รายงานฉบับสมบูรณ์

โครงการ การปรับเปลี่ยนของผิวเซลล์อสุจิโดยโปรตีนในถุงเก็บอสุจิ (thelycum) ของกุ้ง *Penaeus monodon* เป็นขบวนการที่จำเป็นก่อนการปฏิสนธิ

(Modification of Sperm Plasma Membrane by Specific Thelycal Proteins in *Penaeus monodon* Is Essential Before Fertilization)

โดย ดร. วัฒนา วีรชาติยานุกูล และ คณะ

โครงการแล้วเสร็จ วันที่ 17 มิถุนายน 2549

ABSTRACT

Previous evidence has indicated the requirement of sperm modification, as part of capacitation-like process, in a close-typed-thelycum shrimp, however, the molecular mechanism supported this event is scarcely available. Using *Penaeus monodon* as the model, we demonstrated thelycal-dependent sperm modification resulting in an enhanced acrosome reaction (AR) response and the involvement of sperm trypsin-like proteases in induction of AR. Modification of shrimp sperm membrane was mediated through an adsorption or removal of sperm peripheral and integral membrane proteins as indicated by the different profiles of these proteins in spermatophore and thelycal sperm. *In vitro* adsorption of Alexa-488 conjugated thelycal proteins onto the entire S-sperm surface confirmed protein transfer in a time-dependent manner. Anchoring of 83 and 140 kDa proteins to sperm peripheral proteins as well as 53/55 and 60 kDa proteins to sperm lipids suggested that both sperm membrane proteins and lipids served as acceptors for thelycal protein adsorption. Apart from membrane modification, a substantial increase in protein tyrosine phosphorylation was shown to be closely associated with thelycal-dependent sperm modification event. This sperm modification led to an enhanced AR in response to a natural inducer, egg water (EW). We further elucidated that components of EW bound to sperm membrane and initiated AR. This AR process is proven to be trypsin-dependent process coupled with an influx of calcium ion. Ability of denatured EW to induce AR favored the implication that intrinsic sperm trypsin ,rather than that in EW, was a key modulator of AR induction. Taken all together, the results clearly indicate a 3-day-period requirement for sperm modification in female thelycum to gain a full AR response. This AR process is initialized via the binding of EW components to the sperm surface and followed by the proteolytic process of trypsin-like enzyme release from the sperm acrosome.

บทคัดย่อ

จากรายงานที่ผ่านมาพบว่าเซลล์อสุจิของกุ้งที่มีถุงเก็บน้ำเชื้อหน้าท้อง (Thelycum) แบบปิดจำเป็นที่จะต้องผ่านขบวนการพัฒนาความสามารถในการปฏิสนธิ เรียกว่า ขบวนการ Capacitation อย่างไรก็ดี กลไกระดับโมเลกุลของขบวนการดังกล่าวยังไม่มีการรายงานในเซลล์สืบพันธุ์ของกุ้งมาก่อน ดังนั้น ในการศึกษานี้เราใช้กุ้งกุลาดำ (P. monodon) เป็นต้นแบบในการศึกษาขบวนการดังกล่าวเราพบว่าเซลล์อสุจิจะต้องถูกปรับเปลี่ยนโมเลกุลบนผิวเซลล์ ในขณะที่อาศัยอยู่ในถุงเก็บน้ำเชื้อหน้าท้อง ทั้งนี้รวมถึงการกำซาบ (adsorption) ของโปรตีนที่อยู่ในทีไลกัม และการหลุดลอก (removal) ของโปรตีนบนผิวเซลล์ทั้งสองชนิด ได้แก่ ชนิด peripheral และ integral เรายืนยันผลการทดลองดังกล่าวด้วยการใช้โปรตีนของทีไลกัมที่ติดฉลากเรืองแสงย้อมติดบนผิวของเซลล์อสุจิที่นำมาจากตัวผู้ และยังพบว่าโปรตีนของทีไลกัมที่มีขนาด 83 และ 140 kDa จับกับตัวรับชนิด peripheral protein บนผิวเซลล์อสุจิ และโปรตีนขนาด 53/55 และ 60 kDa จับกับตัวรับชนิดไขมันบนผิวเซลล์ นอกเหนือจากการปรับเปลี่ยนผิวเซลล์เรายังพบการเพิ่มระดับของ Tyrosine Phosphorylation ซึ่งจัดว่าเป็นตัวบ่งชี้ที่สำคัญของขบวนการ Capacitation อีกทั้งยังพบว่าเซลล์อสุจิจะมีการเพิ่มความสามารถในการเกิดขบวนการแตกของถุงเอนไซม์ (acrosome reaction, AR) มากขึ้นเรื่อยๆ ตามจำนวนเวลาที่ถูกเก็บไว้ในถุงทีไลกัม โดยความสามารถของการเกิด AR สูงสุด จะพบในวันที่ 3 หลังจากที่เซลล์อสุจิถูกเคลื่อนย้ายเข้ามาในทีไลกัม เราศึกษาต่อไปเกี่ยวกับขั้นตอนเชิงโมเลกุลในการเกิดขบวนการ AR พบว่าโมเลกุลที่อยู่ในน้ำคัดหลั่งจากไข่ (egg water, EW) สามารถจับบนผิวเซลล์อสุจิและเหนี่ยวนำการเกิด AR นอกจากนี้เรายังพบอีกว่าขบวนการ AR ในขั้นตอนเริ่มแรกเกี่ยวกับการหดตัว (depolymerization) ของ anterior spike บนเซลล์อสุจิขึ้นอยู่กับปฏิกิริยาของเอนไซม์ทริปซินที่อยู่ในถุงเก็บเอนไซม์ของเซลล์อสุจิ แต่ไม่ขึ้นอยู่กับเอนไซม์ทริปซินที่อยู่ใน EW อีกทั้งเรายังพบว่าขบวนการ AR นี้ขึ้นอยู่กับการผ่านเข้าสู่เซลล์ของแคลเซี่ยมที่อยู่ภายนอกเซลล์อสุจิอีกด้วย กล่าวโดยสรุปคือ เซลล์อสุจิต้องใช้เวลาอย่างน้อย 3 วัน เพื่อปรับเปลี่ยนโมเลกุลบนผิวเซลล์ให้เกิดการเพิ่มความสามารถในการปฏิสนธิของเซลล์ สำหรับขบวนการ AR นั้น ขั้นตอนที่สำคัญได้แก่ การจับกันของโมเลกุลที่อยู่ใน EW กับผิวเซลล์อสุจิ ซึ่งนำไปสู่การผ่านเข้าสู่เซลล์ของแคลเซียมนอกเซลล์ และเหนี่ยวนำให้เกิดการทำงานของเอนไซม์ทริปซินจากถุงเก็บเอนไซม์ของเซลล์อสุจิ

EXECUTIVE SUMMARY

1. โครงการ: Modification of Sperm Plasma Membrane by Specific Thelycal Proteins in *Penaeus monodon* Is Essential Before Fertilization
(การปรับเปลี่ยนของผิวเซลล์อสุจิโดยโปรตีนในถุงเก็บอสุจิ (thelycum) ของกุ้ง *Penaeus monodon* เป็นขบวนการที่จำเป็นก่อนการปฏิสนธิ)

2. ผู้เสนอ: นายวัฒนา วีรชาติยานุกูล

นักวิจัยที่ปรึกษา ศ. ดร. ประเสริฐ โศภน

3. หน่วยงาน: ภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล

4. ระยะเวลาดำเนินการ: 2 ปี (ก.ค. 46 – มิ.ย. 49) – ขอพักระหว่างโครงการ 1 ปี

5. งบประมาณ: [redacted]

6. ความสำคัญและที่มาของปัญหา

Cultivation of the black tiger shrimp, *Penaeus monodon*, has become a large-scale commercial venture and achieved a very high economic impact of Thailand during this decade. Although shrimp farming has spread rapidly in many countries all over Asia, Australia and some States along the coasts of USA, farmed *P. monodon* from Thailand maintains the leading status among these countries in the last five years. The exported value of shrimp from Thailand to Japan and USA approached 100,000 million baht in year 2000, which accounted for over 25% of the world supply of cultivated shrimps. It should be pointed out here that, up to date, the production of shrimp offsprings (i.e., nauplius, protozoea, mysis and post-larval stage) is much relied on availability of caught broodstocks from the natural habitats. The high productivity of cultured shrimp is limited by an inadequate supply of natural broodstock, both from the scarcity of wild parental stock and increasing viral infection that results in mortality of the offsprings. This immediately causes the major problems for shrimp industry. In addition, the broodstocks obtained become smaller in size with poorer fecundity. In long term shrimp cultivation system needs to be sustainable to obtain a large pool of healthy-domesticated broodstocks. The most ideal way to circumvent this problem is the domestication of in-breed brood stock. The attempt along this line has already been carried out by Dr.

Withyachumnamkul's group (Withyachumnarnkul el al., 1998), with a great success of sustaining four to five disease-free generations of potential brood stocks. However, the size and fecundity of these generations are still lower than those retrieved naturally. Furthermore, the sperm and eggs produced by these domesticated stocks had lower fertilizing capability than those collected from the wild parental stocks. This could be due to the immaturity and/or the improper priming of the gametes before fertilization. With these regards, a multi-disciplinary research gearing to achieve a better understanding of a fundamental knowledge on shrimp fertilization need to be established. In this proposed research, we aim to study the process of sperm modification by female factors which may in turn improve the ability of sperm to undergo an acrosome reaction, thus improve fertilizing capability of the sperm from domesticated parent shrimps.

7. วัตถุประสงค์

a. Overall objective

To study the modification of sperm plasma during the sperm transit from male spermatophore to female thelycom and to compare the ability of thelycal-protein-treated and untreated sperm to undergo the acrosome reaction as well as their in vitro fertilizing ability.

b. Specific objectives

1. To compare the protein profiles of plasma membrane extracted from spermatophore and thelycal sperm.
2. To compare the protein profiles of fluid taken from spermatophore and thelycum.
3. To demonstrate in vitro adsorption of proteins derived from thelycal fluid onto the sperm surface.
4. To monitor the ability of thelycal-protein-treated and –untreated sperm to undergo egg-water-and A23187-induced acrosome reaction.
5. To check the fertilizing ability of sperm taken from spermatophore after thelycal fluid incubation in comparison with the untreated sperm.

8. การดำเนินการทดลอง

1. Protein from plasma membrane of spermatophore and thelycal sperm were extracted and resolved by SDS-PAGE. After silver staining, the protein profiles were analysed and compared.
2. Fluorescently labeled thelycal proteins were incubated with spermatophore sperm. The adsorbed proteins onto sperm surface was viewed under epifluorescent microscope. Alternatively, treated sperm were subjected to plasma membrane extraction followed by Western blotting and the adsorbed-biotinylated proteins was detected by avidin-peroxidase.
3. Physiological functions of sperm transferred into thelycum at different periods which include an increased level of protein tyrosine phosphorylation and the ability of the sperm to undergo acrosome reaction were tested by immunoblotting and EW-induced acrosome reaction, respectively.
4. Thelycal-fluid-treated- and untreated-sperm as well as thelycal sperm were induced to undergo the acrosome reaction using calcium ionophore, A23187, or egg water, a natural inducer. The ability to undergo the acrosome reaction among these sperm samples was analysed statistically.
5. Molecular mechanism of EW-induced acrosome reaction including binding of EW components and proteolytic activity and calcium influx were investigated using incubation assay of fluorescently labeled EW, fluorogenic enzyme assay in conjunction with the presence of inhibitors, and the use of calcium chelators.

9. การดำเนินงาน

In the first six months, we collected both male and female sperm samples for plasma membrane extraction at both Burapha University, Chonburi and at BAFCO farm, Nakhonsrithammarat. Thelycal fluid and EW were collected for biotinylation or fluorescent labeling process in the mean time. Comparison of protein profiles between thelycal and spermatophore fluids as well as membrane proteins of sperm taken from spermatophore and thelycum were carried out.

In the 7^{th} to 12^{th} months, detection of in vitro adsorption of fluorescently labeled or biotinylated thelycal proteins onto sperm surface both with live sperm or plasma membrane extracts were performed. Testing of an increased level of protein tyrosine

phosphorylation, and ability of sperm to undergo EW-induced acrosome reaction were performed. These sets of experiments were repeated at least three times in order to verify its reproducibity to obtain firm conclusion. One manuscript was submitted for publication in Mol Reprod Dev.

In the 13th to 18th month, the induction of the acrosome reaction by egg water in sperm pretreated with thelycal proteins in comparison untreated sperm and thelycal sperm were carried out. In addition, testing of the binding of fluorescent EW components as well as all enzymatic activities that are involved in AR induction including trypsin-like one in both EW and sperm sources were done. We also checked that inhibitors specific to all kinds of proteases could inhibit proteolytic activity in EW and sperm and interfere with AR induction.

In the last six months, we repeated some experiments and cleaned up all of our results to make sure they arereplicable resources. Finally, all of these complete results in the second pieces of work were gathered for manuscript preparation and is currently revised before sending out for publication consideration in Biol Reprod in this few weeks.

10. รายละเอียดการดำเนินการทดลอง และ ผลการทดลอง

I. Molecular Modification of *Penaeus Monodon* Sperm in Female Thelycum and Its Consequent Responses

EXPERIMENTAL APPROACHES:

1. Sample Collection

Broodstock shrimps were caught from Andaman Sea and maintained at Department of Aquatic Science, Faculty of Science, Burapha University, or at BAFCO farm, Nakhon Si Thammaraj, Thailand. Female shrimps were anaesthetized under ice, and then cut through thoracoabdominal segment just below the 5th walking legs where thelycum is situated. The gelatinous sperm masses were physically isolated from thelycums by pairs of fine forceps into 0.22-μm-filtered sea water (SW) containing 2 mM PMSF and 1 mM EDTA. Subsequently, these masses were segregated by vigorous shaking and pipetting. Sperm suspension was then filtered through 212-μm-filter to remove any gross debris. Thelycal (T) sperm in the suspension were pelleted (500g, 5 min) leaving thelycal contents in the supernatant. This supernatant or "thelycal fluid" was cleaned (12,000g, 10 min) and the protein concentration was determined by Bradford protein assay at the wavelength of

595 nm. T-sperm in the pellet were resuspended in SW at the final concentration of 1 x 10^7 sperm/ml before use.

Spermatophores, located at 5th walking legs of male shrimps, were retrieved by electro-stimulation (square wave, frequency = 100 cps, pulse duration = 0.1-0.5 sec, and voltage = 25 mV) applied on the ventral abdominal wall or by deep compression close to the location of spermatophores without sacrificing the shrimps. These spermatophores were processed t spermatophores were processed ttophore (or S) sperm, following the methods described for T-sperm.

