รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาการพัฒนาและการเพิ่มขีดความสามารถของเซลล์อสุจิในกุ้งก้ามกราม

(Study of modifications that are related to maturation and capacitation processes of sperm in the giant fresh water prawn, *Macrabrachium rosenbergii*)

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

Abstract (บทคัดย่อ)

In this study, we aimed to investigate sperm modifications that are related to sperm maturation and capacitation in two shrimp species including the giant freshwater prawn, *Macrobrachium rosenbergii*, and the black tigher shrimp (*Penaeus monodon*). In both species, spermatogenesis takes place in the testis and the testicular sperm are then emptied into male reproductive tract, i.e., vas deferens, and the terminal ampoule containing spermatophores. During mating, the spermatophores are deposited into female thelycum which is sperm storage organ in female. We have shown that modification of *M. rosenbergii* sperm proteins occurred during their storage in male reproductive tract and also in female thelycum. However, sperm protein profiles of sperm taken from the thelycum at 1 h- and 6 h-post insemination were not different, suggesting that modification of sperm proteins in female thelycum may occur rapidly. Moreover, we showed the accumulation of actin in *M. rosenbergii* sperm in the spike and the base of spike of sperm during maturation process, especially when the sperm were stored in the spermatophore. We also showed changes of tyrosine phosphorylation pattern in sperm during translocation from testis into vas deferens, spermatophores and finally into thelycum of both species. The localization pattern of *P. monodon* sperm could be used as one of the markers for sperm capacitation in this shrimp species. Proteolytic activities of sperm were also studied. The results showed that *P. monodon* sperm contained high trypsin-like activities and they gained these activities during storage in female thelycum. The presence of trypsin-like enzymes in sperm of both species was also demonstrated by mean of immunofluorescence staining using soybean trypsin inhibitor. The results suggested the presence of sperm trypsin-like proteases in the subacrosome region of *P. monodon* and the acrosome region of *M. rosenbergii* sperm. The sperm protein modifications and proteolytic activities may be important for sperm maturation and capacitation which is the process that make the sperm ready for fertilization.

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Introduction

Because of increasing domestic and foreign demands, the fresh water prawn *Macrobrachium rosenbergii* is becoming more important economic aquatic animal, and their production has become more depended on the development of efficient aquaculture system. There are many factors that contribute to the successful aquaculture and one way to increase the commercial production of quality prawn is to promote the maturation of viable gametes and to obtain the high quality gametes for production of high quality offsprings. However, it has been shown that spawning success and the number of offsprings produced per spawn are decreased after prolonged captivity of the parent broodstocks. One problem of this decreased fecundity that has not been adequately addressed is the male fertility and sperm quality.

Reproduction of *M. rosenbergii* is different from most other aquatic animals, i.e., male and female mate prior to the female spawns when male deposits its spermatophores on the female abdomen; and then, at the time of spawning, the female releases the eggs to her abdomen and brood fertilized eggs until the eggs hatch as free-swimming larvae in brackish water. It has been shown in the black tiger shrimp *Penaeus monodon*, whose is the close-type thelycum, that the sperm gain their fertilizing ability in the female thelycum and possess high level of sperm protein tyrosine phosphorylation; this process resembles “capacitation” in mammalian sperm. It was also found that *P. monodon* sperm need to reside at least 3 days in thelycum to gain full ability to undergo acrosome reaction which is another marker for sperm capacitation. However, in *M. rosenbergii*, which has the open-type thelycum, little is known about how sperm become functionally developed, and how they achieve fertilizing ability during their production in the testis and transit in male reproductive tract, and while they are in the thelycum. Therefore, in this study, we aimed at investigating the sperm modifications during their production in the testis, transit in spermatic duct and spermatophore, and after their deposit in the female thelycum. Furthermore, as a comparison between close- and open-type thelycum shrimp species, we will determine whether capacitation process occurs in the sperm of *M. rosenbergii* as that happen in *P. monodon*. We also expect to find out some markers denoting sperm with high fertilizing competency which could be applied as markers for selecting high quality male broodstock for aquaculture. Moreover, understanding mechanisms that make the sperm ready to fertilize in this species may lead to the development of an *in vitro* capacitating

method to improve the fertilization and production of larvae for aquaculture of this species.

The project was aimed at investigating sperm modifications during their production in the testis, the transit in the male reproductive tract, and during deposition in female open thelycum which could be used as markers for the sperm maturation and capacitation processes in *M. rosenbergii*. In addition, some biochemical changes in sperm during capacitation in *P. monodon* were also studied.

Literature review

Overview of Reproductive System in Fresh Water Prawn

In crustacean, such as crayfish, the gonads lie dorsally in the thorax between the floor of the pericardial sinus and the hindgut. Their size and appearance depend on the age and reproductive condition of the individuals. In the breeding season, the internal reproductive organs enlarge significantly. The testis shows a milky-white color due to active sperm production whereas the ovary becomes full with yellowish-brown eggs. In crayfish, the testis consists of a paired anterior lobe and elongate unpaired posterior lobe. A highly convoluted spermatic duct or vas deferens arises from each side of the testis and opens at the gonopore on the base of the fifth pereopod. In female, the ovary has trilobes and one straight oviduct that open at the base of the third pereopods. The mature oocytes move down via the gonopores to fertilize during spawning (Holdich, 2002). During fertilization in *M. rosenbergii*, initial attachment to the egg is made through the main body of the sperm with the spike erected. Within a short period, the spike contacts the egg surface and becomes the leading end of the sperm during penetration (Chow and Sandifer, 2001).

Spermatogenesis takes place in testis; testicular sperm are then emptied into vas deferens. Sperm sacs, so called spermatophores, are assembled at the terminal part of the vas deferens. During mating, the spermatophores are transferred to sperm storage organ of female, called thelycum. The spermatophore structure, compositions and complexity are different among species. In general, they are composed of the sperm mass which is embedded in a periodic acid Schiff-positive matrix. The formation and morphology of decapod spermtophores have been described in many species, e.g. *Penaeus setiferus* (King, 1948), *Homarus americanus* (Kooda-Cisco and Talbot, 1982). Upon entry into the vas deferens, the sperm are surrounded by epithelial secretions which aggregate the sperm into a compact mass and form the acellular layers of the spermatophore's wall (Malek and Bawab, 1971). There is variation in the form and complexity of spermatophoric materials being transferred from the male to the female during insemination in penaeoid shrimps (Baurer, 1992). The most complex spermatophores are those attached externally to the genital area, or thelycum, of the female, as in the white shrimp, *Penaeus setiferus*, and other species of the subgenus *Litopenaeus* (Perez Farfante, 1985; Bauer and Cash, 1991; Chow et al., 1991). In contrast, in the rock shrimps, *Sicyonia* species, the spermatophores are simpler and containing only sperm in a seminal fluid; they are transferred to, and stored in the

internal seminal receptacles of the female (Clark et al., 1984; Perez Farfante, 1985; Bauer, 1992). Other penaeoid shrimps, such as *Trachypenaeus* species, show intermediate degrees of spermatophore complexity (Bauer and Min, 1993).

