, Cys261-Cys265 and Cys304-Cys341 as showed in red line of figure 3. The N-glycosylation site was predicted at Asn119.

The amino acid sequences of CAT-D of *M. rosenbergii* (MrCAT-D) from NCBI database was used to compare with other species including *Homo sapiens* (Accession no.AAA51922.1.), *Borus Taurus*

(Accession no. NP001159993.1), *Canis lupus familiaris* (Accession no. NP001020792.1), *Mus musculus* (Accession no. BAE34900.1), *Xenopus laevis* (Accession no. BAC57431.1), *Salmo salar* (Accession no. XP_014032104.1), *Bombus impatiens* (Accession no. XP003489428.1), *Pintada maxima* (Accession no. AEI58896.1), *Homarus americanus* (Accession no. ACV53024.1), *Marsupenaeus japonicas* (Accession no.AIF27797.1), *Macrobrachium rosenbergii* (Accession no. AMQ98967.1) The alignment was done by Clustal Omega (Thomson et al., 1994) as presented in figure 10. The highlight conserved of inhibitor binding site and domain were shown in underline capital letters and blue color. An active site flap was shown in yellow color and the conserved amino acid residues were shown in bold text. The conserved catalytic residue was shown in white text against black shade and catalytic motif was shown in box. Propeptide residues were shown in red color. The deduced amino acid sequence of MrCAT-D showed high amino acid similarity to *Marsupenaeus japonicas* and *Homarus americanus* with 88% and 87% identities, respectively.

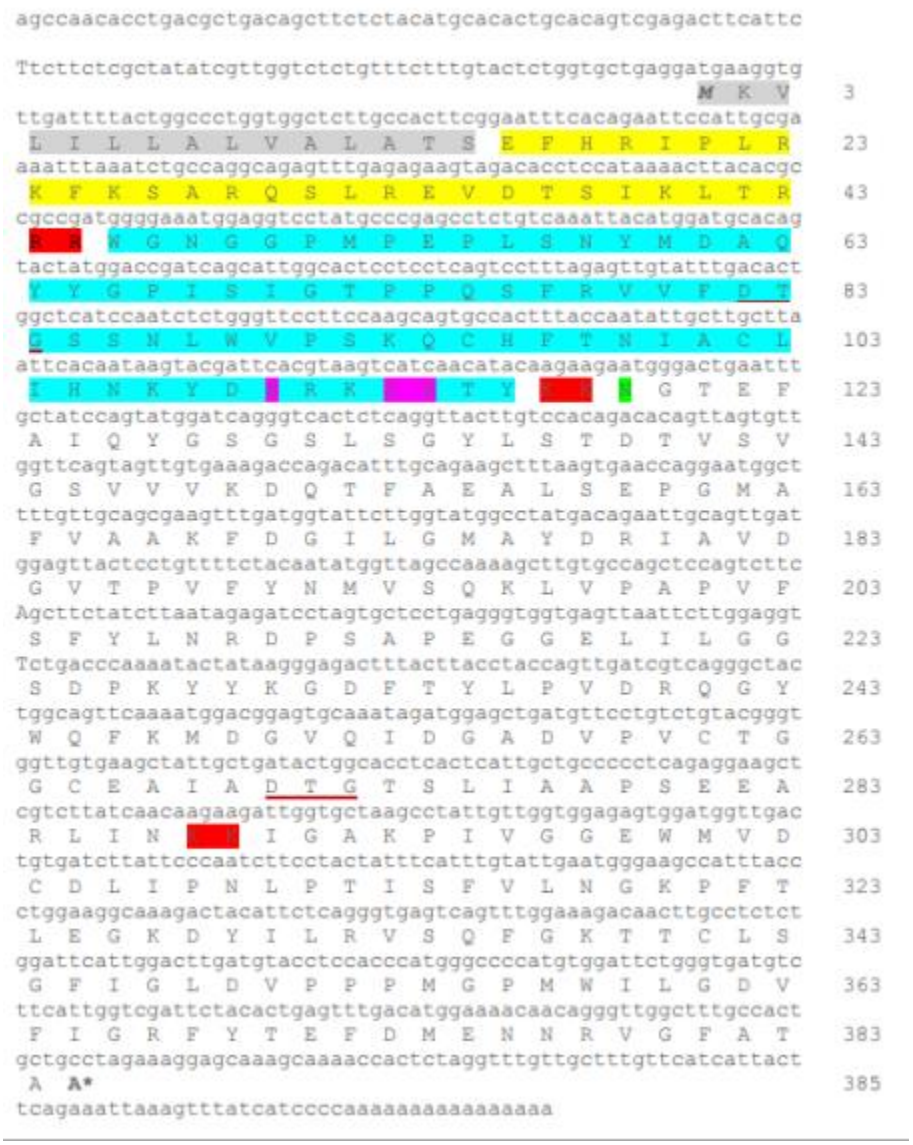

Figure 9. Nucleotide and deduced amino acid sequences in *M. rosenbergii* CAT-D. (GenBank: KP262355.1). the signal peptide is indicated in grey color, the single chain of pCAT-D is indicated in yellow color, the catalytic motif (DTG) are underlined with red line, a potential asparagine glycosylation residue (designated as N) is marked by green, the cleavage site is indicated in red color, the light chain is indicated in blue color. Inhibitor binding site (position 81-172 amino acid residues), catalytic motif (position 81-83 amino acid residues), catalytic residues (position 81 amino), active site flap (position 123-133 amino acid residues and A1_P: propeptide (position17-34 amino acid residues).