2. Extraction of Sperm Peripheral and Integral Membrane Proteins

Peripheral membrane proteins were extracted according to the method described by Frenette and Sullivan (2001). Briefly, the sperm pellets were resuspended with 3 ml of 1 M NaCl and incubated with gentle agitation (30 min, 4°C). Thereafter, the sperm were pelleted (500g, 4°C, 5 min) and the supernatant containing peripheral membrane proteins was collected and cleaned (10,000g, 4°C, 10 min). The proteins were concentrated by MicroCon centrifugation device (Millipore, Bedford, MA) and the protein concentration was determined by Bradford protein assay. The viability of sperm after subjecting to high salt treatment was examined by propidium iodide (PI) staining according to manufacturer's protocol (Molecular Probes, Eugene, OR). The remaining sperm pellets were washed and resuspended in Tris-buffer saline (TBS: 10 mM Tris-HCl, 150 mM NaCl, pH 7.4) for further extraction of sperm plasma membrane by a phase separation of Triton X-114 according to the method described by Bordier (1981). In brief, sperm were reconstituted in a final 1% Triton X-114, thoroughly vortexed and centrifuged (5,000g, 4°C, 10 min) to get rid of the nuclear and cellular debris. The obtained supernatant was overlaid on top of sucrose cushion (6% sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.06% Triton X-114) and allowed to stand at room temperature until a cloudiness was observed in the suspension. The gradient was then subjected to low speed centrifugation (300g, room temperature, 3 min). The upper aqueous and lower detergent phases were gently collected and reconstituted in final 1% Triton X-114 and TBS, respectively. The subsequent phase separation steps and collection of both phases were repeated. Detergent phase containing mainly integral membrane proteins (Clement and Bordier, 1981) was pooled and diluted with TBS before subjecting to protein quantification and SDS-PAGE. A modified method of Lowry's assay using a D_c Protein assay kit (Bio-Rad, Hercules, CA) was applied

because of its compatibility with detergent containing protein samples. The colored reaction product was measured at the wavelength of 750 nm.

3. Delipidation of Integral Proteins and Protein Profiling by SDS-PAGE

To obtain a pure fraction of integral proteins, contaminated lipids in detergent extracts were eliminated according to the delipidation method described by Mastro and Hall (1999). Fourteen milliliters of ice-cold tri-n-butylphosphate: acetone: methanol (1: 12: 1) was added to the diluted detergent extracts to obtain a final of 80% acetone concentration, and incubated at 4°C for 2-3 hr. At this concentration, integral proteins were precipitated leaving membrane lipids still dissolved in organic solvent. The protein precipitate was pelleted (2,800g, 4°C, 15 min) and the supernatant containing lipids and detergent was discarded. Delipidated proteins were washed with acetone twice and finally resuspended in TBS. Protein concentration was determined by Bradford protein assay.

Approximately, 4 μg of total peripheral and integral membrane proteins isolated from S- and T- sperm were resolved by 10% SDS-PAGE under a reducing condition (Laemli, 1970) and further subjected to a silver staining method described by manufacturer (BioRad).

4. In Vitro Adsorption of Alexa-488 Conjugated Thelycal Proteins to Spermatophore Sperm

Thelycal proteins were conjugated to Alexa-488 fluorescent reactive dye (Molecular Probes) or biotinylated (Pierce, Rockford, IL) according to the procedure described by manufacturers. Approximately, 1 million S-sperm were fixed with 4% paraformaldehyde for 1 hr and washed with SW. Subsequently, the sperm were exposed to 5 mg/ml Alexa-488-conjugated thelycal proteins at various time intervals, i.e., 6, 12, 24 hr. Sperm were washed twice (500g, 5 min) with SW to eliminate unbound conjugates. Sperm resuspended in 10 μl PBS/glycerol (1:1, v/v) were plated onto slide, flattened with coverslip and sealed with nail polish. They were then viewed under a Nikon Eclipse epifluorescent microscope equipped with a Nikon DXM 1200 CCD camera using a set of fluorescein filters (excitation/emission wavelength = 488/520 nm). Competition assay was performed by an addition of 50 mg/ml non-labeled thelycal proteins (10 folds) to complete with labeled proteins. S-sperm incubated with Alexa-488-conjugated BSA or T-sperm incubated with Alexa-488-conjugated thelycal proteins served as controls.

In an alternative experiment, aldehyde fixed S-sperm were exposed to biotinylated thelycal proteins under the same conditions described above. After an extensive washes,

sperm were treated either with 1 M NaCl in PBS to extract peripheral proteins (see method below for more details) or with SDS-PAGE loading buffer. They were then subjecting to a Western streptavidin blotting described below.

5. Solid Phase Binding Assay of Labeled Thelycal Proteins with Sperm Membrane Proteins and Lipids

For sperm protein binding assay, ~100 ng of each sample including NaCl- and Triton X-114- (with and without delipidation) protein extracts of S-sperm were dotted onto nitrocellulose membrane. The membrane was submersed in 4% BSA in TBS (1 hr, 4°C) to block any non-specific binding. Then, dotted proteins were incubated (3 hr, 4°C) with biotinylated thelycal proteins at various concentrations (1:250, 1:500, and 1:1,000) and extensively washed with TBS containing 0.1% Tween-20 (TBS-T). Thereafter, the membrane was exposed (30 min, 4°C) to horse radish peroxidase (HRP)-conjugated streptavidin (KPL, Gaithersburg, MD) (1:2500) followed by the detection with an enhanced chemiluminescent method using ECL kit (Amersham Pharmacia, Buckinghamshire, England). The competition assay was performed by addition of an excess amount of non-labeled thelycal proteins (10 folds of labeled proteins) to interfere the binding of labeled proteins.

For sperm lipid binding assay, sperm total lipids were obtained following the chloroform/methanol extraction method described by Bligh and Dyer (1959). Dried lipids were resuspended in TBS, pH 7.4 and coated overnight onto 96-well-plates. Unbound lipids were washed extensively with washing buffer (0.15 M PBS, pH 7.2). Non-specific binding was eliminated with 1% BSA in washing buffer. Subsequently, the plates were washed 3 times and incubated (3 hr, room temperature) with 0.1 mg/ml of biotinylated thelycal proteins. After successive washes to remove unbound proteins, SDS-PAGE loading buffer was added to recover bound biotinylated thelycal proteins followed by streptavidin blotting as described below.

6. Western Streptavidin Blotting

Bound biotinylated thelycal proteins stripped either from whole sperm (by 1 M NaCl in PBS or SDS-PAGE loading buffer) or from micro-wells coated with sperm lipids as stated above were resolved with 10% SDS-PAGE under reducing condition (Laemli, 1970). Thereafter, the protein samples on the gels were electrotransfered (Towbin et al., 1979) onto Hybond N-ECL 0.45 μm nitrocellulose membrane (Amersham Pharmacia) and

further exposed to streptavidin-HRP (1:2500). Visualization of streptavidin-biotin complexes was performed by an enhanced chemiluminescent method using ECL kit.

7. Detection of Protein Tyrosine Phosphorylation

Two of freshly retrieved spermatophores were artificially inseminated (AI) into the thelycum of an individual molting female. After 1, 2 and 3 days post-AI, the sperm were mechanically isolated and washed as the method described above. Total proteins of the whole sperm were quantified by Bradford protein assay. Approximately, 3 μg of total proteins taken from S-sperm (Day 0) and Day 1, 2 and 3 post-AI sperm were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was then subjected to Western immunoblotting using a monoclonal anti-protein tyrosine phosphate IgG (clone P-Try-102: Cell Signaling, Beverly, MA) (1:10,000) followed by goat anti-mouse IgG-HRP (1:2,500) (Zymed, San Francisco, CA). Antigen-antibody complexes were visualized by an enhanced chemiluminescent method using ECL kit.

8. Induction of the Acrosome Reaction of Artificially Inseminated Spermatophore Sperm

To investigate the physiological change of *P. monodon* sperm after translocation into female thelycum at different time points, the ability of AI sperm to undergo AR were monitored by using the natural inducer, egg water (EW) (Clark et al., 1981). S-sperm (Day 0) or Day 1, 2 and 3 post-AI sperm were isolated into filtered SW without protease inhibitors (known to block AR induction). These sperm were treated 5 min with 40 and 100 g/ml EW and without EW (control). Thereafter, a final concentration of 4% paraformaldehyde in SW was added into the sperm suspension. The status of the acrosome was checked under a Nikon phase contrast microscope and >200 sperm were counted for each data point. The acrosome intact sperm exhibited a long anterior spike while that of the acrosome reacted sperm became shorten. Differences among samples tested were analysed statistically using ANOVA.

RESULTS:

1. Comparison of Protein Profiles between S- and T-Sperm

Profiles of NaCl and detergent extracted proteins of S- and T-sperm which represented peripheral and integral proteins were compared in Figure 1. For peripheral proteins, the 72, 83 and 140 kDa proteins were more predominant in T-sperm than S-sperm, however, there appeared the 25, 45 and 70 kDa that were more pronounced in S-sperm than T-sperm (Lane S_{Na} and T_{Na}, arrow heads). Some differences could also be notified in the integral proteins. A set of fast mobility proteins with the M_r ranging from

35 kDa to 43 kDa were apparent in S-sperm while they were sparse in T-sperm. On the other hand, the 53/55, 60, 72 and 83 kDa were still more prominent in T-sperm than S-sperm (lane S_{De} and T_{De}, arrow heads). The 72, and 83 kDa proteins might be the traces of peripheral proteins that were present abundantly in T-sperm surface. These results reflected both the adsorption of additional sperm peripheral and integral proteins as well as the removal of such proteins during sperm membrane modification.

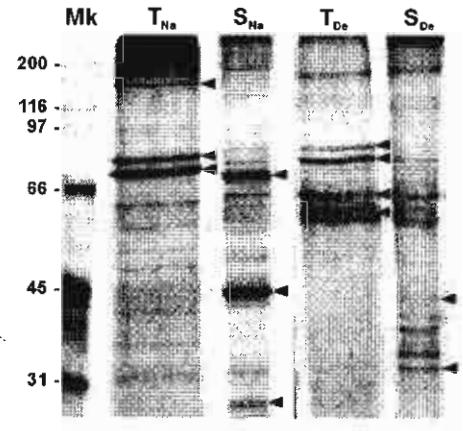


Figure 1 Silver staining of membrane proteins extracted from thelycal (T) and spermatophore (S) sperm using NaCl (Na) and Triton X-114 (De) treatment. Mk denoted standard markers.

2. In vitro Transfer of Labeled Thelycal Proteins to the Surface of Spermatophore Sperm

We mimicked the adsorption of thelycal components onto the sperm surface by incubating S-sperm *in vitro* with Alex-488 conjugated thelycal proteins. The results in Figure 2 revealed an intense-ring-like fluorescent staining around the entire sperm as well as on the anterior spikes (panels a and c). Interestingly, the longer exposure time to thelycal proteins, the higher number of the positively stained sperm detected. The percentages of the stained sperm after 6-, 12- and 24-hr incubation were 25, 40 and 95%, respectively). Moreover, the staining intensity of an individual sperm in all samples studied was also variable from sperm to sperm (panels a and c, arrow heads). These results implicated a time-dependent adsorption of thelycal proteins onto the surface of S-sperm. When an excess amount of non-labeled thelycal proteins were added to interfere the binding of labeled proteins in 24-hr-incubating group, a minimal binding of labeled thelycal proteins was achieved (panel e). Control sperm exposed to labeled BSA showed only background staining (data not shown). In addition, T-sperm exposed to labeled

thelycal proteins did not show positive staining suggesting that these sperm were saturatedly bathed with thelycal components.

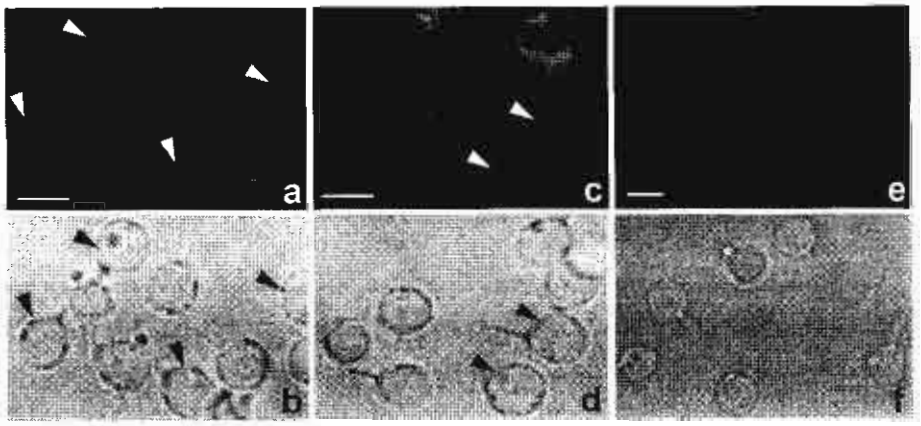


Figure 2 *In vitro* adsorption of Alexa-488 labeled thelycal proteins onto S-sperm surface. Note the increasing number of heterogeneously stained sperm population after 12 hr (a) and 24 hr (b) incubation period. Arrow heads indicated unstained (a) or lightly stained (c) sperm. A background staining was observed when the ten-fold non-labeled proteins were added to interfere the binding (e). a, c and e are fluorescent micrographs: b, d and f are corresponding phase contrast micrographs. Bars = 10 μm.

3. Sperm Peripheral Proteins and Lipids Served as the Targets for Thelycal Protein Anchoring

Dot blot analysis in Figure 3A revealed that S-sperm NaCl extracts (S_{Na}: represented peripheral proteins) had the highest adsorption activity with labeled thelycal proteins whereas the delipidated detergent extracts (S_{De}-delipid: represented pure integral proteins) showed only background adsorption. Sperm detergent extracts (S_{De}) containing both membrane lipids and integral proteins revealed a moderate adsorption activity with thelycal proteins. Control where biotinylated BSA was used in place of labeled thelycal proteins showed minimal adsorption. A complete inhibition of labeled thelycal proteins adsorbed onto sperm peripheral proteins were demonstrated when an excess amount of non-labeled proteins was added to compete the binding (Figure 3B, lower panel). It is therefore implicated from these results that the major extent of thelycal proteins were selectively directed to sperm peripheral proteins and a minor extent to membrane lipids of S-sperm, but not interacted with integral proteins.

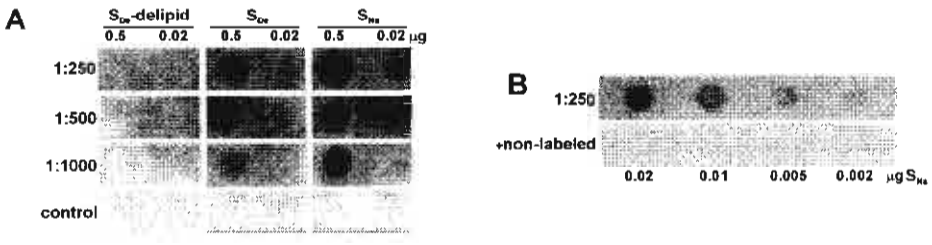


Figure 3 Dot blot analysis of thycal protein binding to the sperm membrane acceptors. A: S-sperm membrane protein extracted by NaCl (S_{Na}) or Triton X-114 with and without delipidation (S_{De}-delipid and S_{De}, respectively) were dotted onto nitrocellulose membrane and exposed to various concentrations (1:250 to 1:1,000) of biotinylated thelycal proteins. Control was those exposed to biotinylated BSA in place of labeled thelycal proteins. B: NaCl extracts of S-sperm incubated with 1:250 labeled thelycal proteins with the present (lower panel) or absence (upper panel) of non-labeled thelycal proteins.