The thelycum of female shrimp is a modification of the sternal plates into protuberances, ridges, grooves or concavities providing for the attachment of the spermatophores (open thelycum), or into an invaginated spermtheca or seminal receptacle (closed thelycum) for sperm storage and protection until the time of spawning (Hudinaga 1942, King 1948, Perez-Farfante 1985). Open thelycum was observed in the subgenus Litopenaeus including *P. setiferus, P. stylirostris*, *P. vannamei, P. occidentalis, and P. schmitti.* The closed thelycum is the characteristics of subgenera Penaeus, Fenneropenaeus, Marcupenaeus, and Melicertus, including *P. monodon, P. japanicus, P. orientalis* and all the other species making up this genus. These differences in thelycal structure make significant differences in shrimp reproductive biology, especially with regards to sperm maturation and capacitation.

Females of the closed thelycum species must mate in the soft-shelled condition immediately following molting. Once the thelycal plates harden sperm transfer cannot take place. Sperm must be kept in the closed thelycum for sometime until the female develops fully mature ovary and then spawns. The sperm undergo capacitation process while being stored in the thelycum (Vanichviriyakit et al., 2004). In the closed thelycum shrimp, sperm from spermatophore can fertilize eggs from several spawning with little or no decrease in the fertilization success (Browdy, 1992). At molting any remaining sperm is lost with the exoskeletons.

The open thelycum shrimp mate in the intermolt hard-shelled condition immediately prior to spawning when ovaries are fully developed (Yano et al., 1988). However, *M. rosenbergii,* which has an open-type thelycum, female is mated in the soft-shelled condition with fully developed ovary. The attached spermatophores are exposed to surrounding water and fertilization takes place as soon as female spawns, and no sperm is retained after spawning of the eggs. Therefore, females of *M. rosenbergii* must mate before each spawning.

Sperm structure of decapod crustacean and cytoskeleton components

Unlike sperm of other animals and most vertebrates, the sperm of decapod crustaceans lack a flagellated tail and mitochondrial midpiece (Talbot and Summers, 1978; Kleve et al., 1980 Pratoomchart et al., 1993). Most decapod sperm are nonmotile and have only the main body with stellate appendages (spikes). In general, this non-

motile sperm comprises of the main body which houses an uncondensed nucleus and a variable number of stellate processes that project from the main body. Decapod sperm have been further categorized as the reptantian type (lobsters, crayfish, and crabs) or natantian type (shrimp) (Talbot and Summer, 1978). The sperm of these two groups differ in the number and structure of their appendages, i.e., reptantian sperm have several stellate appendages (radial arms) which are continuous with the nucleus, in contrast, natantian sperm possess a single appendage (spike) which is not continuous with the nucleus.

The natantians are divided into two sections: the caridea (shrimp that brood their eggs) and the penaeidea (shrimp that are free spawners). Spermatozoa of a penaeid shrimp *Sicyonia ingentis* (Penaeoids: Sigyoniidea) consist of a main body containing the centrally-located uncondensed nucleus, a cap, and a spike (Clark and Griffin, 1988). The cap lies anterior to the main body and contains subacrosomal elements and a portion of the acrosomal vesicle. Spermatozoa of the shrimp *Parapeneus longirostris* (Penaeoids: Penaeoidea) have the typical tack-shaped morphology (Medina, 1994). The main body is composed of the acrosomal cap, the perinuclear cytoplasm and a nucleus containing fibrillar chromatin; no nuclear envelop is observed. The acrosomal complex comprises the acrosomal vesicle, which consists of the spike and acrosomal cap, and the subacrosomal region. The outermost surface of the acrosomal vesicle is bound by a double membrane consisting of the plasma membrane and the membrane of the acrosomal vesicle. *P. monodon* sperm possessed a long anterior spike, acrosome and subacrosomal regions, and the main bodies (Vanichviriyakit, 2007). The acrosome appears as sacs on both side of the anterior spike, which contains hydrolytic enzymes as described in other species including mammalian sperm. The main body contains mainly sperm nucleus, which is filled uniformly with decondensed chromatin fibers.

In *M. rosenbergii,* a sperm appears similar to an inverted umbrella. The sperms are non-motile and nonflagelled, consisting of a 10 μm main body and a single spike extending from the convex surface of the sperm base. However, the acrosomal region is not clearly defined in this specie (Lynn and Clark, 1983). Ultrastructural studies have reported no apparent acrosomal structure in sperm of caridean shrimp (Chow and Sandifer, 2001). In contrast, sperm of other decapod groups, such as Penaeidea, Astacidea, Palinura, Anomura and Brachyura, possess the acrosomal structures which undergo drastic structural changes upon the induction of the acrosomal reaction.

Decapod crustacean sperm showed different characteristic structural components, i.e., appendage(s), a main body, acrosome and subacrosomal region and; in some species, acrosomal filament that is formed after the acrosome reaction (AR). The study in sperm of echinoderm, *Thyone briareus*, showed that profilamentous actin selectively accumulates in the subacrosomal region to form the acrosomal filament during AR (Tilney, 1960; Tilney et al., 1978). Moreover, actins have been reported to be localized in the posterior cortex of the main body of *S. ingentis* sperm surrounding the nucleus (Baldwin et al., 1998). In *P. monodon* sperm, it was shown that the subacrosomal region and the spherical mass of acrosome-reacted sperm were made up of actin (Vanichviriyakit, 2007). Moreover, the evidence obtaining from transmission electron micrography revealed that acrosome-reacted sperm showed the accumulation of the subacrosomal materials to form the spherical mass suggesting that actin components in the subacrosomal region were likely to be the precursor of the spherical mass formed during the AR. In addition, it was found that formation of the subacrosomal region in *P. monodon* sperm is completed while the sperm is stored in the female thelycum (Vanichviriyakit, 2007). These observations suggest the reorganization of actin during sperm maturation and activation.

As state above, cytoskeleton components of sperm, particularly actin, are changed significantly during sperm maturation and activation. Location of certain cytoskeleton components within the sperm could be reorganized during the processes of sperm maturation, capacitation, and activation. Thus, sperm cytoskeleton components may also indicate sperm fertilizing ability.