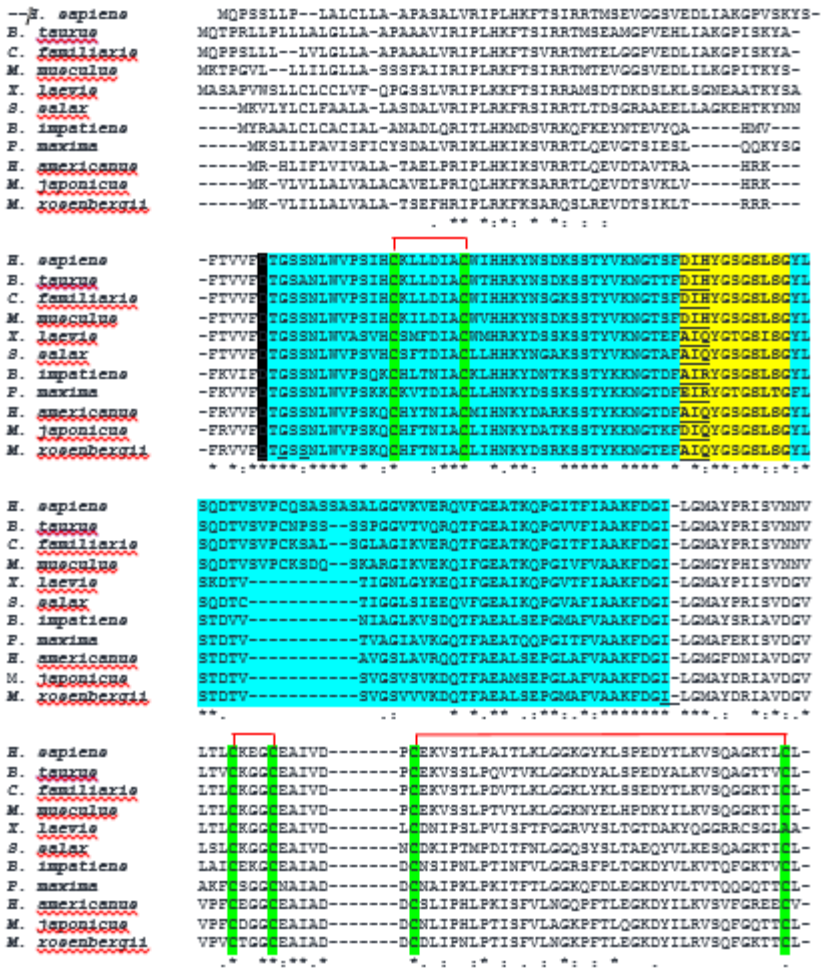

Figure 10. Alignment of amino acid sequences of Cathepsin-D of *M. rosenbergii* with others species- *H. sapiens* (Accession no. AAA51922.1.), *B. Taurus* (Accession no. NP001159993.1), *C. familiaris* (Accession no. NP001020792.1), *M. musculus* (Accession no. BAE34900.1), *X. laevis* (Accession no. BAC57431.1), *S. salar* (Accession no. XP_014032104.1), *B. impatiens* (Accession no. XP003489428.1), *P. maxima* (Accession no. AEI58896.1), *H. americanus* (Accession no. ACV53024.1), *M. japonicas* (Accession no. AIF27797.1), *M. rosenbergii* (Accession no. AMQ98967.1) using Clustal Omega program. The highlight conserved of inhibitor binding site and domain were shown in underline capital letters and blue color. An active site flap was shown in yellow color and the conserved amino acid residues were shown in bold text. The conserved catalytic residue was shown in white text against black shade and catalytic motif was shown in box. Propeptide residues were shown in red color. "*" indicated identical residues, ":" indicated conserved residues, and "." indicated semi-conserved residues. Red lines were indicated disulfide bond is predicted to be residue contact with ligand. " " indicated the N-glycosylation site.

- Phylogenetic analysis of the MrCAT-D peptide

A phylogenetic tree was constructed based on the existence CAT-D amino acid sequence which were CAT-D from vertebrate group (*H. sapiens*, *B. Taurus*, *C. familiaris, M. musculus*, *X. laevis*, *S. salar*), Insecta group (*B. impatiens*), Mollusca group (*P.maxima*) and Crustacea group (*H. americanus*, *M. japonicas, M. rosenbergii*) (Figure 11). The neighbor-joining tree of 11 CAT-D showed the 4 distinct groups: (1) Vertebrate CAT-D, (2) Insecta CAT-D, (3) Mollusca CAT-D and (4) Crustacean CAT-D groups. This result indicated that MrCAT-D was closely related to CAT-D of *M. japonicas* and diverged from a common ancestor.

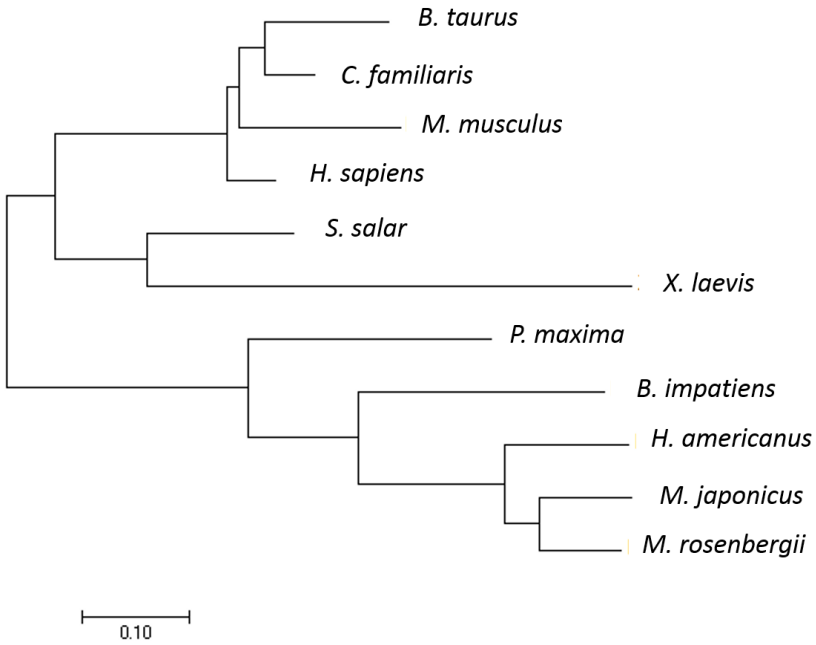

Figure 11. Phylogenetic tree analysis of CAT-D of *M. rosenbergii* with other species. The trees were produced using the Mega 7 program. The trees were based on a multiple sequence alignment of the amino acid sequence using Clustal Omega.