4. Specific Thelycal Proteins Adsorbed onto Sperm Membrane Acceptors

Labeled thelycal proteins stripped from the whole sperm by SDS-PAGE loading buffer which represent the binding to both peripheral proteins and membrane lipids revealed three distinct protein bands, i.e., 60, 83 and 140 kDa (Figure 4, lane 2). Alternatively, thelycal proteins stripping from the entire sperm using 1 M NaCl in PBS which extracted all peripheral proteins revealed the 83 and 140 kDa thelycal proteins (Figure 4, lane 3). Furthermore, there appeared the 60 kDa and with the lesser extent of 53/55 kDa thelycal protein that bound specifically to membrane lipids (Figure 4, lane 4). Control sperm incubated in SW instead of labeled thelycal proteins showed background staining (Figure 4, lane 1).

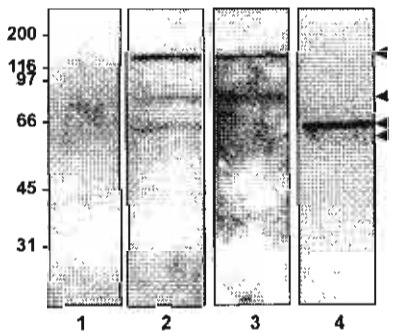


Figure 4 Specific thelycal proteins anchored to S-sperm peripheral proteins or lipids. S-sperm incubated with biotinylated thelycal proteins were treated with SDS-PAGE loading buffer (lane 2) or 1 M NaCl in PBS (lane 3). The sperm extracted lipids were coated onto microwell plates and subsequently incubated with labeled thelycal proteins (lane 4). S-sperm of which labeled thelycal proteins were omittedserve as negative control (lane 1).

5. Sequential Increase of Sperm Protein Tyrosine Phosphorylation in Relevance to the Duration of Thelycal Residence

A relatively low level of protein tyrosine phophorylation could be detected in the sperm directly isolated from spermatophore (comparable to Day 0) (Figure 5, lane 1). The step-wise increase of protein tyrosine phosphorylation became apparent in the sperm taken from Day 1 and 2 post-AI and its level was relatively constant in Day 3 post-AI (Figure 5, lanes 1-3). A drastic increase of 72 and 83 kDa phosphorylated proteins was pronounced in Day 2 and Day 3 post-AI, while proteins with the M_r ranging in between 45-60 kDa also showed higher phophorylated level when compared to Day 0 and Day 1 post-AI (Figure 5, arrow heads). One could postulate from these results that protein tyrosine phosphorylation may have a significant role in regulating *P. monodon* sperm fertilizing capacity as in the case of mammalian models.

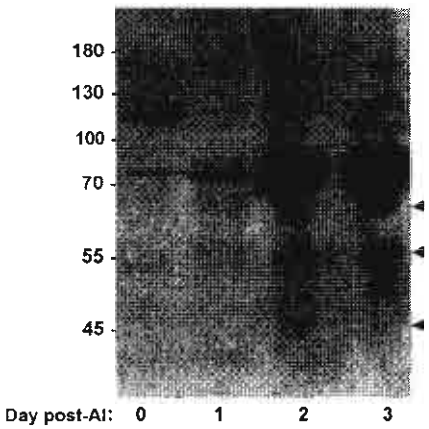


Figure 5 Increase of protein tyrosine phosphorylation in relevance to the duration of thelycal residence. Spermatophores from males were artificially inseminated into thelycums of molting females in the periods of 1, 2 and 3 days. The level of phosphorylation was detected in the total proteins of whole sperm by a monoclonal anti-tyrosine phosphate antibody. Proteins of S-sperm were also loaded as a Day 0 before insemination for comparison.

6. An Enhanced Response of Sperm Acrosome Reaction in Various Timings of Thelycal Storage

Since sperm of *P. monodon* are immotile, we measured an ability of sperm to undergo an EW-induced AR as the only physiological endpoint. When Day 1, 2 and 3 post-AI sperm were treated with 40 μg/ml EW, the percentage of the AR response were 23 ± 5.7, 55 ± 2.6 and 91 ± 4.6%, respectively, while that of S-sperm (Day 0) was 13 ± 1.8% (Figure 6, black bars). At 100 μg/ml EW, there was a slight increase in AR response when

compared to 40 μg/ml in Day 1 and 3 post-AI. The significant difference ($p < 0.01$) of the AR response between 100 μg/ml EW (75 ± 6.2) and 40 μg/ml EW (55 ± 2.6) could be observed at Day 2 post-AI (Figure 6, *). It is therefore suggestive that S-sperm had to be stored in female thelycum for at least 3 days in order to gain a full response of sperm AR, thus fertilizing capacity.

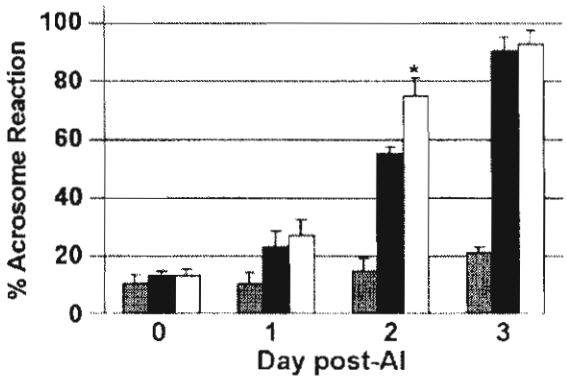


Figure 6 An enhanced ability of inseminated sperm to undergo the acrosome reaction. Day 0-3 post-AI sperm were tested for their ability to undergoAR with 40 μg/ml (black bars) and 100 μg/ml (white bars) egg water or without egg water (grey bars). * denoted significant difference ($p < 0.01$).

II. Induction of Sperm Acrosome Reaction in *P. monodon*

EXPERIMENTAL APPROACHES:

1. Collection of Egg Water (EW) and Sperm

Collection of EW was carried out at BAFCO farm, Nakhonsrithammarat. Mature female shrimp was kept individually in plastic tank and allowed to spawn the eggs into a small volume (~500 ml) of sea water (SW). After swirling, the eggs were allowed to settle and sea water without spawned eggs was collected and centrifuged at 10,000 g for 15 min to remove particulates from the suspension. The protein concentration of EW was determined by Bradford protein assay at 595 nm. EW was stored in –80°C until use.

Sperm were physically isolated from thelycums of naturally caught female shrimps maintained at Department of Aquatic Science, Faculty of Science, Burapha University.

2. Induction and Assessment of AR

In vitro induction of AR was designed in order to rule out whether proteolytic activity in EW and the extracellular Ca^{2+} are involved in this event. Two groups of tested agents used in this experiment included:

1. Agents for testing the role of proteolytic activity

1.1) 1 - 64 μg/ml EW

1.2) 4 μg/ml EW + 2 mM phenylmethylsulfonyl fluoride (PMSF)

1.3) 4 μg/ml EW + 2 mM ethylenediaminetetraacetic acid (EDTA)

1.4) 0.1 - 1% trypsin in SW

1.5) 0.1 - 1% thrombin in SW

1.6) SW (control)

2. Agents for testing the role of extracellular Ca^{2+}

2.1) 4 μg/ml EW in Ca^{2+}-free artificial sea water

2.2) Ca^{2+} ionophore A23187

2.3) 0.1% dimethylsulfoxide (DMSO) (control for A23187)

After 5-min induction, sperm were fixed with 4% paraformaldehyde to stop the reactivity and the percentage of unreacted (having long spike) and reacted (having no spike) sperm were scored with phase contrast microscope. Approximately, 200 sperm were counted for each data point.

RESULTS:

EW has been known to contain components that serve as natural inducers of the acrosome reaction in most of marine invertebrates including crustaceans. However, specific component(s) that is (are) responsible for AR induction are still poorly understood and need further investigation. In some penaeoid shrimps such as *S. ingentis*, the mechanisms underlying two phases of AR are dependent upon extracellular Ca^{2+} influx (for the first phase) and trypsin-like proteolytic activity (for the second phase). In this report, we tested the ability of sperm to undergo AR induced by natural inducer (EW) with or without protease inhibitors and by the condition with and without Ca^{2+} influx in *P. monodon*.

EW collected from a single-spawning into 500 ml sea water contains 64 μg/ml of total protein concentration, and was used directly in this experiment. As expected, the ability of sperm to undergo AR induced by EW is concentration dependent. The AR response of 54.9 ± 15.7 and 76.1 ± 11.0% were observed when 1 and 4 μg/ml of EW was used, respectively (Figure 7). At 16 μg/ml, EW was able to induce AR to reach the maximum response of ~ 91% and the plateau of response was maintained at higher concentration of EW (Figure 14). Approximately 20% of sperm had undergone spontaneous AR when incubated in sea water alone (control). This result suggested that

EW derived from spawned eggs is composed of materials or factors that could induce AR. These materials/factors could be parts of egg extracellular matrice, namely cortical rods, or parts of vitelline envelope (VE).

In order to observe the sudden changes (both increase and decrease) of AR response, the concentration of 4 μg/ml of EW was selected for testing the involvement of proteolytic activity and Ca^{2+} influx in AR induction. Two types of protease inhibitors, i.e. serine protease inhibitor (PMSF), and metalloprotease inhibitor (EDTA) were preincubated with 4 μg/ml EW prior to sperm treatment. Interestingly, both PMSF and EDTA at the concentration of 2 mM were able to drastically inhibited the sperm response to EW to the background level (~ 20%) as that of control (Figure 8A and B), suggesting the involvement of both serine- and metallo-proteases in the process of AR induction. However, purified 0.1 - 1% trypsin and thrombin could not mimic the ability of natural AR inducer as shown by the percent AR response close to the baseline level (Figure 9A and B). The explanation behind this observation could be the prerequisite initial binding of some molecules in EW with sperm surface may be needed to trigger the onset of enzymatic activity present in EW.

To further test if extracellular Ca^{2+} is involved in induction of AR, sperm were incubated in 4 μg/ml EW in Ca^{2+}-free artificial sea water (ASW) or incubated with 30 nM - 100 μM Ca^{2+} ionophore A23187. The percentages of reacted sperm was reduced to the background level when EW was used in the condition where Ca^{2+} is absence from ASW (Figure 10), suggesting that extracellular Ca^{2+} play a role in AR induction. However, this result could be interpreted as the lack of Ca^{2+} influx into the cell or the functional interuption of metalloprotease that may also be involved in AR induction as such a case of serine proteases. The result of sperm treatment with Ca^{2+} ionophore (Figure 18) which showed a background level of AR induction (13 - 30% induction at 30 nM - 100 μM A23187) did not support the former possibility but rather favored the latter one.

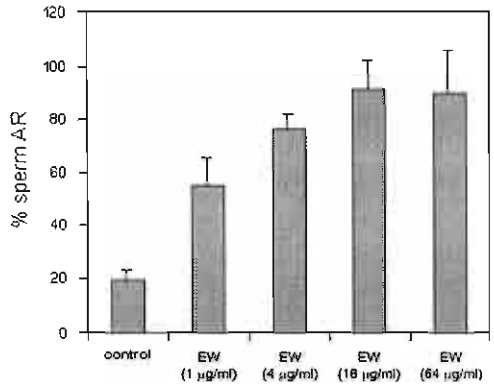
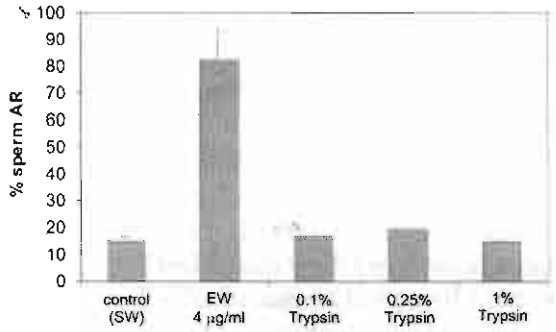


Figure 7 Percentage of AR induced by various concentration of EW



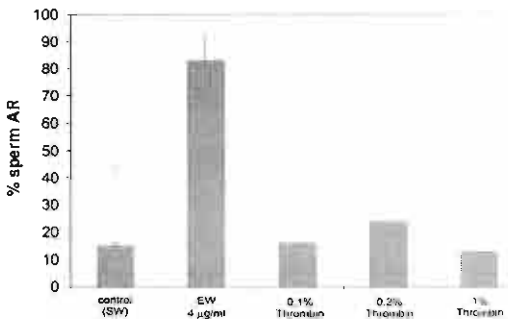
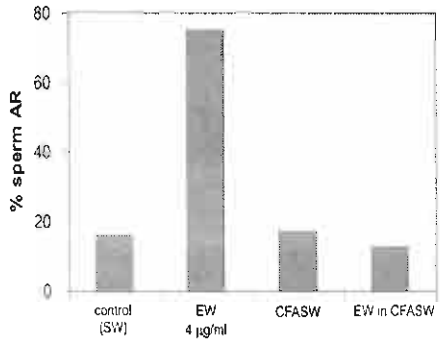


Figure 8 Inhibition of EW-induced AR with PMSF (A) and EDTA (B)

Figure 9 Percentage of AR induced by trypsin (A) and thrombin (B)



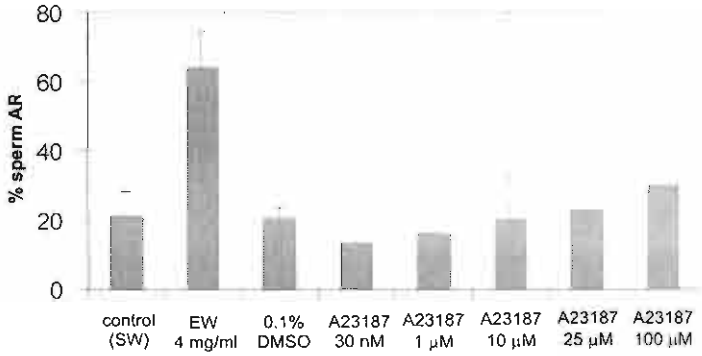


Figure 10 Percentage of AR induced by EW in Ca^{2+}-free ASW

Figure 11 Percentage of AR induced by Ca^{2+} ionophore A23187

11. ผลงานตีพิมพ์ในวารสารนานาชาติ หรือ ประชุมวิชาการ

International Publications

1. Vanichviriyakit R, Kruevaisayawan H, **Weerachatyanukul W***, Tawipreeda P, Withyachumnarnkul B, Pratoomchat B, Chavadej J, Sobhon P. Molecular modification of *Penaeus Monodon* sperm in female thelycum and its consequent responses. *Mol Reprod Dev.* 69(3); 356-63: 2004.
2. Kruevaisayawan H, Vanichviriyakit R, **Weerachatyanukul W***, Withyachumnarnkul B, Basak A, Tanphaichitr N, Sobhon P. Binding Components in Egg Water and Sperm Derived Trypsin Are Essential for Induction of Sperm Acrosome Reaction in Black Tiger Shrimp, *Penaeus monodon. Revised and submitting to Biol Reprod.*

Abstracts in the International Meetings

1. Kruevaisayawan H, Vanichviriyakit R, Murphy M, Hennebold J, Basak A, **Weerachartyanukul W**, Sobhon P, Tanphaichitr N. Characterization of trypsin-like proteolytic activity from the thelycum of *Penaeus monodon* female. Poster presentation at The 38th Annual Meeting for The Society for The Study of Reproduction. July 2005. Quebec, Canada.
2. Vanichviriyakit R, Kruevaisayawan H, **Weerachatyanukul W***, Nernsoungnern P, Tawipreeda P, Withyachumnarnkul B, Sretarugsa P, Chavadej J, Sobhon P Production and changes of intra- and post-testicular sperm of Penaeus monodon. Poster presentation at The 8th Asia-Pacific Conference on Electron Microscopy (8APEM). June 2004. Kanazawa, Japan. pp 55.