Sperm Capacitation

In mammals, before mature sperm can fertilize oocyte, it must undergo a cascade of biochemical and physiological changes that facilitates its binding and penetration into the oocyte (Rajesh and Preeti, 2004). This time-dependent acquisition of fertilizing competence has been defined as "capacitation". In mammals, the sperm capacitation normally takes place in female reproductive tract; however, it can also be achieved *in vitro*. During capacitation, plasma membrane of sperm changes significantly by the release, modifications and adsorptions of proteins and lipids. There are many known proteins being adsorbed onto the sperm surface during capacitation (Jones, 1998; Cooper 1998; Frenette and Sullivan, 2001). Structural reorganization of sperm surface proteins can happen through an adsorption of epididymal proteins onto the sperm surface during epididymal maturation (Jones, 1998; Cooper 1998; Frenette and

Sullivan, 2001). It can also be mediated through removal of the non-functional or masking molecules, so called “decapacitating” factors, to fully expose the egg binding ligands during sperm capacitation (Hunter and Nornes 1969; Oliphant et al., 1985).

One of the key molecular events implicated in the initiation of capacitation includes the removal of cholesterol from the sperm plasma membrane leading to the ion fluxes which results in alteration of the sperm membrane potential and increased tyrosine phosphorylation. Consequently, the sperm show hyperactivated motility and undergo acrosomal reaction (Yanaginachi, 1994; Suarez, 1996; Visconti et al., 2002) which is currently defined as the endpoints of the capacitation. An increased protein tyrosine phosphorylation during sperm capacitation has been reported and should also be considered as another critical indicator to predict the sperm capacitation and fertilizing ability (Visconti and Kopf, 1998; Galantino-Homer et al., 1997; Visconti et al., 1999).

Sperm of some invertebrates undergo gross morphological changes during capacitation. For example, the sperm of flies, nematodes and millipedes undergo numerous morphological reorganization after insemination into females. The sperm of the shrimp, *S. ingentis*, have also been reported to undergo morphological changes during storage in the female thelycum, which include 1) the membrane pouches (a part of acrosome region) of sperm taken from the female thelycum appear swollen and more electron lucent than that of the sperm from male, 2) the crenulated nature of the cap region is greatly reduced in the sperm from female, and 3) the spiral configuration of anterior spike seen in sperm from male is absent (Wikramanayake et al., 1992).

Sperm of some invertebrate species require more subtle interactions with female secretions after insemination, as do mammalian sperm, in order to gain a complete fertilizing competence, e.g., sperm of the hydrozoan *Campanularis flexosa* (O’Rand, 1972), the barnacles *Balanus balanoides* (Walley et al., 1971) and *Pollicipes polymerus* (Lewis, 1975). Post-inseminal changes in sperm of these animals are the prerequisite for fertilization, and these changes have been described as “capacitation process”.

In the shrimp *P. monodon*, it has been reported that the sperm undergo both morphological and biochemical changes during storage in female thelycum (Vanichviriyakit et al., 2004; Vanichviriyakit 2007). These changes include: 1) increase of decondensation stage of the sperm chromatin, 2) adsorption and removal of sperm membrane proteins, both at the levels of peripheral and integral membrane proteins, 3) increase of protein tyrosine phosphorylation of sperm proteins after at least 3 days of

storage in female thelycum, 4) enhanced of the ability of sperm to undergo acrosome reaction.

Sperm proteases

In an ascidian, *Halocynthia roretzi,* both trypsin (acrosin)-like and chymotrypsin-like enzyme activities are necessary for sperm penetration of the egg investments (Hoshi et al., 1981). There are two trypsin-like enzymes in ascidian that have been purified and characterized, acrosin and spermosin (Sawasa et al., 1996). Two kinds of chymotrypsin-like enzymes have also isolated and characterized as high-molecular-weight protease complexes, known as proteasome (Saitoh et al., 1993). These protease complexes comprise several different subunits, which show different protease activities.

Both of trypsin-like and chymotrypsin-like enzymes have been reported to be involved in the ascidian fertilization. However, the studies in the ascidian *H. roretzi* and the sea urchin *Hemicentrotus pulcherrimus* have shown that the chymotrypsin-like enzyme rather than the trypsin-like one shows a lytic activity toward the vitelline coat of eggs (Sawasa et al., 1996; Yamada et al., 1982). In sea urchin, *Strongylocentrotus purpuratus*, spermatozoa also contain a 20 kDa protease that retains its activity toward the trypsin substrate (Resing et al., 1985).

The studies in ascidian species have also shown that both proteases and glycosidase, such as fucosidase and *N*-acetylglucosaminidase which are membrane constituents, are involved in sperm binding (Hoshi et al., 1994). In addition, the multicatalytic proteasome, specifically 20S proteasome found in ascidian *Halocynthia,* is involved in sperm-vitelline coat binding (Takizawa et al., 1993).

Chen and co-workers (1994) have found that *S. ingentis* sperm possessed molecules exhibiting two major types of proteolytic activity, trypsin-like and aminopeptidase-like. Furthermore, they have also shown that sperm penetration of the egg vitelline envelope is due to the activity of a sperm trypsin-like activity. The study in *Rhynchocinetes typus* shrimp has shown the presence of 18 kDa trypsin-like enzyme in the sperm extract (Rios and Barros, 1997). The *in vitro* fertilization is inhibited when the sperm are treated with soybean trypsin inhibitor (SBTI), *p*-aminobenzamidine or phenylmethanesulphonyl fluoride (PMSF), suggesting that this trypsin-like enzyme is involved in sperm penetration through the egg-coat. A similar inhibitory effect has been reported during *in vitro* fertilization of ascidian (Hoshi et al., 1981).

Eventhough, the acrosomal region, which is believed to contain hydrolytic enzymes, is not clearly defined in *M. rosenbergii*, sperm enzymatic activities, especially proteases, would be expected to be also necessary and play an important role during fertilization process in this species. Since the sperm are non-motile, sperm proteolytic activity must play even a more important role during fertilization especially in sperm penetration. Thus, sperm protease enzymes may also indicate sperm fertilizing ability and could be used as a marker denoting capacitation.