- The distribution of the CAT-D expression in various tissues of M. rosenbergii by using RT-PCR method

The various tissues of *M. rosenbergii*, including testis, vas deferens, spermatophores, muscle, hepatopancreas, stomach, heart and gill were collected and the total RNA were isolated by using Trizol reagent following the manufacturer's protocol. The purity and quantification amount of the RNA was measured by Nanodrop at 260 nm and 280 nm. RT-PCR amplification was performed according to

the procedure described in Materials and Methods using CAT-D primers designed and constructed from *M. rosenbergii* CAT-D nucleotides sequences to give a deduced RT-PCR product length of 323 bp. The results in figure 12 revealed a single, intense PCR amplifying band at 323 bp in testis, vas deferens, spermatophores as well as other tissues. This results suggested that CAT-D transcripts were synthesized in testis, vas deferens, spermatophores and other tissues. A β-actin used as positive control were detected in all tissues (Figure12).

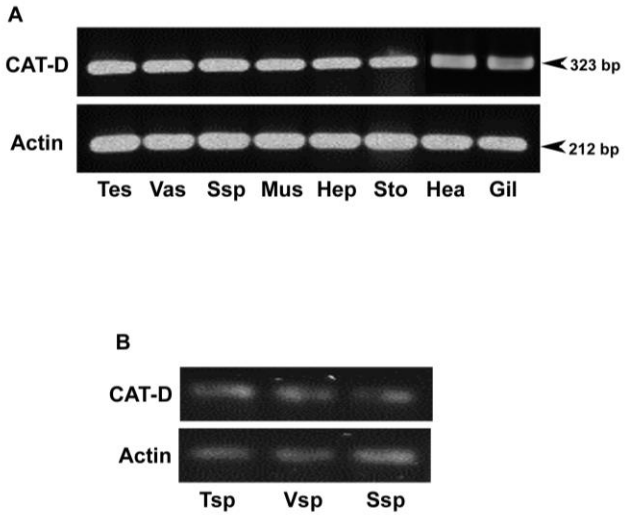

Figure 12. The PCR product of CAT-D gene using the total RNA of the *M. rosenbergii* tissues as template and CatD-F and CatD-R as primers. The size of the interested band was 323 bp.

3.3 CAT-D is a secreted protein existing in the fluid of male reproductive tract.

Sperm proteins extracted from testis (Tsp), vas deferens (Vsp), spermatophores (Ssp) and their fluid samples which are testicular fluid (TF), vas deferens fluid (VF) and spermatophore fluid (SF) were separated on 12.5% SDS-PAGE under a reducing condition. The result showed the different protein profiles of Tsp, Vsp, Ssp (Figure 13A) and TF, VF, SF (Figure13B). These protein profiles showed wide range of protein bands from >150 to < 10 kDA. Both sperm and their fluid protein profiles, the bands that interested at molecular weight about 42 kDA, 28 kDA and 9 kDA may be the molecular weight of predicted CAT-D. 42 kDA, 28 kDA and 9 kDA may be the intermediate form, the heavy chain, and light chain, respectively (Fig.5). These results suggested that there are the modification of sperm and their fluid proteins during sperm's transit from testis into vas deferens and spermatophores in male reproductive tract of *M.rosenbergii.*

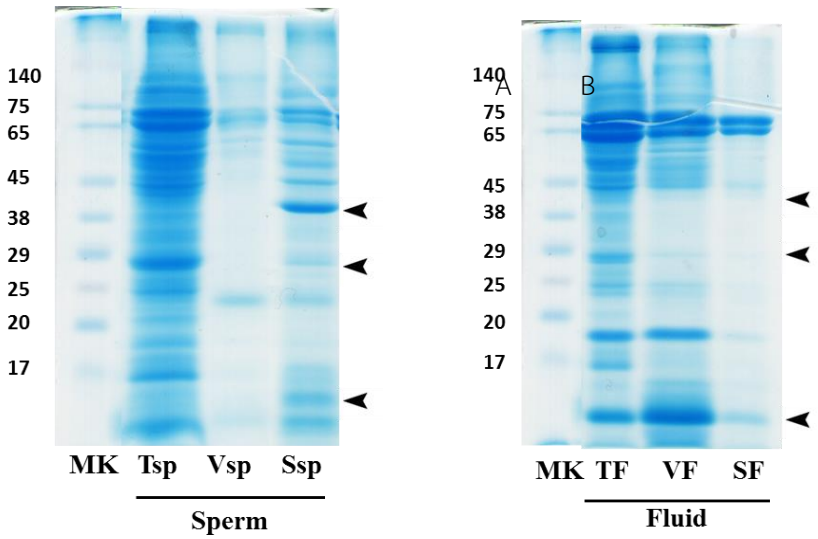

Figure 13. Sperm proteins (A) extracted from testis (Tsp), vas deferens (Vsp), spermatophores (Ssp) and their fluid (B) samples which are testicular fluid (TF), vas deferens fluid (VF) and spermatophore fluid (SF) were separated on 12.5% SDS-PAGE. The interested bands at molecular weight about 42 kDA, 28 kDA and 9 kDA (arrows) may be the molecular weight of predicted CAT-D. 42 kDA, 28 kDA and 9 kDA may be the intermediate form, the heavy chain, and light chain, respectively. MK: protein molecular weight marker.