MOLECULAR REPRODUCTION AND DEVELOPMENT 69:356–363 (2004)

Molecular Modification of *Penaeus monodon* Sperm in Female Thelycum and Its Consequent Responses

RAPEEPUN VANICHVIRIYAKIT,[1] HATHAIRAT KRUEVAISAYAWAN,[1]
WATTANA WEERACHATYANUKUL,[1]* PATTIRA TAWIPREEDA,[1,2]
BOONSIRM WITHYACHUMNARNKUL,[1,2] BOONYARATH PRATOOMCHAT,[3]
JITTIPAN CHAVADEJ,[1] AND PRASERT SOBHON[1]

[1]*Department of Anatomy, Mahidol University, Bangkok, Thailand*
[2]*Research Center of Excellence (Centex Shrimp), Faculty of Science, Mahidol University, Bangkok, Thailand*
[3]*Department of Aquatic Science, Faculty of Science, Buraphar University, Chonburi, Thailand*

ABSTRACT Using *Penaeus monodon* as the model, we demonstrated the molecular changes and the mechanism of thelycal-dependent sperm modification resulting in an enhanced acrosome reaction (AR) response. Attention was paid to the modification of the sperm plasma membrane which was mediated through an adsorption or removal of sperm peripheral and integral membrane proteins as indicated by the different profiles of these proteins in spermatophore (S) and thelycal (T) sperm. In vitro adsorption of Alexa-488 conjugated T proteins onto the entire S-sperm surface confirmed protein transfer in a time–dependent manner. Specific anchoring of 83 and 140 kDa proteins to sperm peripheral proteins as well as 53/55 and 60 kDa proteins to sperm lipids was demonstrated. Apart from membrane modification, a substantial increase in protein tyrosine phosphorylation was shown to be closely associated with T-dependent sperm modification event. The physiological significance of this sperm modification in enhancing sperm AR response, which required at least 3 days of T residence in order for the sperm to gain a complete AR response, was also elucidated. *Mol. Reprod. Dev. 69: 356–363, 2004.* © 2004 Wiley-Liss, Inc.

Key Words: *Penaeus monodon*; sperm; thelycum; tyrosine phosphorylation; capacitation; acrosome reaction

INTRODUCTION

Upon their synthesis and release from the testis, the sperm of most marine invertebrates, including crustaceans, do not need post-testicular modification to acquire their fertilizing ability. They are readily capable of fertilization after a "broadcasting" release from males into the surrounding seawater where fertilization takes place (Clark and Griffin, 1988; Ohlendieck and Lennarz, 1996; Vacquier, 1998). However, this event does not occur throughout all species of marine invertebrates, particularly in certain species of penaeid shrimp possessing a close-typed-thelycum or sperm receptacle whose function or mechanism is still poorly understood. In *Sicyonia ingentis*, spermatophores (or sperm sacs) from a male shrimp need to be translocated into the female thelycum during mating (Wikramanayake et al., 1992; Clark et al., 1994). Post-insemination changes of the sperm, both morphological and functional [gauged from their ability to undergo the acrosome reaction (AR)], have been addressed (Clark and Griffin, 1988; Wikramanayake et al., 1992). Nevertheless, biochemical data for this sperm modification event are still scarce. Supporting the essence of post-testicular modification in penaeid sperm is very low (~10%) in vitro fertilization rate, compared to that occurs naturally where sperm in the thelycum are released to fertilize the spawned eggs (Clark et al., 1973). In addition, the ability to undergo an egg–water-induced AR, a prerequisite step in fertilization, of spermatophore (S) sperm is far inferior (~1–3%) to that of thelycal (T) sperm (>93%) (Clark et al., 1981).

Sperm molecular modification has been extensively reported in mammals. This process is accomplished through both sperm maturation and capacitation in male and female reproductive tracts, respectively, by which the sperm achieve their fertilizing capacity (Yanagimachi, 1994; Cooper, 1998). Similar information in the sperm of marine invertebrates, however, is less readily available. Attention has been paid to sperm plasma membrane modification. Structural reorganization of sperm surface proteins can happen through an adsorption of epididymal proteins, some of which are relevant to the egg interaction, onto the sperm surface during epididymal maturation (Cooper, 1998; Jones, 1998; Frenette and Sullivan, 2001). It can also be mediated through the removal of nonfunctional or marking molecules, so called "decapacitating" factors,

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*Correspondence to: Wattana Weerachatyanukul, Department of Anatomy, Faculty of Science, Mahidol University, Rama 6 Rd., Payathai, Bangkok, Thailand 10400. E-mail: scwwy@mahidol.ac.th

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o fully expose the egg binding ligands during sperm apacitation (Hunter and Nornes, 1969; Oliphant et al., 1985). Modification and redistribution of sperm membrane lipids, both correlated with egg binding, for example, sulfogalactosylglycerolipid (SGG) in the case of higher mammals (White et al., 2000; Weerachatyanukul et al., 2001), and with regulation of membrane fluidity through the efflux of cholesterol or reorganization of phospholipids (Cross, 1998; Flesch and Gadella, 2000; Manjunath and Therian; 2002) are also well documented. Interestingly, a well defined signal transduction pathway, a cAMP-dependent protein tyrosine phosphorylation, has been reported to be a consequence of this membrane cholesterol removal (Galantino-Homer et al., 1997; Visconti and Kopf, 1998; Visconti et al., 1999). Furthermore, sperm plasma membrane modification during maturation/capacitation also leads to at least two physiological responses, that is, the activation of sperm motility from nonmotile to hypermotile and the enhanced ability of sperm to undergo AR (Yanagimachi, 1994; Suarez, 1996).

In this study, we provided the evidence regarding the molecular modification of *P. monodon* sperm membrane through both peripheral proteins and lipids by female T components. As a consequence, the sequential increases of protein tyrosine phosphorylation coupled with an enhanced response of the sperm AR (the only physiological endpoint that can be monitored in *P. monodon* sperm since they are immotile) are of particular interest, and are suggestive of the significance of the T-dependent modification of *P. monodon* sperm prior to fertilization.

MATERIALS AND METHODS

Sample Collection

Broodstock shrimp were caught from the Andaman sea and maintained at the Department of Aquatic Science, Faculty of Science, Buraphar University, or at BAFCO farm, Nakhon Si Thammaraj, Thailand. Female shrimp were anesthetized under ice and then cut through the thoracoabdominal segment just below the 5th pair of walking legs where thelycum is situated. The gelatinous sperm masses were physically isolated from the dissected thelycums into 0.22-μm-filtered sea water (SW) containing 2 mM PMSF and 1 mM EDTA. Subsequently, these masses were segregated by vigorous shaking and pipetting. The sperm suspension was then filtered through a 212-μm-filter to remove any gross debris. T-sperm in the suspension were pelleted (500*g*, 5 min), leaving T fluid as a supernatant (S1). T-sperm were resuspended in SW at a final concentration of 1×10^7 sperm/ml before use. The S1-supernatant was centrifuged (12,000*g*, 10 min, 4°C) to clean any gross debris and the resulting supernatant (S2), termed "thelycal proteins," was kept frozen until use. Protein concentration of the T proteins was determined by Bradford's protein assay at the wavelength of 595 nm.

Spermatophores, located at the 5th pair of walking legs of male shrimp, were retrieved by the application of an electrostimulation (square wave, frequency = 100 cps, pulse duration = 0.1–0.5 sec, and voltage = 25 mV) to the ventral abdominal wall or by deep compression close to the location of the spermatophores, without sacrificing the shrimp. These spermatophores were processed to obtain sperm, so called S sperm, following the methods described for T-sperm.

Extraction of Sperm Peripheral and Integral Membrane Proteins

Peripheral membrane proteins were extracted according to the method described by Frenette and Sullivan (2001). Briefly, the sperm pellets were resuspended with 3 ml of 1 M NaCl and incubated with gentle agitation (30 min, 4°C). Thereafter, the sperm were pelleted (500*g*, 4°C, 5 min) and the supernatant containing peripheral membrane proteins were collected and cleaned (10,000*g*, 4°C, 10 min). The proteins were concentrated using a MicroCon centrifugation device (Millipore, Bedford, MA) and the protein concentration was determined by Bradford's protein assay. The viability of the sperm after being subjected to high salt treatment was examined by propidium iodide (PI) staining, according to manufacturer's protocol (Molecular Probes, Eugene, OR). The remaining sperm pellets were washed and resuspended in Tris buffered saline (TBS: 10 mM Tris-HCl, 150 mM NaCl, pH 7.4) for further extraction of sperm plasma membrane by a phase separation of Triton X-114, according to the method described by Bordier (1981). In brief, sperm were reconstituted in a final 1% Triton X-114 solution, thoroughly vortexed and centrifuged (5,000*g*, 4°C, 10 min) to get rid of the nuclear and cellular debris. The obtained supernatant was overlaid on top of a sucrose cushion (6% sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.06% Triton X-114) and allowed to stand at room temperature until a cloudiness was observed in the suspension. The gradient was then subjected to low-speed centrifugation (300*g*, room temperature, 3 min). The upper aqueous and lower detergent phases were gently collected and reconstituted in final 1% Triton X-114 and TBS, respectively. Subsequently, the phase separation steps and collection of both phases were repeated. The detergent phase containing mainly integral membrane proteins (Bordier, 1981) was pooled and diluted with TBS before being subjected to protein quantification and SDS–PAGE. A modified method of Lowry's assay using a Dc Protein assay kit (Bio-Rad, Hercules, CA) was applied because of its compatibility with the detergent containing protein samples. The colored reaction product was measured at a wavelength of 750 nm.

Delipidation of Integral Proteins and Protein Profiling by SDS–PAGE

To obtain a pure fraction of integral proteins, contaminated lipids in detergent extracts were eliminated according to the delipidation method described by Mastro and Hall (1999). An ice-cold tri-*n*-butylphosphate:acetone:methanol (1:12:1) was added to the diluted detergent extracts to obtain a final 80% acetone concentration, and incubated at 4°C for 2–3 hr. At this

concentration, the integral proteins were readily precipitated leaving membrane lipids still dissolved in the organic solvent. The protein precipitate was pelleted (2,800*g*, 4°C, 10 min) and the supernatant containing lipids and detergent was discarded. The delipidated proteins were washed with acetone twice and finally resuspended in TBS. Protein concentration was determined by Bradford's protein assay.

Approximately 4 μg of total peripheral and integral membrane proteins isolated from S- and T-sperm as well as T proteins were resolved with 10% SDS–PAGE under a reducing condition (Laemmli, 1970) and further subjected to a silver staining method, as described by the manufacturer (Bio-Rad).

In Vitro Adsorption of Alexa-488 Conjugated Thelycal Proteins to Spermatophore Sperm

T proteins were conjugated to Alexa-488 fluorescent reactive dye (Molecular Probes) or biotinylated (Pierce, Rockford, IL) according to the procedure described by the manufacturers. Approximately, one million S-sperm were fixed with 4% paraformaldehyde for 1 hr and washed (500*g*, 5 min) with SW. Subsequently, the sperm were exposed to 5 mg/ml Alexa-488-conjugated T proteins at various time intervals, that is, 6, 12, and 24 hr, and washed twice (500*g*, 5 min) with SW to eliminate unbound conjugates prior to viewing under a Nikon Eclipse epifluorescent microscope equipped with a Nikon DXM 1200 CCD camera using a set of fluorescein filters (excitation/emission wavelength = 488/520 nm). A competition assay was performed by the addition of 50 mg/ml nonlabeled T proteins (10 fold) to compete with the labeled proteins. S-sperm incubated with Alexa-488- conjugated BSA or T-sperm incubated with Alexa-488-conjugated T proteins served as controls.

In an alternative experiment, aldehyde-fixed S-sperm were exposed to biotinylated T proteins under the same conditions described above. After extensive washing, sperm were treated either with 1 M NaCl in PBS to extract peripheral proteins or with SDS–PAGE loading buffer. They were then subjected to Western streptavidin blotting as described below.

Solid Phase Binding Assay of Labeled Thelycal Proteins With Sperm Membrane

Proteins and lipids. For the sperm protein binding assay, ~2–500 ng of each sample, including NaCl and Triton X-114 (with and without delipidation) protein extracts of S-sperm, was dotted onto a nitrocellulose membrane. The membrane was submersed in 4% BSA in TBS (1 hr, 4°C) to block any nonspecific binding. Then, dotted proteins were incubated (3 hr, 4°C) with biotinylated T proteins at various concentrations (1:250, 1:500, and 1:1,000) and extensively washed with TBS containing 0.1% Tween-20 (TBS-T). Thereafter, the samples were exposed (30 min, 4°C) to horseradish peroxidase (HRP) conjugated streptavidin (KPL, Gaithersburg, MD) (1:2,500) and visualized by means of an enhanced chemiluminescent method using an ECL kit (Amersham Pharmacia, Buckinghamshire, England). The competition assay was performed by the addition of an excessive amount of nonlabeled T proteins (10× that of labeled proteins) to interfere with the binding of labeled proteins.

For the sperm lipid binding assay, total sperm lipids were obtained following the chloroform/methanol extraction method described by Bligh and Dyer (1959). Dried lipids were resuspended in TBS, pH 7.4 and coated overnight onto a 96-well-plate. Unbound lipids were washed extensively with washing buffer (0.15 M PBS, pH 7.2). Nonspecific binding was eliminated with 1% BSA in washing buffer. Subsequently, the samples were incubated (3 hr, room temperature) with 0.1 mg/ml of biotinylated T proteins. After successive washes to remove unbound proteins, SDS–PAGE loading buffer was added to recover bound biotinylated T proteins followed by streptavidin blotting as described below.

Western Streptavidin Blotting

Bound biotinylated T proteins stripped either from whole sperm (by 1 M NaCl in PBS or SDS–PAGE loading buffer) or from micro-wells coated with sperm lipids as described above were resolved with 10% SDS–PAGE under a reducing condition (Laemmli, 1970). Thereafter, the protein samples on the gels were electrotransfered (Towbin et al., 1979) onto a Hybond N-ECL 0.45 μm nitrocellulose membrane (Amersham Pharmacia) and further exposed to streptavidin-HRP (1:2,500). Visualization of the streptavidin–biotin complexes was performed by an enhanced chemiluminescent method using an ECL kit.

Detection of Protein Tyrosine Phosphorylation

Two of the freshly retrieved spermatophores were artificially inseminated (AI) into the thelycum of an individual molting female shrimp. After 1, 2, and 3 days post-AI, the sperm were mechanically isolated and washed, according to the method described above. The total proteins of the whole sperm were quantified by Bradford's protein assay.

Approximately, 3 μg of total proteins taken from the S-sperm (Day 0) and Day 1, 2, and 3 post-AI sperm were resolved by 10% SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was then subjected to Western immunoblotting using a monoclonal anti-protein tyrosine phosphate IgG (clone P-Try-102, Cell Signaling, Beverly, MA) (1:10,000) followed by a goat anti-mouse IgG-HRP (1:2,500) (Zymed, San Francisco, CA). Antigen–antibody complexes were detected by an enhanced chemiluminescent method using an ECL kit. In control experiments, retrieved spermatophores were incubated with SW for 1 day at 4°C and processed likewise as described for experimental samples.