From the existing data, detailed information regarding sperm maturation and capacitation, which is well-studied in mammalian species and some shrimp species and shown to be a prerequisite for fertilization success is still lacking in *M. rosenbergii*. Therefore, in this study, we aim at investigating modifications of *M. rosenbergii* sperm in testis, male reproductive tract, and after their deposit in the female thelycum which are related to the maturation and capacitation processes. Changes of the sperm were examined in two populations of sperm, i.e., sperm retrieved from male reproductive tract, and from the female thelycum. The parameters studied included sperm protein profile and the level of sperm protein tyrosine phosphorylation, which are the key maker of capacitation. In addition, sperm actin which has been reported to be involved in sperm capacitation and acrosome reaction in many aquatic species was also studied. Moreover, sperm protease activities were also studied in *P. monodon*.

Materials and methods

1. Sample collection and sperm preparation

Sexually mature male giant freshwater prawns, *M. rosenbergii*, were anesthetized on ice for 2-3 minutes and then testis, vas deferens, and spermatophores were dissected out. The gelatinous sperm masses were physically isolated in phosphate buffer saline (PBS), and filtered through 0.22-μm-filter to remove any gross debris. Subsequently, the sperm suspension was centrifuged twice (500 g, 5 min) to wash the sperm. For female prawn, sperm masses that were inseminated to the female thelycum were collected after mating at 1, and 6 h. Then, the sperm were isolated sperm as described for sperm from male prawns.

2. Protein profiling of sperm

This experiment was designed to see if there was any change in sperm proteins during maturation and/or capacitation processes. Sperm proteins from different groups of samples, including sperm taken from testis, vas deferens, spermatophroes, and thelycum (1 h- and 6 h-post insemination), were extracted using lysis buffer containing protease inhibitor cocktails. The protein concentration was determined using Bradford assay. Twenty microgram of sperm proteins were separated on 10% SDS-PAGE under a reducing condition. After electrophoresis, the gels were stained with Coomassie blue solution. The protein profiles of sperm taken from were compared. The sperm samples were collected and pooled together from 5 prawns and used in one experiment; three different groups of samples were performed.

2. Determination of sperm protein tyrosine phosphorylation and actin by Western blot analysis

In order to determine capacitation status of sperm after insemination into female thelycum and change of sperm actin, Western blot analysis was performed using antibody to protein tyrosine phosphorylation and actin, respectively. Twenty microgram of sperm proteins were separated on 10% SDS-PAGE under a reducing condition. After electrophoresis, sperm proteins were transferred onto nitrocellulose membranes. The membranes were blocked non-specific binding by incubating with 2% BSA and 3% skim milk. After that, the membranes were incubated with 1) a monoclonal anti-protein tyrosine phosphate IgG (clone P-Try-102: Cell Signaling, Beverly, MA, USA) followed by

a goat anti-mouse IgG-HRP (Zymed Labolatories Inc.) 2) rabbit anti-actin antibody (amino residues 20-33, Sigma) followed by a goat anti-rabbit IgG-HRP (Zymed Labolatories Inc.). Antigen-antibody complexes were detected by an enhanced chemiluminescent method using an ECL kit.

3. Immunostaining of protein tyrosine phosphorylation and actin in sperm

In order to study the organization of actin in sperm taken from different locations (testis, vas deferens, spermatophore, and thelycum) and pattern of sperm protein tyrosine phosphorylation, isolated sperm were fixed with 2% paraformaldehyde in PBS for 1 h at room temperature. Then, the sperm were washed with PBS by centrifugation for 3 times. The samples were incubated with 2% BSA and 5% normal goat serum in PBS and then with 1) a monoclonal anti-protein tyrosine phosphate IgG (clone P-Try-102: Cell Signaling, Beverly, MA, USA) followed by Alexa 594 conjugated goat anti-mouse IgG (Molecular probe, Eugene, OR, USA) 2) rabbit anti-actin antibody (amino residues 20-33, Sigma) followed by Alexa 488 conjugated goat anti-rabbit IgG (Molecular probe, Eugene, OR, USA). The sperm were also stained with nuclear staining dye, Topro 3 (Molecular probe). After washing, the sperm samples were resuspended in PBS:Glycerol (1:1), mounted onto slides and viewed under a confocal microscope using sets of filters of excitation/emission wavelength = 488/520 nm and 590/617 nm for the samples incubated with Alexa 488 and Alexa 594 conjugated secondary antibodies, respectively. The signal from Topro 3 was captured using the filter of 642/661 nm. Exclusion of the primary antibodies in the incubation of sperm samples will be used as a control.

4. Protease assay

The enzyme assay was performed according to the method described by Zimmerman et al. (1977) with some modifications. Fluorogenic-4-methylcoumarin-7-amide (MCA) substrates were used to quantitate enzyme activities in sperm samples. Substrates to be included are: 1) trypsin specific substrates: Boc-QAR-MCA, Boc-FSR-MCA, Boc-VPR-MCA, Boc-LRT-MCA and Boc-LSTR-MCA (Peptides International, Louisville, KY, USA); 2) chymotrypsin specific substrate, Suc-AAPF-MCA (Bachem, CA, USA); 3) proprotein convertase specific substrates; pERTKR-MCA (Peptides International) and Boc-RVRR-MCA (Bachem). The enzyme assays were performed in a black 96-well flat bottom plate at room temperature. 7-amino-4-methylcoumarin (AMC) which conjugated to a short peptide sequence will be released upon cleavage by a

protease in the samples. The released free AMC is fluorescent and can be monitored spectrofluorometrically at various time point using a spectro-fluorometer with excitation and emission wavelengths of 360 and 470 nm, respectively. The enzymatic activity was calculated using the AMC standard curve. Moreover, specific inhibitors were used in the assay to confirm sperm enzymatic activity. The protease inhibitors included soybean trypsin inhibitor (SBTI), amidino phenylmethanesulfonyl fluoride (AMPSF), N_{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK).

5. Gelatin Zymography

Proteolytic activities in sperm and fluid in thelycum (T-fluid) were investigated by zymography according to the method described by Caballero et al. (2001) with some modifications. Sperm proteins and T-fluid were separated electrophoretically under a non-reducing condition in 10% polyacrylamide gel containing 0.3% (w/v) gelatin. The gel was immersed twice, for 30 min each, in 50 mM Tris-HCl, pH 8.0 containing 3% (w/v) Triton X-100, washed in 50 mM Tris-HCl, pH 8.0 and incubated at 37°C, overnight in the incubation buffer (50 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, 50 mM $CaCl_2$, pH 7.6) with gentle shaking. The gel was then stained with 0.025% Coomassie Brilliant Blue R-250. Protein bands containing protease activity appeared translucent against the intensely blue background. Purified bovine pancreatic trypsin was also co-electrophoresed as a positive control.