3.4 The distribution of the CAT-D expression in various tissues of *M. rosenbergii*

The various tissues of *M. rosenbergii*, including testis, vas deferens, terminal ampoule, muscle, hepatopancreas, stomach, heart and gill were collected and the total RNA were isolated by using Trizol reagent following the manufacturer's protocol. The purity and quantification amount of the RNA was measured by Nanodrop at 260 nm and 280 nm. RT-PCR amplification was performed according to the procedure described in Materials and Methods using CAT-D primers designed and constructed from *M. rosenbergii* CAT-D nucleotides sequences to give a deduced RT-PCR product length of 323 bp. The results in Figure 5 revealed a single, intense PCR amplifying band at 323 bp in testis, vas deferens, spermatophores as well as other tissues. This results suggested that CAT-D transcripts were synthesized in testis, vas deferens, terminal ampoule and other tissues. β-actin used as positive control were detected in all tissues. Quantitative PCR analyses of CAT-D, normalized to actin, revealed CAT-D mRNA abundance in the vas deferens and terminal ampoule versus no significantly quantities in the testis (Figure 14). Moreover, the CAT-D mRNA was relatively high in small male and orange claw male (Figure 15), especially in the testis (Figure 16).

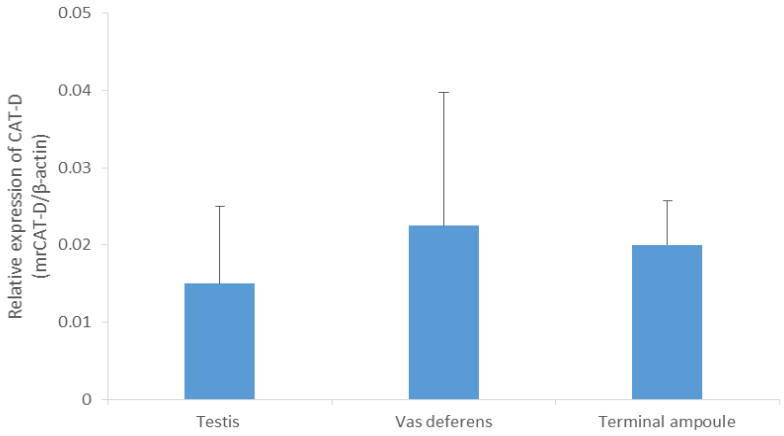

Figure 14. Relative expression of CAT-D in reproductive tissues. Quantitative PCR amplification from total RNA isolated from different reproductive tissues, including testis, vas deferens and terminal ampoule.

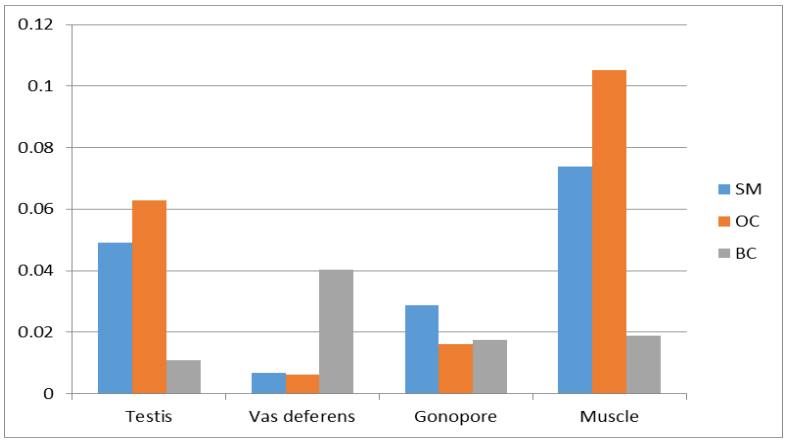

Figure 15. Relative expression of CAT-D in reproductive tissues and muscle. Quantitative PCR amplification from total RNA isolated from different reproductive tissues and muscle of small male (SM), orange claw male (OM) and blue claw male (BC).

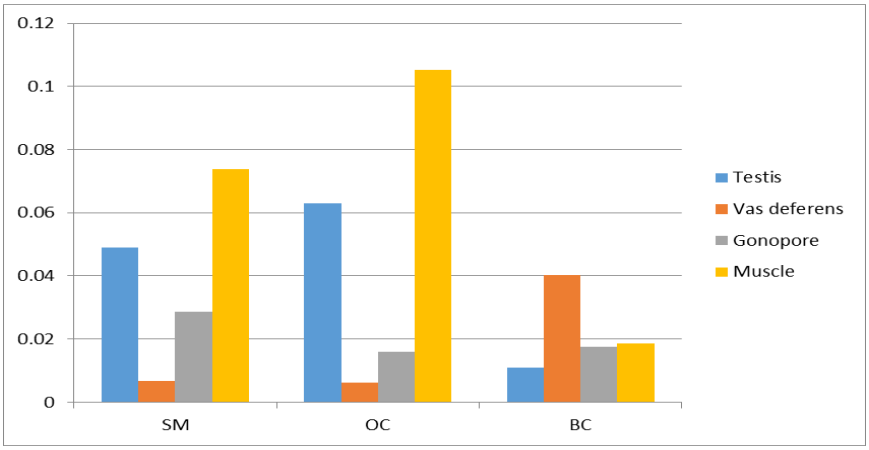

Figure 16. Relative expression of CAT-D in small male (SM), orange claw male (OM) and blue claw male (BC). Quantitative PCR amplification from total RNA isolated from different reproductive tissues and muscle.

- *In situ* hybridization of CAT-D in male reproductive tissues

Using *in situ* hybridization with the specific DIG-labeled oligonucleotide probes for CAT-D, the mRNA of CAT-D was detected in epithelial lining cells of testis and vas deferens and terminal ampoule. An intense signals representing the mRNA expression of CAT-D in testis, vas deferens and spermatophores were detected throughout the cytoplasm of epithelium lining cells (Figure 17). This result will show the existence of CAT-D transcripts in the male reproductive tract of *M. rosenbergii*. This might suggest that CAT-D is synthesized by epithelial cells of male reproductive tissues.