Induction of the Acrosome Reaction of Artificially Inseminated Spermatophore Sperm

To investigate the physiological change in *P. monodon* sperm after translocation into the female thelycum at

different time points, the ability of AI sperm to undergo AR was monitored using a natural inducer, egg water (EW; collected as described previously by Griffin et al. (1987) with some modifications). Shrimp was allowed to spawn into a container containing 500 ml SW. After a gentle shaking for 5 min, eggs were settled at the bottom of the container and the flocculent SW or "egg water" was collected without disturbing the eggs. This EW was centrifuged (10,000*g*, 15 min) to remove any debris and the protein concentration was measured by Bradford protein assay.

S-sperm (Day 0) or Days 1, 2, and 3 post-AI sperm were isolated into filtered SW without protease inhibitors (known to block AR induction). These sperm were treated for 5 min with 40 and 100 μg/ml EW and without EW (control). Thereafter, a final concentration of 4% paraformaldehyde in SW was added to the sperm suspension. The status of the acrosome was checked under a Nikon phase contrast microscope and >200 sperm were counted for each data point. The acrosome intact sperm exhibited a long anterior spike while the acrosome reacted sperm showed a shortened spike. Differences among the samples tested were analysed statistically using ANOVA.

RESULTS

Comparison Between the Protein Profiles of S- and T-Sperm and the Profile of Thelycal Proteins

Profiles of NaCl- and detergent-extracted proteins of S- and T-sperm representing peripheral and integral proteins were compared in Figure 1. For peripheral proteins, the 72, 83, and 140 kDa proteins are more predominant in T-sperm than S-sperm; however, the 25, 45, and 70 kDa appeared to be more pronounced in S-sperm than T-sperm (Lane S_{Na} and T_{Na}, arrow heads). Some differences were also be notable in the integral proteins. A set of fast-mobility proteins with the M_r ranging from 35 to 43 kDa were clearly apparent in S-sperm while they were barely visible in T-sperm. On the other hand, the 53/55, 60, 72, and 83 kDa were more prominent in T-sperm than S-sperm (lane S_{De} and T_{De}, arrow heads). The 72 and 83 kDa proteins might be the traces of peripheral proteins that were abundantly present on the T-sperm surface. These results reflect both the adsorption of additional sperm peripheral and integral proteins as well as the removal of such proteins during sperm membrane modification. T proteins contained major protein bands with the molecular mass ranged from 50 to 200 kDa.

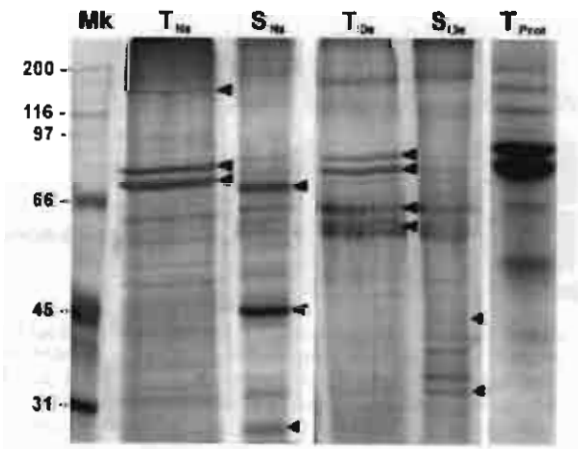

Fig. 1. Protein profiles of membrane extracts from thelycal (T) and spermatophore (S) sperm using NaCl (Na) and Triton X-114 (De) treatment, as well as thelycal proteins. Mk denotes standard markers.

In Vitro Transfer of Labeled Thelycal Proteins to the Surface of Spermatophore Sperm

We mimicked the adsorption of T components onto the sperm surface by incubating S-sperm in vitro with Alex-488 conjugated T proteins. The results in Figure 2 revealed an intense ring-like fluorescent staining around the entire sperm as well as on the anterior spikes (panels a and c). Interestingly, the longer the exposure to T proteins, the higher the number of positively stained sperm detected. The percentages of the stained sperm after 6-, 12-, and 24-hr incubation were 25, 40, and 95%, respectively. Moreover, the staining intensity of an individual sperm in all samples studied also varied from sperm to sperm (panels a and c, arrow heads). These results indicated a time-dependent adsorption of T proteins onto the surface of S-sperm. When an excess amount of nonlabeled T proteins was added to interfere with the binding of labeled proteins in the 24 hr incubating group, a minimal binding of labeled T proteins was achieved (panel e). Control sperm exposed to labeled BSA showed only background staining (data not shown). In addition, T-sperm exposed to labeled T proteins did not show positive staining, suggesting that these sperm were saturated with T components (data not shown).

Sperm Peripheral Proteins and Lipids Served as the Targets for Thelycal Protein Anchoring

Dot blot analysis as shown in Figure 3a revealed that NaCl extracts of S-sperm (S_{Na} represents peripheral proteins) had the highest adsorption activity with labeled T proteins, whereas the delipidated detergent extracts (S_{De}-delipid represents pure integral proteins) showed only background adsorption. Sperm detergent extracts (S_{De}) containing both membrane lipids and integral proteins revealed a moderate adsorption activity with T proteins. The control, where biotinylated BSA was used in place of labeled T proteins, showed minimal adsorption. A complete inhibition of the adsorption of labeled T proteins onto sperm peripheral proteins was demonstrated when an excess amount of nonlabeled proteins was added to compete with the binding (Fig. 3B, lower panel). These results indicate that, to a greater extent, T proteins were selectively directed to sperm peripheral proteins, and, to a lesser extent, to membrane lipids of S-sperm, but they did not interact with the integral proteins.

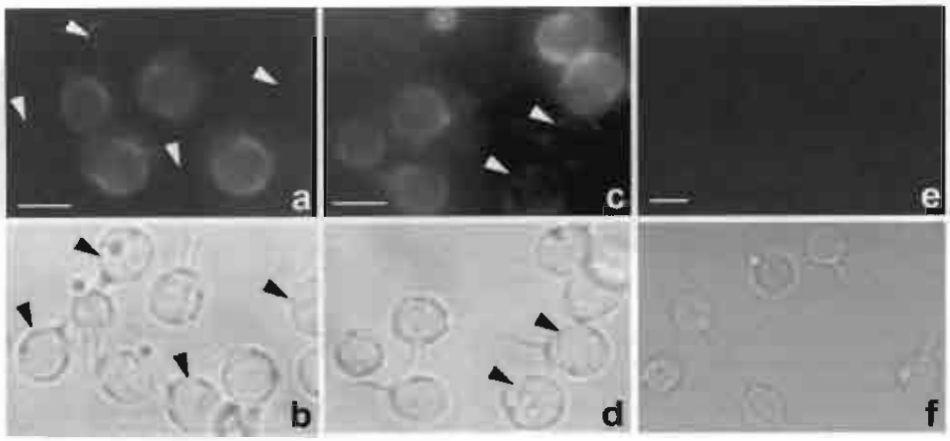

Fig. 2. In vitro adsorption of Alexa-488 labeled T proteins onto S-sperm surface. Note the increasing number of heterogeneously-stained sperm population after 12 hr (**a**) and 24 hr (**c**) incubation period. Arrow heads indicate unstained (a) or lightly stained (c) sperm. Background staining was observed when the 10-fold nonlabeled proteins were added to interfere with the binding (**e**). Above, a, c, and e are fluorescent micrographs; **b, d,** and **f** below are corresponding phase contrast micrographs. Bars = 10 μm.

Specific Thelycal Proteins Adsorbed Onto Sperm Membrane Acceptors

Labeled T proteins stripped from the whole sperm by SDS–PAGE loading buffer (which extracted the entire membrane components) revealed three distinct protein bands, that is, 60, 83, and 140 kDa (Fig. 4, lane 2). Alternatively, T proteins stripped from the entire sperm using 1 M NaCl in PBS (which extracted all peripheral proteins) revealed the 83 and 140 kDa T proteins (Fig. 4, lane 3). Furthermore, it appeared that the 60 kDa and, to the lesser extent, 53/55 kDa T proteins bound specifically to membrane lipids (Fig. 4, lane 4). Control sperm incubated in SW in place of labeled T proteins showed background staining (Fig. 4, lane 1).

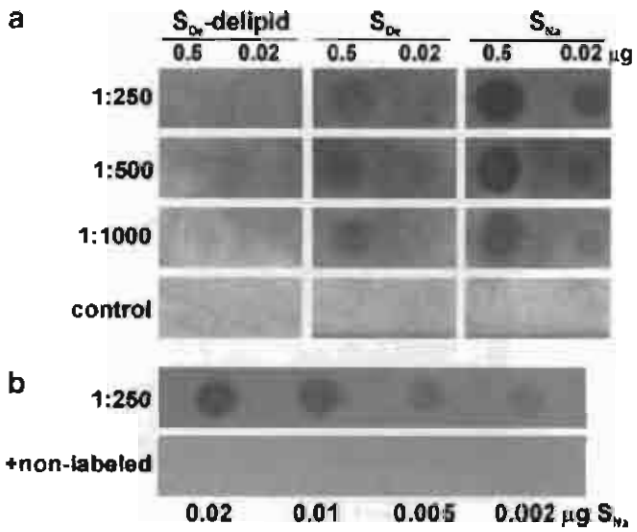

Fig. 3. Dot blot analysis of T protein binding to the sperm membrane acceptors. **a**: S-sperm membrane protein extracted by NaCl (SNa) or Triton X-114 with and without delipidation (SDe-delipid and SDe, respectively) were dotted onto a nitrocellulose membrane and exposed to various concentrations (1:250–1:1,000) of biotinylated T proteins. The control was those exposed to biotinylated BSA in place of labeled T proteins. **b**: NaCl extracts of S-sperm incubated with 1:250 labeled T proteins with the presence (**lower panel**) or absence (**upper panel**) of nonlabeled T proteins.

Sequential Increase of Sperm Protein Tyrosine Phosphorylation in Relation to the Duration of Thelycal Residence

A relatively low level of protein tyrosine phosphorylation was detected in the sperm directly isolated from the spermatophores (comparable to Day 0) (Fig. 5, lane 1). A step-wise increase of protein tyrosine phosphorylation was clearly apparent in the sperm taken from Day 1 and 2 post-AI and its level was relatively constant in Day 3 post-AI (Fig. 5, lanes 1–3). A drastic increase of 72 and 83 kDa phosphorylated proteins was observed on Days 2 and 3 post-AI, while proteins with the M_r ranging between 45 and 60 kDa also showed higher phosphorylated levels, compared to Day 0 and 1 post-AI (Fig. 5, arrow heads). Sperm isolated from spermatophores that were exposed to SW for 1 day revealed a background level of protein tyrosine phosphorylation similar to that of Day 0 sperm. The results suggest the significant role of T components in the sperm modification process, which in turn, results in an increased level of protein tyrosine phosphorylation.

Enhanced Response of Sperm Acrosome Reaction in Various Timings of Thelycal Storage

Since the sperm of *P. monodon* are immotile, we measured the ability of sperm to undergo an EW-induced AR as the only physiological endpoint. When Days 1, 2, and 3 post-AI sperm were treated with 40 μg/ml EW, the percentages of the AR responses were 23 ± 5.7, 55 ± 2.6, and $91 \pm 4.6\%$, respectively, while that of the S-sperm (Day 0) was $13 \pm 1.8\%$ (Fig. 6, solid bars). At 100 μg/ml EW, there was a slight increase in the AR response when

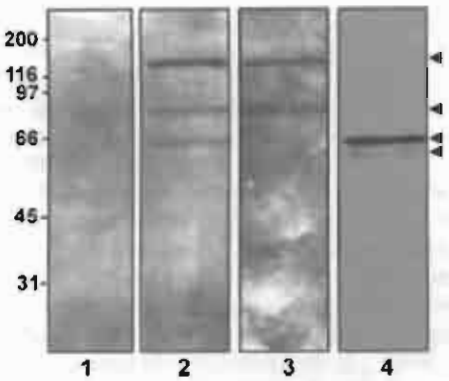

Fig. 4. Specific T proteins anchored to S-sperm peripheral proteins or lipids. S-sperm incubated with biotinylated T proteins were treated with SDS-PAGE loading buffer (**lane 2**) or 1 M NaCl in PBS (**lane 3**). The sperm extracted lipids were coated onto micro-well plate and subsequently incubated with labeled T proteins (**lane 4**). S-sperm from which labeled T proteins were omitted serve as negative control (**lane 1**).

compared to 40 μg/ml on Days 1 and 3 post-AI (Fig. 6, gray bars). A significant difference ($P < 0.01$) in the AR response between 100 μg/ml EW (75 ± 6.2) and 40 μg/ml EW (55 ± 2.6) was observed on Day 2 post-AI (Fig. 6, *). These results suggest that S-sperm have to be stored in the female thelycum for at least 3 days in order to gain a full response of sperm AR, thus increasing the fertilizing capacity.

DISCUSSION

Although a post-testicular modification of the close-thelycum penaeid shrimp has long been suggested to be crucial prior to fertilization (Clark and Griffin, 1988; Wikramanayake et al., 1992), the molecular mechanism

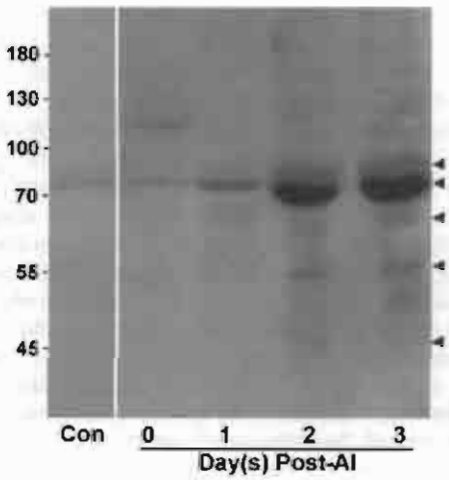

Fig. 5. Increase of protein tyrosine phosphorylation in relation to the duration of T residence. Spermatophores from male shrimp were artificially inseminated into thelycums of molting female shrimp in periods of 1, 2, and 3 days. The level of phosphorylation was detected in the total proteins of whole sperm by a monoclonal anti-tyrosine phosphate antibody. Proteins of S-sperm were also loaded as Day 0 before insemination for comparison. Protein extracts of sperm isolated from spermatophores which were exposed to SW for 1 day served as negative control.