6. Localization of trypsin-Like enzymes on sperm using Alexa 488 conjugated soybean trypsin inhibitor (SBTI)

Sperm were fixed with 4% paraformaldehyde for 1 h and washed several times with ASW by centrifugation (500 ×g, 5 min). Subsequently, the sperm were incubated with 4% BSA for 1 h followed by 2 μg/ml Alexa 488-conjugated SBTI (Invitrogen, Eugene, OR, USA) for 2 h at room temperature. After washing, the sperm were mounted onto a glass slide and viewed under a confocal laser scanning microscope using a set of filters of excitation/emission wavelength for 488/520 nm.

Results

1. Modification of *M. rosenbergii* sperm proteins during storage in male reproductive tract and female thelycum

To investigate sperm protein profiles during storage in testis, vas deferens, spermatophores, and thelycum that could indicate maturation and/or capacitation status of *M. rosenbergii* sperm, SDS-PAGE and Coomassie blue staining were performed. Protein profiles of *M. rosenbergii* sperm retrieved from testis (Tes), vas deferens (Vsp), spermatophores (Ssp) and female thelycum (Tsp) at 1h- and 6h-post insemination were different. The sperm proteins loaded into the gels were from 5 pooled samples (Fig. 1). Tes showed wide range of protein profile ranging from >200 to ~10 kDa. Vsp showed similar protein profile compared with Tes. However, Vsp and also Ssp, and Tsp showed the additional band at ~25 kDa; this band was not shown in Tes. Significantly, Ssp showed some unique protein bands at molecular weight of ~175 and ~48, ~45, ~38 kDa, and these protein bands were prominent. Interestingly, some protein bands in Tsp showed an increase in their intensity when compared with that of Ssp, i.e., the band at molecular weight ~200, ~120, ~100, ~15, and ~12 kDa. Moreover, the band at molecular weight of ~30 kDa showed decrease in its intensity and the bands at ~48, ~45, ~38 kDa that were abundant in Ssp were not present in Tsp. The results suggested that sperm proteins of *M. rosenbergii* were modified during their translocation from the testis into the vas deferens, the spermatophore, and the female thelycum. These changes may be important for sperm maturation and capacitation. However, sperm protein profiles of Tsp at1h- and 6h-post insemination were not different, suggesting that modification of sperm proteins in female thelycum may occur rapidly.

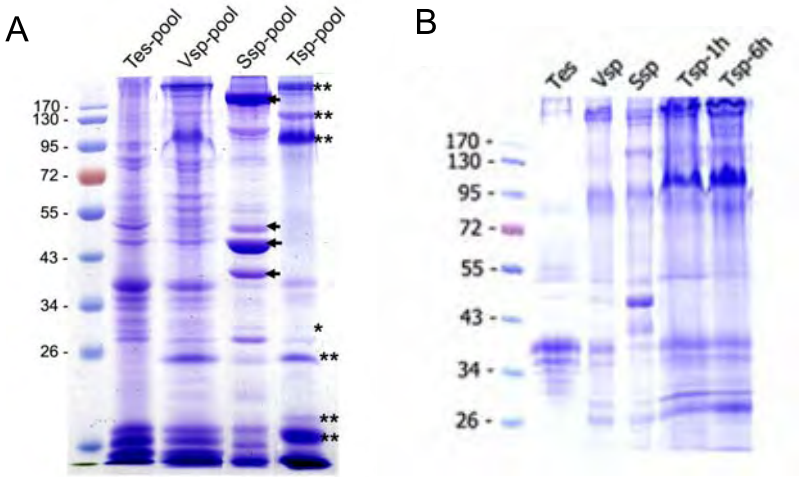

Fig. 1 (A-B) Protein profiles of *M. rosenbergii* sperm including testicular sperm (Tes), vas deferens sperm (Vsp), spermatophoric sperm (Ssp) and thelycal sperm (Tsp). Black arrows indicated the band that specifically presented in Ssp, whereas two asterisks (**) showed the protein bands of Tsp that were increased in their intensity. One asterisk (*) indicated the protein bands that were decreased in their intensity.

2. Accumulation of actin in *M. rosenbergii* sperm during storage in spermatophore and presence of actin in sperm anterior spike and base of spike

Actin, which plays an important role during sperm acrosome reaction and sperm-egg reaction in many species, was also detected in *M. rosenbergii* sperm by Western blot analysis. The result demonstrated that only Ssp showed prominent positive protein band at molecular weight of ~42 kDa (Fig. 2B). This protein band was also shown prominently in Coommasie blue staining (Fig. 2A). This result suggested that actin accumulation was occurred during sperm traveled along the male reproductive tract, thus we can see highest amount of actin in Ssp. However, sperm actin was decreased when sperm were deposited onto the thelycum.

Immunolocalization of actin showed that actin was localized in the spike and the base of spike of sperm (Fig. 3).

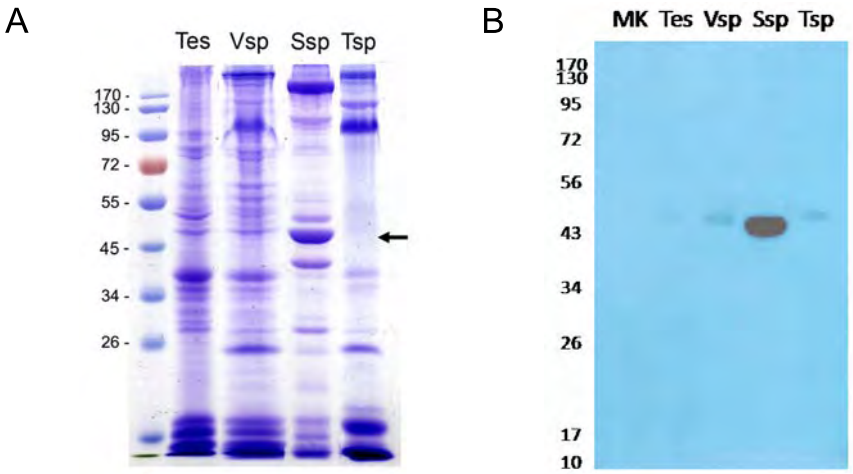

Fig. 2 (A) *M. rosenbergii* sperm protein profiles including testicular sperm (Tes), vas deferens sperm (Vsp), spermatophoric sperm (Ssp), and thelycal sperm (Tsp). (B) Westerm blot analysis of sperm proteins immunostained with anti-actin antibody.