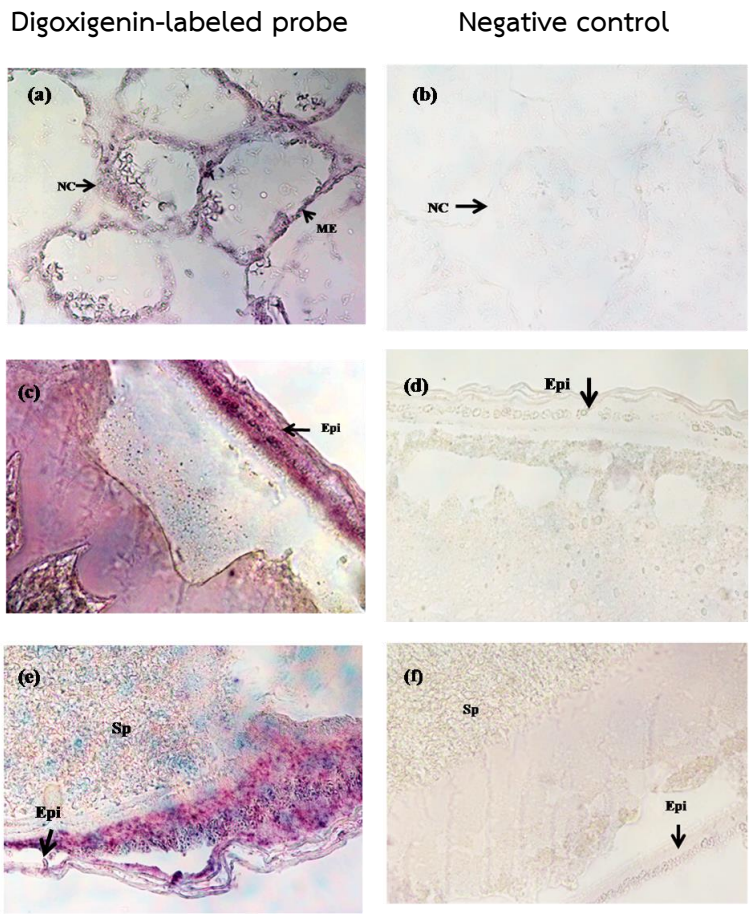

Figure 17. In situ hybridization analysis of CAT-D transcripts in reproductive tissues of M.rosenbergii. (a,b) Testis, (c,d) Vas deferens, (g,h) Terminal ampoule. Epi, epithelium; ME, myoepithelial lining; NC, nurse cells; Sp, sperm.

- Immunocytochemistry of CAT-D

Using the anti-CAT-D, the intense dark brown enzymatic products making the localization of CAT-D protein was found in the epithelial cells of testis, vas deferens and terminal ampoule. The immunoreactivity was reveal in the cytoplasm of epithelial cells. From these results, it is possible that CAT-D may be involved in sperm maturation. Moreover, CAT-D may be synthesized from these epithelial lining (Figure 18).

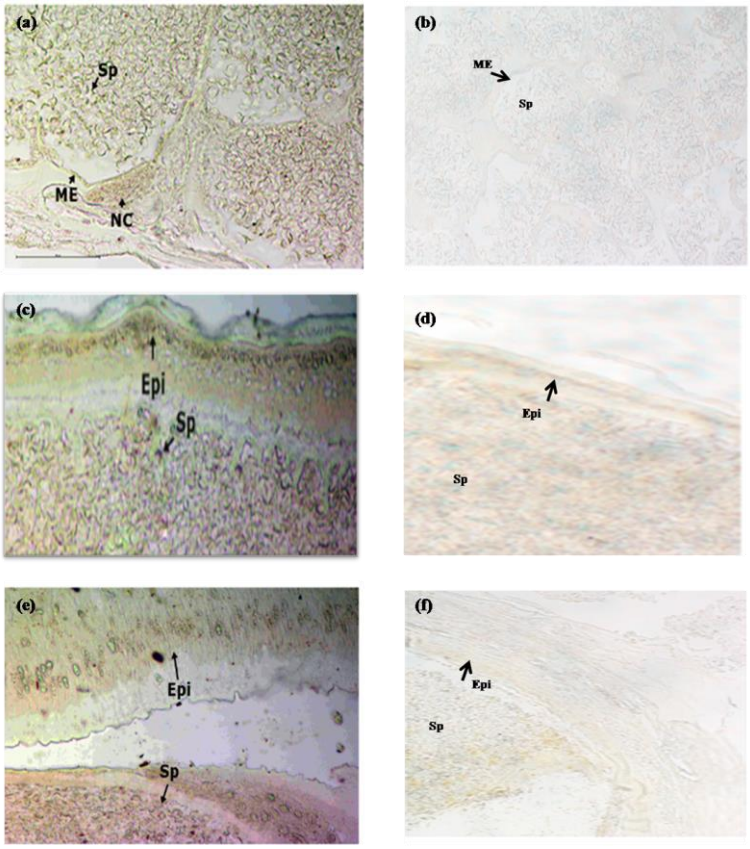

Figure 18. Immunohistochemical staining of CAT-D (left panel) compare to negative control (right panel). The intense immunoreactivity in the nurse cells, myoepithelial lining layer and sperm in testis as well as sperm and epithelium of vas deferens and terminal ampoule. (a, b) Testis, (c, d) Vas deferens, (g, h) Terminal ampoule. Epi, epithelium; ME, myoepithelium; NC, nurse cells; Sp, sperm.

- Immunoblotting with anti-CAT-D

CAT-D was detected in sperm, fluids, vesicles and epithelium of *M. rosenbergii* by western blot analysis. The results revealed that an intense immunoreactive bands of a 100 kDa, 70 kDa and 55 kDa were detected in Tsp, a 70 kDa and 55 kDa in Vsp and Ssp (Figure 19). Interesting, the expression levels of CAT-D in sperm gradually decrease from testis to spermatophores. In VF and SF, there was an intense immunoreactive band of a 130 kDa and 55 kDa that were detected in SF but it is not present in VF (Figure 19.) The vesicles (supernant of the fluid), there were the band of an 130 kDa that were detected in vesicle from vas deferens fluid (Vv) and the bands of 130 kDa and 75 kDa in vesicle from spermatophore fluid (Sv). Moreover, there were the bands of 130 kDa and 55 kDa that were detected in epithelium of vas deferens and spermatophores (Figure 20).