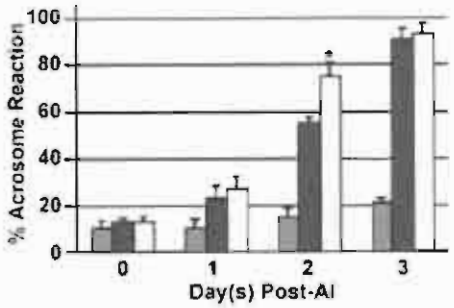

Fig. 6. Enhanced ability of inseminated sperm to undergo the acrosome reaction (AR). Day 0–3 post-artificial insemination (AI) sperm were tested for their ability to undergo AR with 40 μg/ml (solid bars) and 100 μg/ml (empty bars) egg water or without egg water (gray bars). Significant difference ($P < 0.01$) is denoted by *.

of this modification is still poorly understood. In this communication, we thus provided the information of the molecular modification of S-sperm by T components in another close-typed thelycum shrimp, *P. monodon*. The molecular basis of this modification was, to a certain extent, comparable to the mammalian sperm maturation and capacitation process (Yanagimachi, 1994; Cooper, 1998). Our silver staining profiles of both peripheral and integral membrane proteins, which revealed a relative difference between the extracted proteins of S- and T-sperm (Fig. 1), strongly indicated sperm plasma membrane modification. This process could have been accomplished through an adsorption of the T components onto the sperm plasma membrane or a removal of originally existing molecules from the sperm surface. Our following experimental evidence confirmed T protein adsorption: (1) the more pronounced 72, 83, and 140 kDa peripheral proteins, as well as 53/55 and 60 kDa integral proteins that were detected in T-sperm but were sparse in S-sperm; (2) the in vitro adsorption of Alexa-488-labeled T proteins onto the surface of S-sperm, but not T-sperm, which were saturated with T components (Fig. 2). This adsorption was a time-dependent process as indicated by a gradual increase in sperm population positively stained with labeled T proteins as well as the heterogeneity of sperm staining in all experimental groups (Fig. 2, a and c). The removal of certain protein species originally existing on S-sperm, for instance, the 25, 45, and 70 kDa peripheral proteins and 35–43 kDa integral proteins, were also revealed (Fig. 1). Mobilization of the sperm proteins that have less physiological relevance (so called "masking" molecules) in order to expose the buried egg-binding ligands, or the sperm proteins that serve more purposes during fertilization, is also crucial for the sperm to achieve maximum fertilizing ability (Hunter and Nornes, 1969; Oliphant et al., 1985).

The transfer of proteins from an epididymal source towards the sperm surface during mammalian sperm maturation has been extensively reported. One of the most well-studied examples is the adsorption of the epididymal carbonylreductases [i.e., hamster P26h (Legare et al., 1999); bull P25b (Frenette and Sullivan,

2001); monkey P31m (Lamontagne et al., 2001); human P34H (Boue et al., 1996)]. These proteins are transported to the sperm surface via multilamellar lipid vesicles known as prostasomes and become glycosylphosphatidylinositol (GPI)-linked proteins (Legare et al., 1999; Frenette and Sullivan, 2001). Other proteins, on the other hand, have been shown to be peripherally adhered to the existing sperm surface molecules and can thus be dissociated from their anchoring sperm ligands by a highly concentrated salt treatment. Some examples are β-galactosidase conjugated via fructose-6-phosphate (Grimalt et al., 1995), β-glucuronidase via mannose-6-phosphate (Barbieri et al., 1995), and our recently described arylsulfatase-A (AS-A) presumably via an interaction with the sulfated moiety of its existing substrate on the sperm surface, sulfogalactosylglycerolipid (SGG) (Carmona et al., 2002; Weerachatyanukul et al., 2003). Our findings in this article that the adsorbed 83 and 140 kDa T proteins which were extractable from the sperm surface by a high-strength NaCl treatment (Fig. 4) corresponded with the sperm peripheral membrane modification model.

Apart from the modification of peripheral molecules, we also reported here the interaction between the 53/55 and 60 kDa T proteins and the sperm membrane lipid extracts (Figs. 3 and 4), which was a part of the membrane lipid modification. The interaction was presumably mediated via hydrophobic conjugation or via direct incorporation of a prostasome-like carrier, as in the case of the carbonylreductases mentioned above. Modification and reorganization of sperm membrane lipids, particularly during sperm capacitation can drastically influence sperm physiological functions. It has been suggested that a decrease in cholesterol/phospholipid ratio of capacitated sperm up-regulates membrane fluidity, thus facilitating the fusion of the sperm plasma membrane and the outer acrosomal membrane during AR (Davis, 1981; Cross, 1998; Gadella and Harrison, 2000). A massive efflux of cholesterol during sperm capacitation seems to be a key point of membrane fluidity alteration (Wolf and Cardullo, 1991; Benoff, 1993; Cross, 1998). Reorganization of membrane phospholipids via many mediators has also been reported (Gadella et al., 2001; Rathi et al., 2001; Manjunath and Therien, 2002). While albumin, an enriched component of female reproductive tract and its functional agonistic compound, β-cyclodextrin, have been shown to play a crucial role in cholesterol sequestering (Davis et al., 1979; Cross, 1999; Visconti et al., 1999), a series of bovine seminal proteins (BSPs) and a scramblase enzyme have also been demonstrated to interact with phophatidylcholine and nonspecific phospholipid species, respectively, thus removing/reorganizing membrane phospholipids during capacitation (Gadella and Harrison, 2000; Manjunath and Therien, 2002). Cholesterol removal has recently been shown to trigger a cAMP-dependent protein tyrosine phosphorylation, a keynote event marking mammalian sperm capacitation (Galantino-Homer et al., 1997; Visconti and Kopf, 1998; Visconti et al., 1999). Among all marine invertebrates, we have demonstrated here, for the first time, a sequential increase of protein tyrosine phosphorylation during the T residence of shrimp sperm (Fig. 5). In fact, the results also indicate the conservation of this well-defined signal transduction pathway, at least from crustaceans to mammals. Furthermore, the increased response in the sperm AR, which required at least 3 days of T residence to achieve the maximum response (Fig. 6), added more fundamental detail to that previously reported in *S. ingentis* (Griffin et al., 1987). This would conclusively indicate the essence of T-dependent sperm modification as an indispensable process before gamete fertilization in this species.

Sets of experiments are being conducted in our laboratory to explore the molecular mechanism, how the membrane modification initiates a signal transduction and protein tyrosine phosphorylation in the sperm of penaeid species.

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Binding Components in Egg Water and Sperm Derived Trypsin Are Essential for Induction of Sperm Acrosome Reaction in Black Tiger Shrimp, *Penaeus monodon*

Hathairat Kruevaisayawan[1], Rapeepun Vanichviriyakit[1], Wattana Weerachatyanukul[1*], Boonsirm Withyachumnarnkul[1, 2], Ajoy Basak[3], Nongnuj Tanphaichitr[4, 5], Prasert Sobhon[1]

[1]Department of Anatomy and [2]Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Bangkok 10400 Thailand; [3]Diseases of Aging Program and [4]Hormones/Growth/Development Research Group, Ottawa Health Research Institute, Ottawa, Ontario K1Y 4E9 Canada; [5]Department of Biochemistry/Microbiology/Immunology and Department of Obstetrics and Gynecology, University of Ottawa, Ottawa, Ontario K1H 8M5 Canada

*Correspondence should be addressed to Dr. Wattana Weerachatyanukul, Department of Anatomy, Faculty of Science, Mahidol University, Rama 6 Rd., Payathai, Bangkok, Thailand 10400. Fax: (66) 2-354-7168. E-mail: scwwy@mahidol.ac.th

Running title: Sperm acrosome reaction in *P. monodon*
Key words: *P. monodon*, acrosome reaction, egg water, trypsin

Abstract

The acrosome reaction (AR) in the black tiger shrimp, *Penaeus monodon,* is an extremely short process in which the two well-established AR phases as reported in *Sicyonia ingentis* are not distinguishable; only the shortening of the sperm anterior spike in conjunction with the rupture of the acrosomal vesicle is recognized. In this study, we demonstrated that an AR natural inducer, egg water (EW), at 16 μg protein/ml possessed a high competency in inducing (I would rather say "effectively induced " the sperm AR to the maximal level of >90%. Fluorescently labeled EW was shown to bind to the entire sperm surface. EW components that bound to the sperm surface included proteins in the broad range of 26-93 kDa with the 87 and 93 kDa being the major proteins. You should give a logic why you wanted to study the roles of proteases in the AR induction. We further investigated the involvement of proteases in AR induction and the results demonstrated that among various groups of proteases studied, serine proteases in particular trypsin-like enzymes were key mediators in AR induction. Although the trypsin-like activity was detected in both EW and sperm, the trypsin-like enzymes involved in AR induction were not derived from the EW but rather from sperm , presumably from the acrosome (no evidence at all –with this statement, the reviewers may want to tell you to do more work to localize proteases in sperm- I would delete it). This was based on the finding that the AR inducing ability of heat- or APMSF-inactivated EW was the same as that of the untreated EW. Our results indicated the importance of EW binding to sperm in the *P. monodon* AR induction; this binding may then trigger the release of the sperm trypsin-like proteases for spike depolymerization during the AR.

Introduction

In closed thelycum penaeoid shrimp, sperm in the male spermatophores have to be inserted into the female seminal receptacles (called thelycum) before being released with the eggs during spawning [1]. We have shown previously that sperm gain their fertilizing ability during their storage in the thelycum [2]. Unlike sperm of other shrimp species, penaeoid sperm are non-flagellated and immotile [3, 4]. The success of fertilization in the penaeoid shrimp thus relies on two key steps: contact of sperm anterior spike with the egg outer vestment and the acrosome reaction (AR). Generally, the AR process involves a triggered fusion of the sperm plasma membrane with the outer acrosomal membrane leading to the release of the acrosomal contents and the exposure of the inner acrosomal membrane to bind to and fuse with the oolemma. In *Sicyonia ingentis*, AR is shown to be a biphasic process. During the first phase, the anterior spike of the sperm becomes depolymerization upon contacting the vitelline envelope (VE) thus leading to acrosomal exocytosis. The second phase involves the formation of the acrosomal filament, which binds to the egg plasma membrane followed by gametes plasma membrane fusion [4 – 6]. These two distinct events are temporally separated and can be subsequently induced by two different inducers [6]. While the event in the first AR phase is common in all penaeoid sperm, formation of the acrosomal filament during the second AR phase cannot be detected in some penaeoid shrimp including *P. aztecus*, *P. setiferus*, and *P. stylirostris* [7].

In *P. monodon*, information of the AR has not been reported. Our preliminary work has indicated that the AR in *P. monodon* differs drastically from that of *S. ingentis* in both timing and morphological alteration. The AR during natural fertilization of *P. monodon* occurs within a few minutes [8] compared with ~45-60 minutes in *S. ingentis* [9]. This rapid event makes it difficult to distinguish the stepwise morphological changes (if any) during the AR in this species. Structural alterations during the AR in *P. monodon* at the light microscopic level include depolymerization of the anterior spike, in conjunction with the swelling and rupturing of the acrosomal vesicle. The formation of the acrosomal filament during the second AR phase, in *P. monodon* still requires further verification.

Egg water (EW), a flocculent material in seawater that is generated during egg spawning. has been commonly used for in vitro induction of sperm AR in several species, i.e., sea urchin [10, 11], sturgeon [12] and shrimp [9]. Putatively, EW of penaeoid shrimp is made up of

components which are released during spawning including the outmost layer of the eggs, the vitelline envelope (VE), the egg jelly precursor embedded in the egg extracellular crypts, cortical rods (CR), and some thelycal substances. It should be noted that trypsin-like proteolytic activity in EW is important for the acrosomal filament formation during the second AR phase in *S. ingentis*, [6, 13]. The given explanation is that trypsin may hydrolyse enzymes or channels in the inner acrosomal membrane thus activating filament formation [13]. Whether or not these EW enzymes are involved in any steps of the AR induction in *P. monodon* is a question to be answered. In this study, we thus ask whether the EW endows to the AR induction in this species by providing these trypsin-like enzymes, or is it rather involved in the initial part of the AR induction, simply through its binding to the sperm surface? Furthermore, since trypsin-like enzyme is also known to be derived from the sperm source (Ref.), its possible involvement in the AR induction is also investigated.

Materials and Methods

Sample collection

Collection of egg water (EW) was carried out at the Bangkok Aquaculture Farm Company, Nakhon Sri Thammarat province, Thailand. Mature female broodstocks with the mature ovaries (stage IV) were individually placed in a 500-litre plastic tank. The shrimp that have not been inseminated were use in this study in order to avoid the contamination from the sperm factors. The shrimp were held firmly over a 500-ml container and allowed to spawn their eggs into filtered seawater. After swirling gently to settle the eggs to the bottom, seawater without spawned eggs was collected and designated as EW. This EW was centrifuged (10 000 × *g*, 15 min, 4°C) to remove particulates and kept in –80°C until use. Protein concentrations of the EW were determined by Bradford's protein assay [14] using Sigma's Bradfordf reagent (Sigma, St. Louis, MO, USA).

To collect thelycal (T-) sperm , female broodstocks were anesthetized under ice and the thelyca located at the 5th-pair walking legs were carefully removed and placed in a calcium-free artificial seawater (CFASW: 423 mM NaCl, 9 mM KCl, 23 mM Mg Cl_2, 9.3 mM $MgSO_4$, 2.1 mM $NaHCO_3$, pH 7.8). Subsequently, the sperm masses and contents inside the thelycum were physically isolated using dissecting forceps. T-sperm suspension was filtered through a 212-μm-

metal sieve (Endecotts, London, UK) to remove aggregates, washed (500 × *g*, 5 min) and resuspended in CFASW at the final concentration of 1×10^7 sperm/ml before use. T-fluid was also collected from female shrimp without spermatophore insemination following the physical method described above until achieving T-sperm suspension. This sperm suspension was centrifuged (500 × *g*, 5 min) to remove sperm and re-centrifuged (12 000 × *g*, 10 min, 4°C) to remove any small particulates, checked protein concentration and kept frozen until use.

Cortical Rods (CR) were isolated from mature ovaries according to the method described by Lynn and Clark (1987). Briefly, pieces of ovaries were homogenized in an isolation medium (IM; 500 mM NaCl, 9 mM $CaCl_2$, 14 mM KCl, 15 mM $MgCl_2$, and 10 m*M* Tris, pH 7.6, containing 30% sucrose) and subjected to centrifugation (1 000 × *g*, 4°C, 5 min). The pellet was resuspended in IM, and the suspension was overlaid onto a 60% sucrose in IM and centrifuged (8 000 × *g*, 4°C, 60 min). The white pellet containing mainly CR was washed four times with IM and used immediately or stored at –80°C until use.

Induction of sperm acrosome reaction (AR) by EW

Isolated T-sperm in CFASW were pelleted and resuspended in ASW. The sperm were then treated with EW in the concentration-dependent manner (1-64 μg/ml) for 5 min or with 16 μg/ml EW in the time-dependent manner (0 – 600 sec) to induce AR. Treated sperm were fixed with 4% paraformaldehyde and the percentage of unreacted (with an intact long anterior spike) and reacted (without the anterior spike) sperm were scored under a phase contrast microscope. The percentage of spontaneous AR was assessed from the sperm treated with ASW. Approximately 200 sperm were counted for each data point. Each experiment was repeated at least three times using different sperm samples.

Electron microscopy of acrosome intact and reacted sperm

Sperm were fixed with 2% glutaraldehyde and 4% paraformaledehyde in artificial seawater, pH 7.8 (2 hr, 4°C). They were then postfixed in 1% OsO_4 in ASW, dehydrated in an increasing percentages of cold ethanol, and finally embedded in Spurr's resin (EMS, USA). Thin sections (~70 nm) were mounted on formvar coated copper grids and counterstained with lead citrate and uranyl acetate before viewing under a Philips Tecni-200 transmission electron microscope at 80 kV.