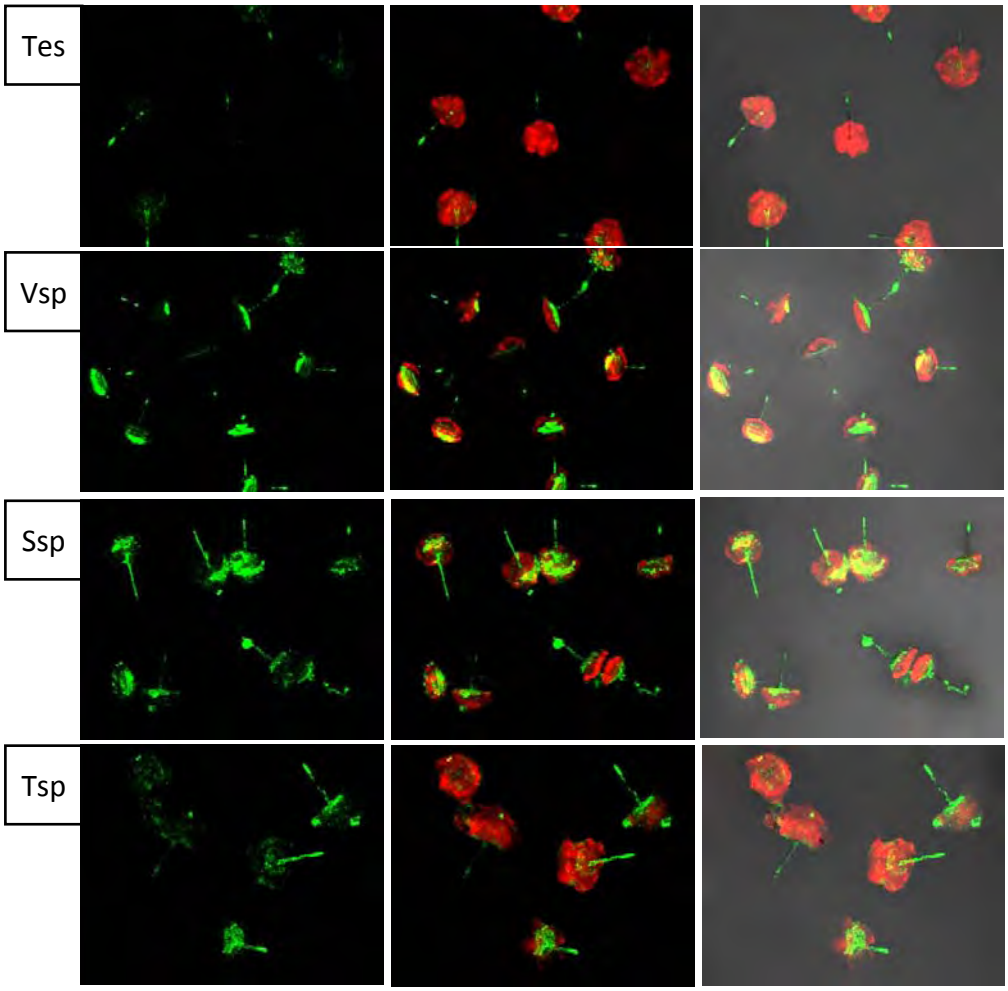

Fig. 3 Immunofluorescence staining of sperm actin showing actin localization in the anterior spike and the base of spike of *M. rosenbergii* sperm.

3. Change of sperm tyrosine phosphorylation localization pattern during sperm storage in male reproductive tract

To investigate capacitation status of *M. rosenbergii* sperm using protein tyrosine phosphorylation as a marker, Western blotting using anti-protein tyrosine phosphorylation was performed. The result showed that several protein bands of Tes, Vsp, Ssp, and Tsp (1 h- and 6 h-post insemination) showed tyrosine phosphorylation ranging from the bands at molecular weight of ~45-100 kDa (Fig. 4). However, there was a little decrease in level of sperm tyrosine phosphorylation in Ssp and Tsp, when compared with that of Tes and Vsp. Specifically, the bands at 72, 50, and 45 kDa of Sspa and Tsp showed a decrease in level of sperm tyrosine phosphorylation. Interestingly, Tsp at 6 h-post insemination showed an increase in level of sperm tyrosine phosphorylation. Ssp and Tsp showed Control experiment where the anti-tyrosine phosphorylation was omitted showed no positive signal on the film.

In order to localize the tyrosine phosphorylation in *M. rosenbergii* sperm, indirect immunofluorescence staining was performed. Sperm retrieved from testis (Tes), vas deferens (Vsp), spermatophore (Ssp), and thelycum (Tsp) were stained with anti-tyrosine phorphorylation antibody followed by goat anti-mouse Alexa 488. The fluorescent signal was detected under a fluorescence microscope. The result demonstrated that Tes showed the green positive staining in the body of sperm (Fig. 3 and 4, respectively). Interestingly, Vsp, Ssp, and Tsp showed the green positive staining at the anterior spike of sperm (Fig. 5). These results suggested changes of tyrosine phosphorylation pattern of sperm during translocation from testis into vas deferens and finally into spermatophores which may be important for sperm maturation process. However, the level and localization pattern of sperm tyrosine phosphorylation could not be used as a capacitation marker in *M. rosenbergii*, since Tsp did not show any change in tyrosine phosphorylation compared with Ssp.

We also performed immunofluorescence staining of tyrosine phosphorylation in *P. monodon* sperm. The result showed that the localization of tyrosine phosphorylation in sperm was changed from spike and base of spike of Vsp and Ssp to the body of Tsp (Fig. 6). Therefore, the localization pattern of *P. monodon* sperm could be used as one of the markers for sperm capacitation in this shrimp species.

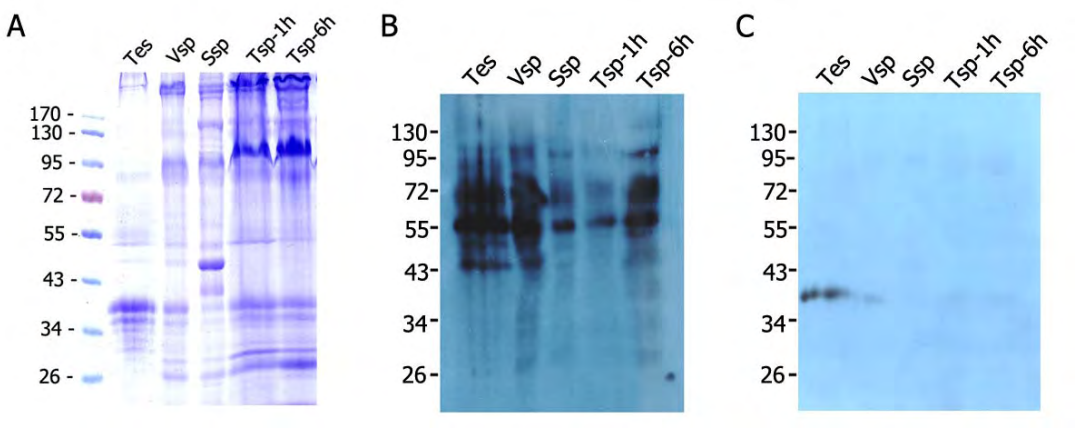

Fig. 4 (A) Coommassie blue stained gel of *M. rosenbergii* sperm proteins including testicular sperm (Tes), vas deferens sperm (Vsp), and spermatophoric sperm (Ssp). (B) Western blot analysis of *M. rosenbergii* sperm proteins immunostained with anti-tyrosine phosphorylation antibody. (C) Control experiment of Western blotting where the anti-tyrosine phosphorylation was omitted from the incubation.