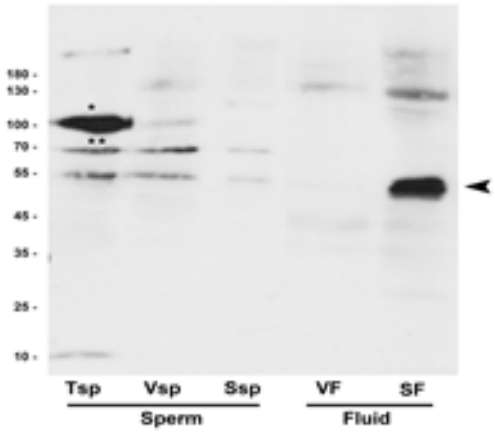

Figure 19. Presence of CAT-D on sperm protein extracted from testis (Tsp), vas deferens (Vsp), spermatophores (Ssp) and their fluids: vas deferens fluid (VF) and spermatophore fluid (SF) by western blotting.

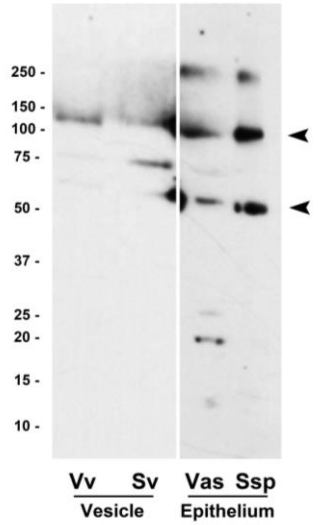

Figure 20. Presence of CAT-D on vesicles isolated from vas deferens (Vv) and spermatophores (Sv) and epithelium of vas deferens (Vas) and spermatophores (Ssp) by western blotting.

3.5 Identification, structural characterization and distribution of MrNPC2 transcript in male reproductive tract of *M. rosenbergii*

- Identification of MrNPC2

Besides the loss of cholesterol in sperm plasma membrane both in *M. rosenbergii* and *P. monodon* during sperm transferred from testis to vas deferens, further studies were performed to identify the factors that are involved in cholesterol removal. Previous studies showed that the protein, namely, Niemann pick type 2C (NPC2)/Epididymal secretory protein 1 (HE1) is involved in cholesterol transport in the cells and the authors found this protein is more abundant in epididymis (Okamura et al., 1999). We asked whether it could be detected in *M. rosenbergii* and may play a role in cholesterol removal from sperm plasma membrane during sperm maturation. To identify MrNPC2 gene, we determined the fraction cDNA sequences from transcriptomes analysis of *M. rosenbergii* that retrieved from NCBI database. The full-length of MrNPC2 was consisted of 1620 nucleotides and 151 amino acid residues (Figure 21), including a 19 amino acid of signal peptides at N-terminus, 132 amino acids of a mature peptide including 35 amino acids lipid binding domain and terminal codon (Figure 21). This predicted protein was small secreted protein with the predicted molecular weight at 16.333 kDa and an isoelectric point (PI) of 8.60 (Gasteiger et al., 2005). The signal peptide was shown in the black underline and the putative cholesterol/lipid binding site was shown in yellow underline analyzed by

BLASTP 2.3.1 Program (Altschul et al., 1990). It was indicated that the sequence at 87-122 was predicted to be the site of cholesterol/lipid binding. The essential residues that formed the pocket cavity were F87, V118 and Y122 (Figure 21).

The deduced amino acid of MrNPC2 was aligned to compare the identity with other species including *Canis lupus familiaris* (NCBI Reference Sequence: NP001003242), *Bos Taurus* (NCBI Reference Sequence: NP776343), *Mus musculus* (NCBI Reference Sequence: NP075898), *Homo sapiens* (NCBI Reference Sequence: BAG34872), *Xenopus laevis* (NCBI Reference Sequence: NP001083475), *Danio rerio* (NCBI Reference Sequence: NP775331), *Anopheles gambiae* (NCBI Reference Sequence: EAA07758), *Drosophia melanogaster* (NCBI Reference Sequence: NP608637), *Daphnia pulex* (NCBI Reference Sequence: EFX84782) and *Penaeus monodon* (NCBI Reference Sequence: ALG64704). The alignment was done by Clustal Omega Program (Thomson et al., 1994) as showed in Figure 22. MrNPC2 protein was in agreement with other NPC2 family's protein. This secreted protein was consisted of six cysteine residues that were conserved position in NPC2 family gene as showed in the white text against shading. The six cysteines form three disulfide bonds, connecting residues Cys_28-Cys_143, Cys_44-Cys_51and Cys_97-Cys104 of MrNPC2 as showed red line in Figure 22. The essential amino acid residues that generated the cholesterol pocket were shown in the boxes. There was hydrophobic amino acid and conserved among species. The amino acid sequences of NPC2 in Crustacea including *D. pulex* and *P. monodon* homologues were ~35.57% and~ 57.12% identical to MrNPC2, respectively.

- Phylogenetic analysis of the MrNPC2 peptide

A phylogenetic tree was constructed based on all existence NPC2 amino acid sequence which were NPC2 from vertebrate group (*H. sapiens*, *D. rerio*, *B. Taurus*, *C. familiaris*, *M. musculus*, *X. laevis*), Insecta group (*D. melanogaster*, *A. gambiae*), and Crustacea group (*D. pulex*, *P. monodon*, *M. rosenbergii*) (Figure 22). The neighbor-joining tree of 11 NPC2/HE1s showed the 3 distinct groups: Vertebrate NPC2/HE1, Insecta NPC2/HE1, and Crustacea NPC2/HE1 groups. This result indicated that MrNPC2/HE1 was closely related to PmNPC2 and diverged from a common ancestor (Figure 23).