Binding of fluorescently labeled EW to the sperm surface

EW was conjugated to a Alexa-488 fluorescent reactive dye (Molecular Probes, Eugene, OR, USA) according to the manufacturer's procedures. Since EW initiated AR in an extremely short timing (see Fig. 1C), we used aldehyde fixed acrosome intact sperm for the EW-sperm binding experiments. T-sperm collected in CFASW were fixed with 4% paraformaldehyde in ASW (1 hr, room temperature). Fixed sperm were washed twice with ASW and blocked with 4% bovine serum albumin (BSA) containing 0.1 M glycine in ASW (30 min, room temperature). The sperm were incubated (15 min, room temperature) with 16 μg/ml fluorescently labeled EW, washed twice (500 × *g*, 10 min) to eliminate unbound EW, plated on a slide and flattened with a coverslip before being observed under a Nikon Eclipse epifluorescent microscope equipped with a Nikon DXM 1200 CCD camera. T-sperm incubated with Alexa-488 EW in the presence of a 50-fold non-labeled EW to compete with the binding of fluorescent EW were also performed.

Determination of EW components that bound to the sperm surface

To characterize the EW components that bound to the surface of the acrosome intact sperm, ~7 million fixed T-sperm (prepared and blocked as described above) were incubated (15 min, room temperature) with various concentrations (4-32 μg/ml) of biotinylated EW, prepared according to the manufacturer's procedure (Sigma). Subsequently, sperm were washed twice, lysed with sample buffer, resolved by 10% SDS-PAGE and electrotransfered onto a Hybond N-ECL 0.45 μm nitrocellulose membrane (Amersham Bioscience, UK). The membrane was submersed in 5% skimmed milk in TBS and further exposed to streptavidin-HRP (1: 10,000, 1 hr, roomtemperature). The streptavidin-biotin complexes were detected by an enhanced chemiluminescent method using an ECL detection kit (ECL, Amersham Bioscience). Moreover, the addition of an excess amount (50-fold) of non-labeled EW to compete with Alexa-488 EW was also performed.

Effects of proteases on the EW-induced acrosome reaction

To test whether AR induction was dependent on protease activities, various concentrations of protease inhibitors including 1) serine protease inhibitors: phenylmethylsulfonyl fluoride (PMSF; 0.1 – 1 mM), amidinophenylmethanesulfonyl fluoride

(APMSF; 0.2 – 1 mM), soybean trypsin inhibitor (SBTI; 0.1 – 0.4 mM), 2) cysteine protease inhibitor: E-64 (1 – 100 μM), 3) aspartic protease inhibitor: pepstatin A (1 10 μM) and 4) metalloprotease inhibitor: EGTA (0.25 – 1 mM) were pre-added into EW prior to co-incubation with the T-sperm resuspended in ASW. The percentage of AR response was analyzed according to the aforementioned protocol.

To prove whether EW-derived proteases, especially trypsin-like proteases, were mediators for AR induction, heat-inactivated EW (HI-EW) or APMSF pretreated EW (APMSF-EW) was used in place of native EW to treat sperm. HI-EW was prepared by heating EW at 100°C for 2 hr and kept chilled at -20°C for overnight. The activity of this HI-EW was tested using the trypsin specific fluorogenic substrate (as described below) in parallel with native EW and the percent activity was expressed as the remaining activity in comparison to that of native EW. APMSF-EW was prepared by pre-incubating EW with 0.5 mM APMSF at 4°C for 2 hr. The mixture was washed and concentrated (10 000 × *g*, 15-30 min) through Microcon YM-10 (Millipore, Bedford, MA, USA) to remove unbound APMSF into the flow-through fraction. Native EW washed and concentrated in the similar fashion as APMSF-EW was also conducted as a sham experimental control. These HI-EW, APMSF-EW and sham-EW were used for sperm-ASW treatment in place of native EW and the number of the acrosome reacted sperm was scored as described above.

Furthermore, to differentiate the function of trypsin-like enzyme on the sperm surface and in the acrosome that may attribute to the AR induction, sperm surface enzymes were preblocked with either 0.4 mM SBTI or 1 mM APMSF. The excess inhibitors were washed away (500 × *g*, 5 min) twice from the sperm. Thereafter, trypsin activity on the surface of the live-intact cells was tested in the inhibitor-treated sperm compared to that of non-treated sperm. These sperm samples were also subjected to16 μg/ml EW-ASW treatment. The percentage of AR response was then analysed.

Enzyme assays

The serine protease assay was performed according to the method described by Zimmerman [16] with some modifications. Fluorogenic-4-methylcoumarin-7-amide (MCA) substrates used to determine serine protease activities in EW and T-sperm lysate included Boc-Gln-Ala-Arg-MCA (Peptide Institute, Louisville, KY, USA) for trypsin-like enzymes and Suc-Ala-Ala-Pro-Phe-MCA (Bachem, Torrance, CA, USA) for chymotrypsin-like enzymes. All

assays were performed at room temperature in a final volume of 100 µl in a 96-well flat bottom black plate (Corning Inc., Corning, NY, USA). T-sperm lysate was prepared by thorough vortexing followed by sonication at 100 watts (30 sec, 3 bursts, 4°C). The reaction mixture was comprised of 5 µl of each sample (EW or T-sperm lysate) or ASW (blank), 10 µl of the 0.2 mM substrate solution in dimethyl sulfoxide (DMSO) and 85 µl of buffer A (10 mM $CaCl_2$, 0.001% Triton X-100 in 50 mM Tris-HCl, pH 7.5) for the trypsin assay, or buffer B (10 mM $CaCl_2$ in 50 mM Tris-HCl, pH 8.0) for the chymotrypsin assay. Fluorescent 7-amino-4-methylcoumarin (AMC), the product from the substrate hydrolysis was monitored spectrofluorometrically at various time points using a Spectra Max Gemini XS (Molecular Dynamics, Sunnydale, CA, USA) with excitation/emission wavelengths of 360 and 470 nm, respectively. The enzymatic activity was quantitated using the AMC standard curve. One unit of the enzyme activity is defined as a picomole of AMC released per hr at 25°C. Specific activity of the enzyme is defined as unit activity per µg protein.

Results

Induction of acrosome reaction with EW

Figure 1A displayed the morphology of the acrosome intact and acrosome reacted *P. monodon* sperm. Transmission electron microscopy revealed the presence of an electron-dense materials in the anterior spike and a moderately electron dense materials in the acrosome and subscrosomal region of the acrosome intact sperm (panel a). Following 5 min of the AR induction, the anterior spike completely disappeared along with the rupture of the acrosomal vesicle (panel b). The subacrosomal region of the acrosome reacted sperm then became somewhat larger and more electron dense (panel b). There was no evidence of the formation of the acrosomal filament (as seen in *S. ingentis*) even at the longer incubation time period (>10 min) (data not shown). At the light microscopic level, the disappearance of the anterior spike and the presence of a dark subacrosomal materials in the acrosome reacted sperm were evident, which were thus used as criteria for differentiating the acrosome reacted sperm from the acrosome intact ones in all experiments described below.

The ability of a natural inducer, EW, to induce AR in P. monodon sperm was both concentration- and time-dependent (Fig. 1B and C). The AR levels were 54.9 ± 15.7 and 76.1 ±

11.0% at 1 and 4 μg/ml of EW, respectively. At 16 μg/ml of EW, the induction reached the maximum with the AR level of ~90.1 ± 9.9%. Therefore, the EW concentration at 16 μg/ml was selected for subsequent experiments. When artificial seawater was used in place of EW, approximately 20% of sperm underwent spontaneous AR.

We also investigated the effect of EW at 16 μg/ml on the AR of *P. monodon* sperm as a function of time (Fig. 1C). The result revealed that within 15 sec of the sperm-EW incubation, 71.9 ± 0.9% of sperm underwent AR, whereas 25.0 ± 2.0% of sperm treated with ASW became acrosome reacted. The percentage of sperm AR increased to ~89.3 ± 4.9% at 30 sec incubation time and this percent AR was maintained up to 3 min of the incubation time. The number of acrosome reacted sperm slightly increased at 5 min incubation time (~91.2 ± 2.3%) and remained constant in the longer incubation period (10 min). Sperm exposed to ASW showed a constant level (~25%) of spontaneous AR up to 5 min. The spontaneous AR response was slightly increased to 31.3 ± 5.6% at the 10-min incubation period. We therefore chose the 5-min incubation period for the subsequent experiments.

Binding of EW to the sperm surface

We hypothesized that the AR induction involved the initial binding of EW components to the sperm membrane receptor(s). Our results showed that fluorescently labeled EW bound to the entire sperm surface, with the high fluorescent intensity at the anterior spike (Fig. 2a) in all sperm population. A slight difference in fluorescent intensity was noted among individual sperm. A background level of fluorescent staining was observed when an excess amount of non-labeled EW was included in the sperm-EW coincubates (Fig. 2c), indicating the specific interaction of fluorescently labeled EW to the sperm surface.

To characterize the given EW components that bound to the sperm surface, biotinylated EW was used followed by.Western streptavidin blotting. The results revealed a broad spectrum of EW proteins ranging from ~26 – 98 kDa with the major proteins being 98 and 87 kDa (Fig. 3; arrowheads). The binding of these EW proteins to the sperm surface was concentration-dependent (Fig. 3; lane 1 - 3). The sperm bound EW proteins were drastically diminished when a 50-fold non-labeled EW was included to interfere with the biotinylated EW binding (Fig. 3; lane 4). Sperm incubated with biotinylated BSA revealed a minimal binding (Fig. 3; lane 5).

Serine proteases mediated AR induction in shrimp sperm

We investigated the possible involvement of proteases in *P. monodon* sperm AR induction. Results shown in Fig. 4 indicated that serine proteases inhibitors (PMSF, APMSF and SBTI) significantly inhibited EW-induced AR. Notably, SBTI at 0.4 mM decreased EW-induced AR to only 12% of the control values. PMSF and APMSF at 1 mM also exerted a marked inhibition (75-85%) of the EW-induced AR. Inclusion of EGTA, a specific Ca^{2+} chelator and metalloprotease inhibitor, also gave a high inhibitory effect (~80% inhibition) as serine proteases inhibitors. E-64 and pepstatin, a cysteine and an aspartic protease inhibitor, i.e., respectively, at any concentrations used did not exert a marked inhibitory effect on AR induction. Pepstatin at 10 μM also showed a basal level of inhibition (~27% inhibition).

Existence of trypsin-like enzymes in EW and sperm

Among serine proteases, trypsin and chymotrypsin have been shown to be significant for inducing AR [19 – 22]. We thus focused our attention to investigate the existence and physiological function of these two proteases in *P. monodon*. Results shown in Table 1 indicated that EW and sperm lysates selectively hydrolysed the trypsin-specific substrate (Boc-Gln-Ala-Arg-MCA) with the specific activity of 30.7 and 264.5 units/μg protein, respectively. Chymotrypsin-like activity (testing with Suc-Ala-Ala-Pro-Phe-MCA) was minimal in both EW (0.16 units/μg protein) and sperm lysates (5.36 units/μg protein), both of which were only ~0.5 and 2.0%, respectively, as compared with the trypsin activities.

Further attempt was made to determine the contributing source of trypsin-like activity in two major components of EW, cortical rods (CR) and TF. Apparently, trypsin-like activity in TF (170.97 units/μg protein) was ~70 fold higher than that of isolated CR (2.38 units/μg protein) (Table 1). This result strongly suggested that enzymatic activity detected in EW was chiefly derived from TF rather than CR.

EW trypsin-like enzymes were not involved in the AR induction

EW trypsins have been shown to mediate the AR induction in *S. ingentis* [6]. This prompted us to prove whether this mechanism also held true in *P. monodon* sperm. Activity of trypsin-like enzyme in EW was abolished by either heat-inactivatation (HI-EW) or pretreatment with an irreversible trypsin inhibitor, APMSF (APMSF-EW). The remaining trypsin-like

activities of HI-EW and APMSF-EW were 1.5 and 1.9%, respectively, as compared to that of native EW (Table 1). Surprisingly, treatment of sperm with HI-EW or APMSF-EW revealed relatively similar AR responses (84 and 82%, respectively), compared with sperm treated with native EW (~90%) (Fig. 5). The percentage of spontaneous AR was ~16%. This result indicated that trypsin-like activity involved in AR induction was not derived from EW but rather from the sperm source.

Despite the presence of trypsin in sperm acrosome, the existence of the sperm surface trypsin-like enzyme was also checked on the live isolated sperm using trypsin-specific fluorescent substrate. It was found that a considerable amount of trypsin-like activity (250 Units/sperm) was detected on the live T-sperm (Fig. 6A). In order to differentiate which source of sperm trypsins that is involved in AR induction, surface trypsin activity was thus diminished by pretreatment of sperm with SBTI (impermeable to sperm membrane) prior to the AR induction. Following EW treatment, the percentage of AR sperm in the inhibitor treated group (85%) was comparable to that of untreated group (90%). Taken all together, these experimental evidence allow us to claim the significance of sperm acrosomal trypsin-like enzyme in *P. monodon* sperm AR induction.

Discussion

We reported here the essence of EW binding components and the sperm-derived trypsin-like activity in *P. monodon* sperm AR induction. In spite of an extremely short period of AR in this species, two morphological changes, spike depolymerization and the rupture of the acrosome vesicle (Fig. 1A), were still recognized and would therefore be referred to as a "complete AR" in this study. EW used in this study possessed a high competency in inducing AR (Fig. 1B), consistent to those reported in other penaeoid shrimp [2, 6, 23]. In *S. ingentis*, the egg VE, possessing the primary sperm binding property and initiating the acrosomal exocytosis [24, 25] is expelled during spawning [8] to form part of the EW components. It is, therefore, reasonable to propose that EW components possessing the binding ability to *P. monodon* sperm membrane (Fig. 2 and 3) might be part of the VE components. However, components of CR (egg jelly precursor) that may bind and trigger sperm AR should also be taken into account, as these jelly substances have been extensively demonstrated to be an AR inducer in echinoderms. This

deduction is based upon the comparable ultrastructural feather and biochemical compositions of shrimp's and sea urchins' jelly substances, although their difference in the egg's outer coat arrangement should also be concerned.

We further demonstrated herein the physiological relevance of serine proteases in the AR induction (Fig. 3). In fact, the significance of these proteases in the AR has been documented in *S. ingentis* sperm [6,13]. However, two discrepancies were observed: 1) the proteolytic activity in *S. ingentis* specifically mediated the acrosome filament formation while such morphological change was not notable in *P. monodon*, and 2) proteases involved in the AR induction in *S. ingentis* was derived from EW while those of *P. monodon* was from the sperm acrosome. These discrepancies could thus be considered as the species specific events. Our results also indicated that, between the two serine proteases shown to be significant for inducing AR [19 – 22], trypsin-like enzyme was a key mediator for the AR induction, whereas chymotrypsin-like enzyme had a much less significant role (Fig. 4). This was based on two lines of evidence. First, AR inhibitory levels were comparable between PMSF (inhibiting all serine proteases) and APMSF (inhibiting all serine proteases, except chymotrypsin)(Fig. 4). Second, a negligible levels of chymotrysin activity were detected in both EW and sperm lysates (Table 1). The predominant role of trypsin-like enzymes in the sperm AR induction in *P. monodon* corroborated a number of similar reports in lower invertebrates, e.g., sea urchin [28], ascidian [21], and penaied shrimp [6, 13]. Although the inhibitory effect of EGTA on the sperm AR may partly reflect the involvement of metalloproteases in the AR, however, it could also be due to the effect of EGTA on chelating the extracellular calcium. In fact, many reports indicating the crucial role of the extracellular Ca^{2+} ions and their influx during the AR process have favorably supported this latter possibility [18 need more ref!!!; our unpublished results].