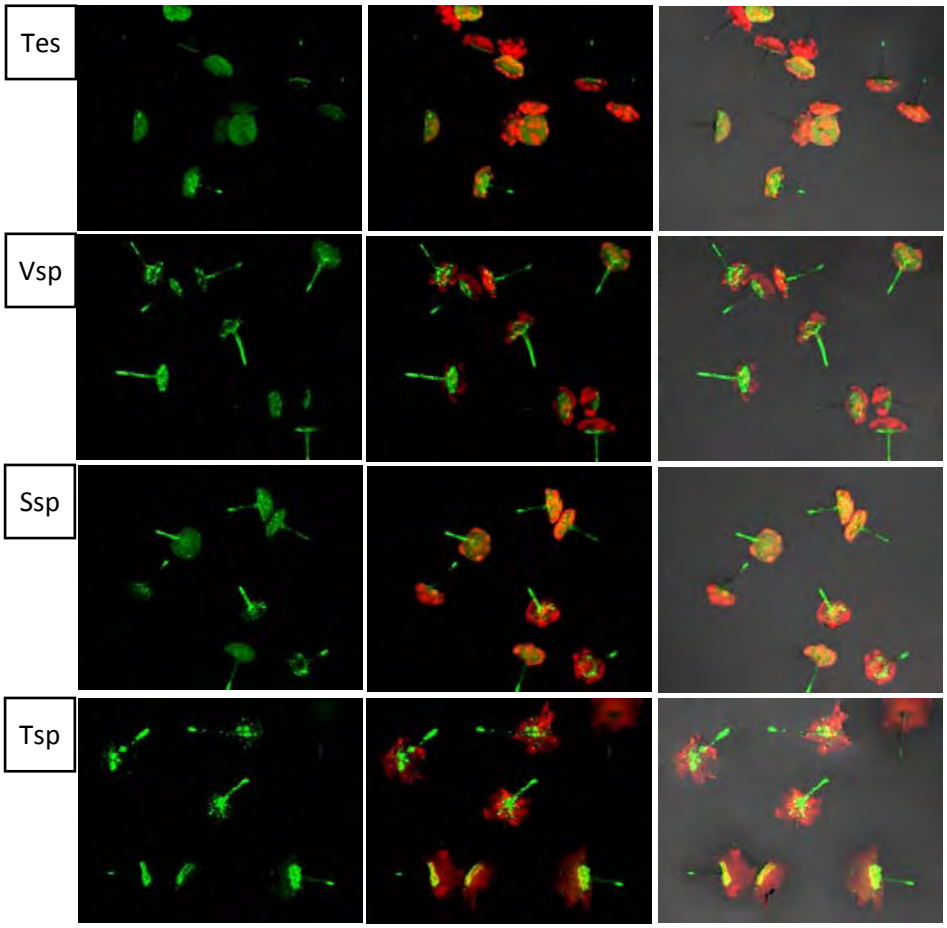

Fig. 5 Immunofluorescence staining of P-tyr in sperm of *M. rosenbergii*. Tes-sp = testicular sperm, Vsp = vas deferens sperm, Ssp = spermatophoric sperm, and Tsp = thelycal sperm.

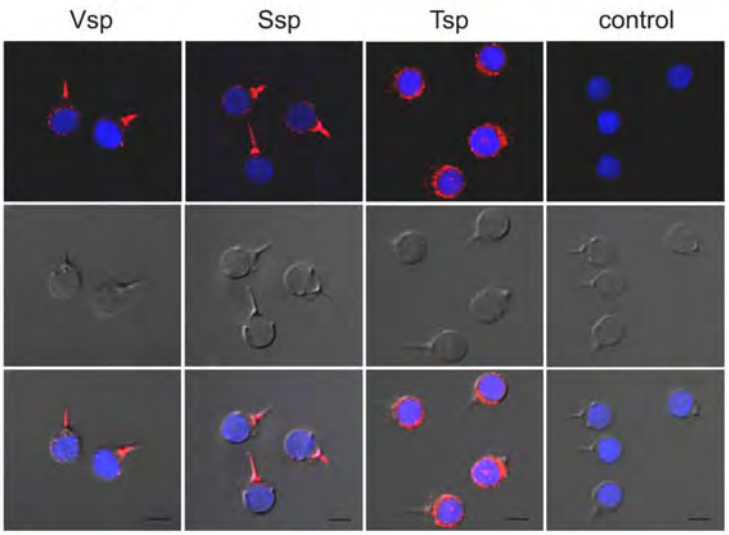

Fig. 6 Immunofluorescence staining of P-tyr in sperm *P. monodon*. Tes-sp = testicular sperm, Vsp = vas deferens sperm, Ssp = spermatophoric sperm, and Tsp = thelycal sperm. Bar = 10 µm.

4. Protease activities in *P. monodon* sperm

Four fluorogenic peptidyl-MCA substrates were used to demonstrate the activities of trypsin-like, PC-like and chymotrypsin-like enzymes in sperm collected from different reproductive organs including the vas deferens (V-sperm), spermatophore (S-sperm) and thelycum (T-sperm). Results in Table 1 revealed that T-sperm extracts showed the highest hydrolytic activity toward trypsin substrate (Boc-QAR-MCA) and some small hydrolytic activities toward PC substrate (pERTKR-MCA). In contrast to T-sperm, V- and S-sperm showed very low proteolytic activities for all substrates tested. These results suggested that only T-sperm possessed a high trypsin-like activity.