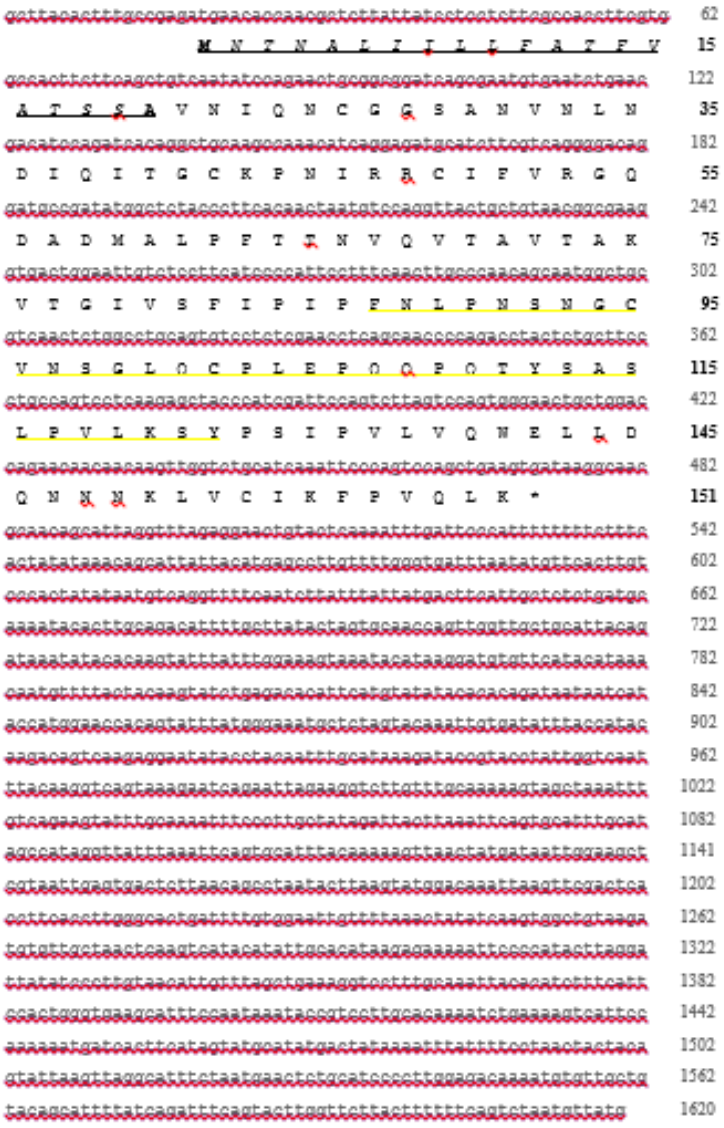

Figure 21. MrNPC2-like nucleotide and deduced amino acid sequence of full length *M. rosenbergii*. Amino acids were shown in capital letters. The signal peptide and the cleavage site, predicted by SignalP-4.1 program, were indicated by italics underlined text and an end text, respectively. The italics sequence represent the associated peptide. An asterisk was indicated the stop codon. The number on the right was indicated nucleotide and amino acid sequence.

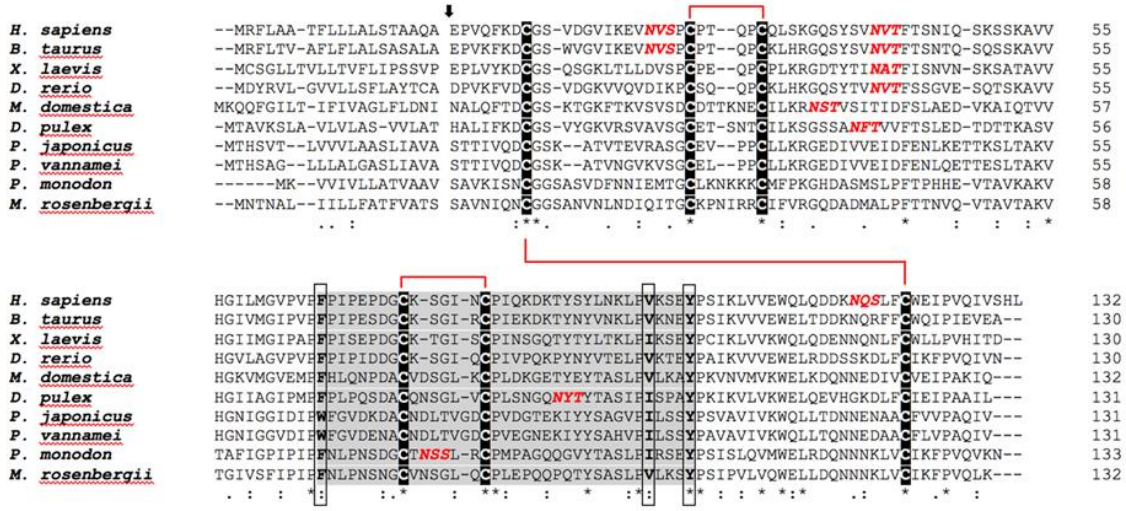

Figure 22. Alignment of amino acid sequences of NPC2 of *M. rosenbergii* with other species - *Canis lupus familiaris* (accession no. NP001003242), *Bos Taurus* (accession no. NP776343), *Mus musculus* (accession no. NP075898), *Homo sapiens* (accession no.BAG34872), *Xenopus laevis* (accession no. NP001083475), *Danio rerio* (accession no. NP775331), *Anopheles gambiae* (accession no. EAA07758), *Drosophia melanogaster* (accession no. NP608637), *Daphnia pulex* (accession no. EFX84782) and *Penaeus monodon* (accession no. ALG64704) using Clustal Omega program. The highly conserved cysteine residues were shown in white text against shade background. The cholesterol binding site were shown in blue shade color. The conserved amino acid residues of cholesterol binding pocket were shown in the boxes. The red line showed three disulfide bond. "*" indicated identical residues, ":" indicated conserved residues, and "." indicated semi-conserved residues.