Regarding to the source of trypsin-like enzymes that are involved in the AR induction, we showed herein for the first time that trypsin-like enzymes involved in the AR were not derived from EW but rather from the sperm acrosome. Evidence supported this finding was the comparable ability of the denatured EW (HI-EW) or inhibitor-inactivated EW (APMSF-EW) to induce an AR response as that of native EW (Fig. 5). One of the reasons that may explain this finding was the proteolytic activity detected in EW was chiefly contributed by TF proteases (Table 1). It should be noted that, this protease-rich TF has solely bathed the sperm surface during their thelycal storage without initiating a premature AR. Whether these trypsin-like

enzymes in TF have complexed with the surrounding inhibitors in the thelycum to keep them inactivated as in the case of, or the TF trypsins in EW have different isoforms than those in the sperm acrosome which may render them recognizing different native enzyme substrates remains to be resolved.

Conclusively, we have experimentally demonstrated the significance of EW component binding to the sperm surface and trypsin-like activity in *P. monodon* sperm AR as summarized in Fig. 6. Initially, the components of EW bound specifically to the sperm membrane receptors (Fig. 6A). This ligand-receptor binding would likely induce downstream signaling events, leading to the fusion of sperm plasma membrane and the acrosomal membrane. The trypsin-like contents are then released to the environment and act to depolymerize the sperm anterior spike as observed in the acrosome reacted sperm (Fig. 6B). Whether *P. monodon* sperm in this species undergo an acrosomal filament formation (during the second AR phase) as in *S. ingentis*, or are devoid of this process as in some other penaeoidean shrimp is a matter of our current investigation.

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Figure Legends

Fig. 1 Panel A) Transmission electron micrographs of acrosome intact (a) and reacted (b) sperm after 5 min induction with EW. Insets in each figure represent the corresponding phase contrast micrographs. Increases in the AR were dependent on concentrations (panel B) and incubation time with EW (panel C, square symbol). Triangles in panel C represented the use of ASW in place of EW for sperm incubation. In all AR induction experiments, at least 200 sperm were counted for each data point. Data in this figure and the remainder were expressed as means ± standard deviations from at least three experiments performed on different days.

Fig. 2 Binding of Alexa-488 labeled EW to aldehyde fixed sperm. Sperm were incubated with 16 μg/ml Alexa-488 labeled EW (panels a and b) plus 100x excess on unlabeled EW (panels c and d). Background staining was observed when sperm were exposed only to Alexa-488 (panels e and f). Panels a, c and e = fluorescent micrographs;panels b, d and f = corresponding phase contrast micrographs. Bars = 10 μm.

Fig. 3 Streptavidin blotting showing binding of biotinylated EW to sperm. Aldehyde fixed sperm were incubated with various concentrations of biotinylated EW (lane 1 = 4 μg/ml, lane 2 = 16 μg/ml, lane 3 = 64 μg/ml) and subjected to Western streptavidin blotting. Lanes 4 and 5 = sperm incubated with 16 μg/ml biotinylated EW in the presence of a 50-fold excess of non-labeled EW or hemolymph proteins, respectively. Lane 6 = sperm exposed to streptavidin-HRP only without prior incubation with biotinylated EW, serving as a negative control.

Fig. 4 Effects of several protease inhibitors on the EW-induced AR. Egg water at 16 μg/ml was pretreated with various concentrations of the inhibitors (see Materials and Methods) prior to sperm incubation. Data were expressed as % control. The AR levels obtained from sperm treated with control EW (untreated with protease inhibitors) were assigned as 100%.

Fig. 5 Effects of heat-inactivated EW (HI-EW) and APMSF pretreated EW (APMSF-EW) on the AR induction. HI-EW or APMSF-EW was added to the T-sperm suspension to initiate AR in comparison to native EW. Notably, HI-EW and APMSF-EW were able to induce AR at comparable levels as native EW. Percent spontaneous AR was observed in sperm exposed to ASW.

Fig. 6 Pictorial diagram postulating the temporal events of the AR in *P. monodon*. (A): Components in EW (+) initially first bind to the sperm surface, this may trigger the fusion of the sperm membranes creating porosity of the sperm plasma membrane overlying the acrosome (Ac). This would lead to a leakage of trypsin-like enzymes (•) in the sperm acrosomal vesicle (a funny word to use here). Depolymerization of the anterior spike (AS) may then be induced to occur due by the hydrolytic activity of the released trysin-like enzymes (B). Shortly afterwards, the anterior spike was completely depolymerized along with the rupturing of the entire acrosomal vesicle(C). N = nucleus, SAc = subacromal region.

Table 1. Trypsin- and chymotrypsin-like activities

	Specific Activity (Units/μg Protein)[a]	
	Trypsi n (Boc-Gln-Ala-Arg-MCA)	**Chymotrypsin** (Suc-Ala-Ala-Pro-Phe-MCA)
T-sperm	264.50 ± 61.41 (100%)	5.36 ± 2.59 (2.0%)[b]
EW	30.69 ± 0.47 (100%)	0.16 ± 0.13 (0.5%)[b]
TF	170.97 ± 4.05	N/A
CR	2.38 ± 0.05	N/A
HI-EW	1.5 ± 1.9 (4.9%)[c]	N/A
APMSF-EW	1.9 ± 0.1 (6.2%)[c]	N/A

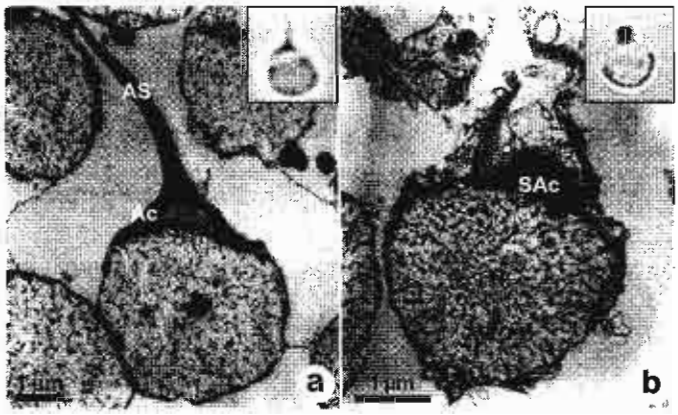
[a] One unit of enzyme activity is defined as picomoles of AMC released per hr at 25°C

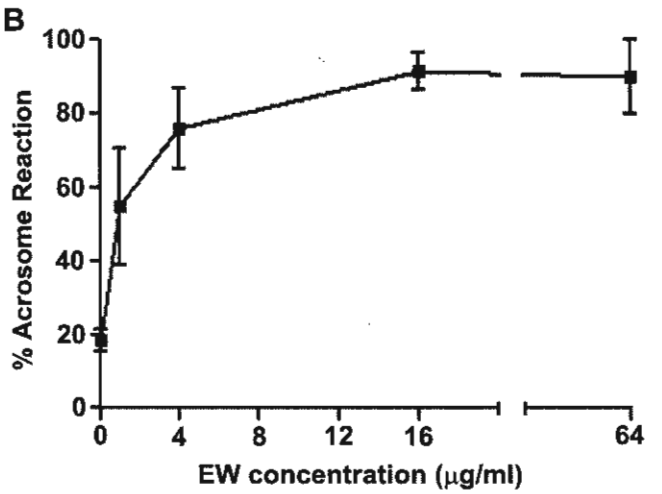
[b] Chymotrypsin activity in comparison to trypsin activity

[c] The remaining activity in comparison to that of native EW

Fig. 1

A


AS
Ac
a
SAc
b


B
% Acrosome Reaction
100
80
60
40
20
0
0
4
8
12
16
64
EW concentration (μg/ml)

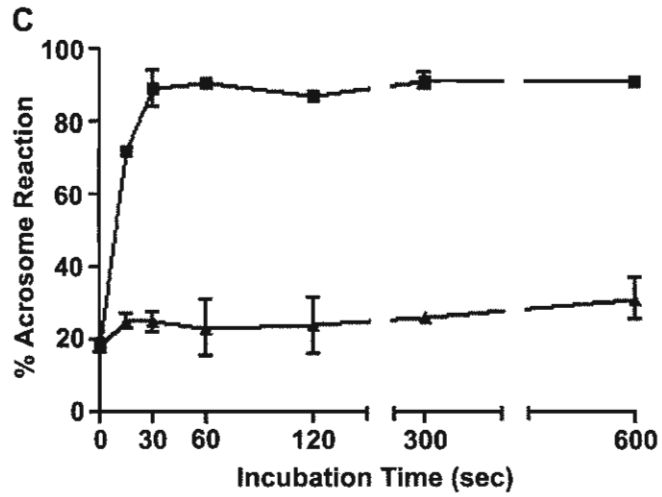

C
% Acrosome Reaction
100
80
60
40
20
0
0
30
60
120
300
600
Incubation Time (sec)

Fig. 2

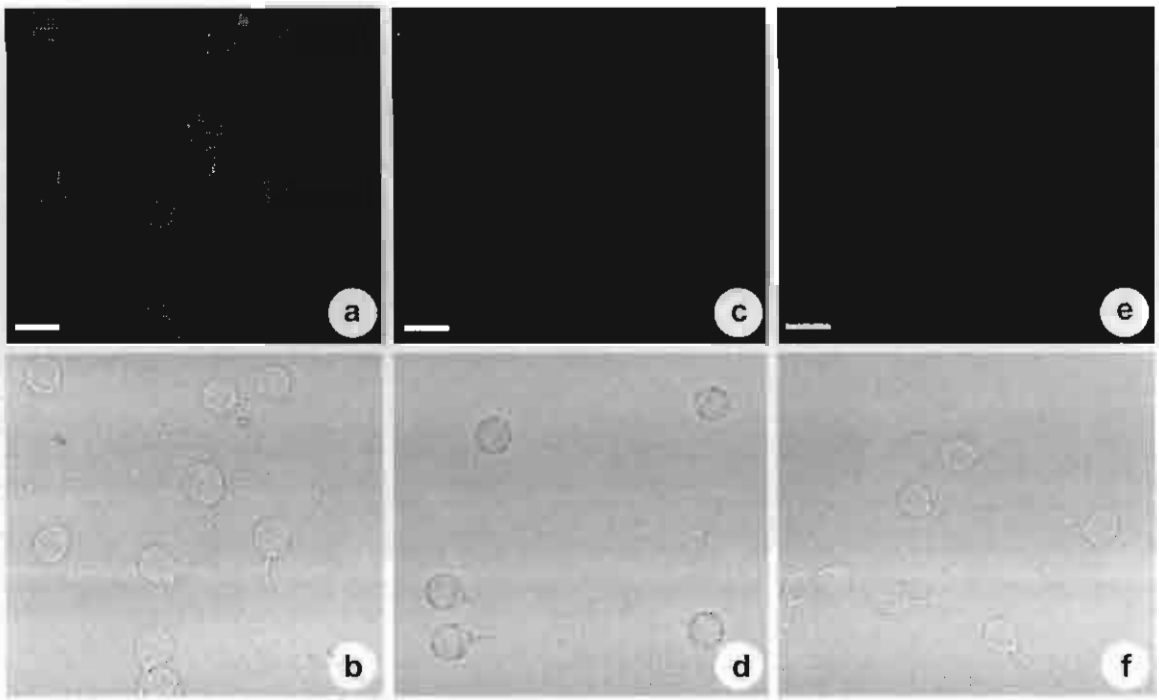


Fig. 3

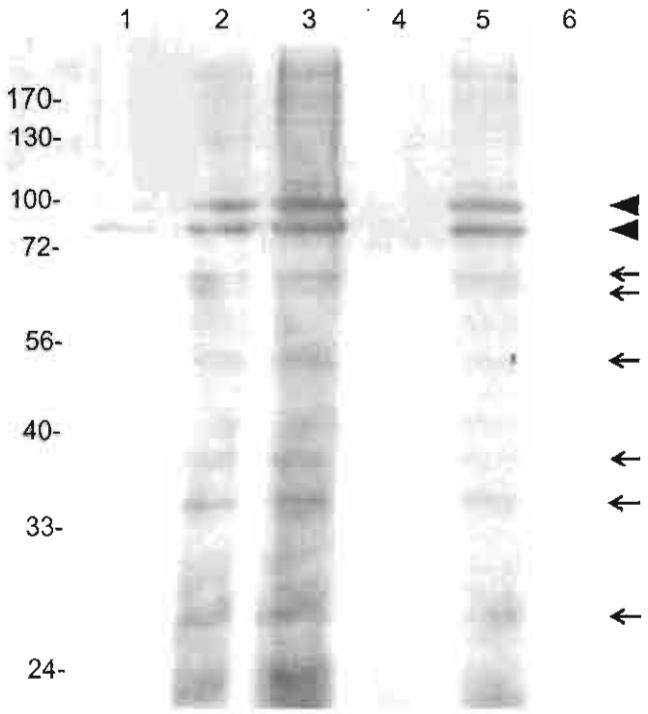


Fig. 4

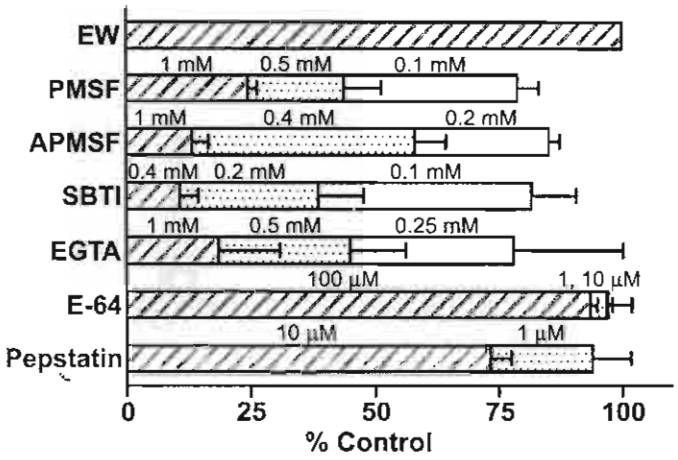

EW
PMSF
1 mM
0.5 mM
0.1 mM
APMSF
1 mM
0.4 mM
0.2 mM
SBTI
0.4 mM
0.2 mM
0.1 mM
EGTA
1 mM
0.5 mM
0.25 mM
E-64
100 µM
1, 10 µM
Pepstatin
10 µM
1 µM
0
25
50
75
100
% Control

Fig. 5

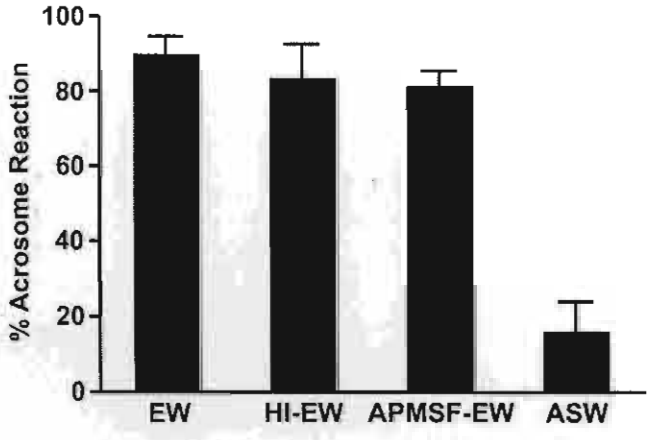

% Acrosome Reaction
100
80
60
40
20
0
EW
HI-EW
APMSF-EW
ASW

Fig. 6