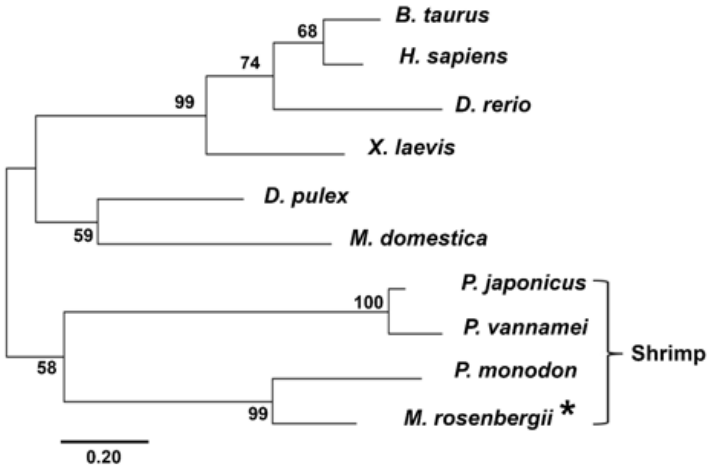

Figure 23. The cladogram of NPC2/HE1 protein. The phylogenic tree was conducted based on neighbor-joining method. The number at branch nodes indicated the present bootstrap confidence values obtained from 1000 replications.

- Crystal 3D structure prediction of MrNPC2

The predicted structure of MrNPC2 was generated by RaptorX program (Kallberg et al., 2012); PmNPC2 by Phyre2 and bNPC2 from protein data bank: 1nep. The structure-like of MrNPC2 was an immunoglobulin-like β-sandwich fold consisting of seven β-strands arranged in two β-sheets. This folding conformation contained a large cavity in the interior of the protein that was stabilized by three disulfide bonds. The overall structure of MrNPC2 was similar to bovine NPC2 (bNPC2) and PmNPC2 as showed in Figure 24. The structure of MrNPC2 showed that the ligand binding pocket was line exclusively with hydrophobic amino acid residues which were Phenylalanine (Phe-87), Valine (Val-118) and Tyrosine (Tyr-122) as in bNPC2 whereas in PmNPC2 was Phe, Iie and Tyr. These amino acid residues in NPC2 were identified as being able to bind with cholesterol 1:1 stoichiometry (Okamura et al., 1999, Friedland et al., 2003). In addition, Phe-87 and Tyr-122 are the aromatic residues that located at the gate opening of the cholesterol cavity and they were believed in stabilizing the conformational change of the cholesterol cavity.

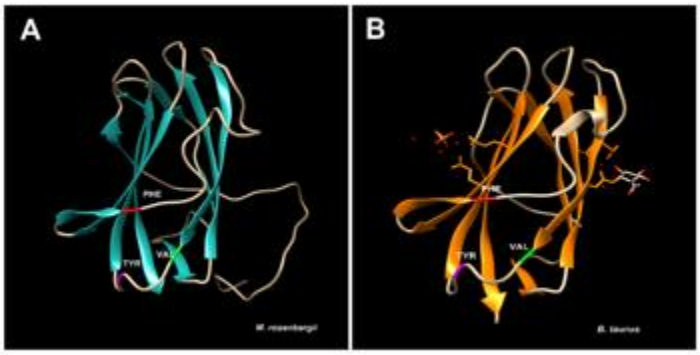

Figure 24. Comparison of a predicted crystal structure of *M. rosenbergii* NPC2 (A), and bovine NPC2 (Protein Data Bank: 1nep) (B). A solid ribbon style, colored by secondary structure type, PHE (F87), VAL (V118) and TYR (Y122) amino acids of *M. rosenbergii* (A) and PHE (F66), VAL (V96) and TYR (Y100) of *B. taurus* (B) were highlighted; these residues in NPC2 was identified as being able to bind cholesterol.

- Tissue expression of MrNPC2

MrNPC2 transcript was detected by reverse-transcription PCR in various tissues including reproductive tracts which were testis, vas deferens and spermatophore. Moreover, MrNPC2 transcript was also found in thoracic ganglion (Tg), brain (Br), stomach (Sto), Gill (gil) and hepatopancreas (Hep) as showed in Figure 16. The ß-actin, positive control, was detected with equal intensity in all samples.

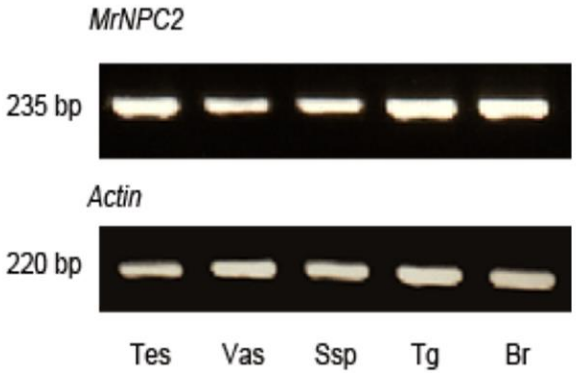

Figure 25. Tissue expressions of MrNPC2 using reverse-transcription PCR. (A) Expression of MrNPC2 was detected in various tissues (i.e., Testis (Tes), Vas deferens (Vas), Spermatophore (Ssp), thoracic ganglion (Tg), Brain (Br). β-Actin was evaluated as an internal control.

4. Output

1. Timklay W, Magerd S, Sato C, Somrit M, Watthammawut A, Senarai T, Weerachatyanukul W, Kitajima K, **Asuvapongpatana S.** N-linked mannose glycoconjugates on shrimp thrombospondin, pmTSP-II, and their involvement in the sperm acrosome reaction. Molecular Reproduction and Development 2019; 86(4):440-449. DOI: 10.1002/mrd.23122
2. Sakulset C, Surinlert P, Thongsum O, Watthammawut A, Somrit M, Nakiem J, Weerachatyanukul W, **Asuvapongpatana S.** Cathepsin D in prawn reproductive system and its function in actin digestion as part of testicular spermiation. In submission to Zoological Science.
3. Surinlert P, Sakulset C, Kongka T, Chotwiwatthanakun C, Vanichviriyakit R, Weerachatyanukul W, **Asuvapongpatana S**. Existance and distribution of Niemann Pick type 2C (NPC2) in shrimp reproductive tracts and its binding ability to sperm cholesterol. In preparation.
4. ผลิตนักศึกษาที่จบปริญญาเอกจากโครงการนี้ จำนวน 2 คน, นักศึกษาปริญญาโท กำลังดำเนินการวิจัย 1 คน และนักศึกษา Senior project จำนวน 4 คน