Table 1 Proteolytic activities in live sperm collected from the vas deferens (V-), spermatophore (S-), and thelycum (T-)

Substrates	Specific Activity (Units/μg Proteins)		
	V-Sperm	S-Sperm	T-Sperm
Boc-QAR-MCA (trypsin)	0.07 ± 0.06	0.28 ± 0.17	589.19 ± 253.42
Suc-AAPF-MCA (chymotrypsin)	0.01 ± 0.01	0.05 ± 0.09	5.40 ± 4.77
pERTKR-MCA (PC)	0.00 ± 0.00	0.61 ± 0.79	31.57 ± 24.29
Boc-RVRR-MCA (PC)	0.03 ± 0.06	0.10 ± 0.08	3.51 ± 3.41

5. Inhibitor study of trypsin-like enzymes in *P. monodon* sperm

To investigate the substrate preference for the trypsin-like enzymes in T-sperm, we used various trypsin specific substrates including Boc-QAR-MCA, Boc-FSR-MCA, Boc-VPR-MCA, Boc-LRT-MCA and Boc-LSTR-MCA. The results showed that Boc-QAR-MCA was the most preferred trypsin-specific substrate for T-sperm (Fig. 7).

T-sperm trypsin-like enzymes were also determined whether the specific trypsin inhibitors, including SBTI, APMSF and TLCK, could inhibit their activities. The hydrolytic activity of T-sperm against Boc-QAR-MCA was dramatically inhibited when SBTI was included (Fig. 8). The inhibitory effect of APMSF was concentration dependent. When 1, 10 and 100 μM APMSF were added to the reaction, the percent inhibition ranged from ~8% to ~77 %, respectively. Slight inhibition of the hydrolytic activity in T-sperm against Boc-QAR-MCA was noted for TLCK as only 32% inhibition was obtained with

100 μM TLCK while the minimal inhibition level was observed with the lower TLCK concentrations.

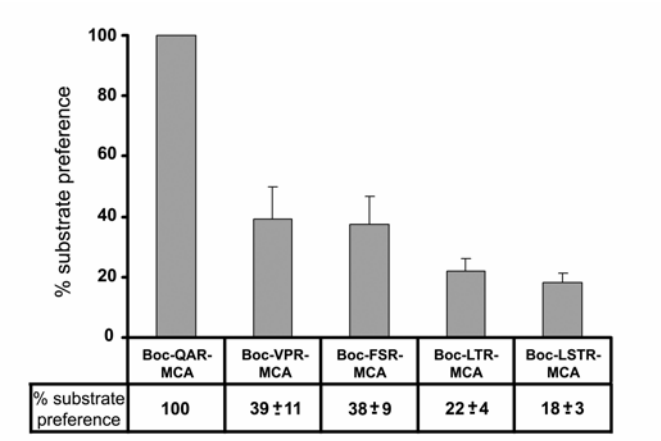

Fig. 7 Substrate preference for T-sperm trypsin-like enzymes. Fluorogenic trypsin-specific substrates used were Boc-QAR-MCA, Boc-VPR-MCA, Boc-FSR-MCA, Boc-LRT-MCA and Boc-LSTR-MCA.

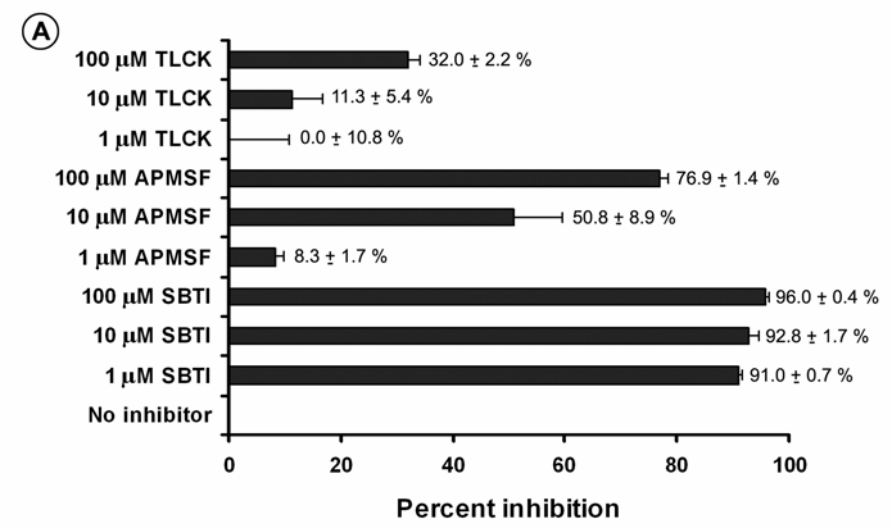

Fig. 8 Inhibition of SBTI, APMSF and TLCK on T-sperm trypsin-like proteolytic activity. T-sperm were incubated with Boc-QAR-MCA in the presence and absence of trypsin inhibitors. Hydrolase activity without trypsin inhibitor was considered as 0% inhibition (or 100% activity) and used as the baseline for expression % inhibition by each inhibitor.

6. Zymography of *P. monodon* sperm

We further identified the trypsin-like enzymes in T-sperm by gelatin zymography. Zymogram in Fig. 9 revealed that T-sperm exhibit a broad spectrum of proteolytically active bands, ranging from 28-48 kDa. The bands with the most intense protease

activity were positioned at 46 and 48 kDa, while those with moderate protease activity were positioned at 30 and 33 kDa. Moreover, S-sperm showed no proteolytically active bands. Interestingly, thelycal fluid (T-fluid) contained three major active protease bands positioned at the molecular masses of 35, 46 and 48 kDa (Fig. 9). This result suggested that the active protease bands present in T-sperm may be derived from enzymes in T-fluid.

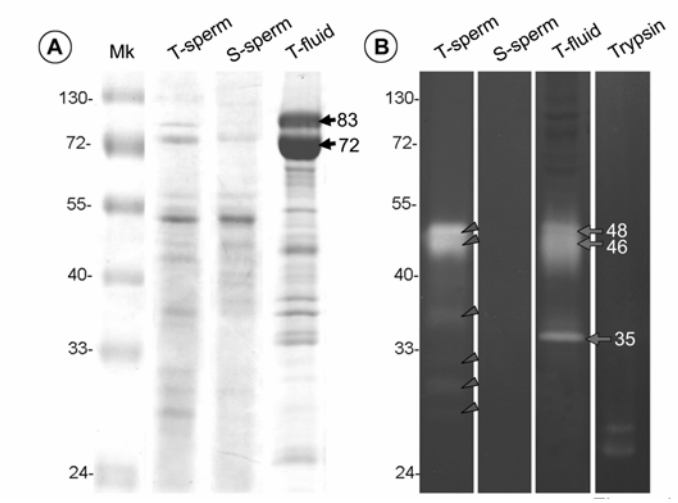

Fig. 9 Coomassie blue-stained gel (A) and gelatin zymogram (B) of T-sperm, S-sperm and T-fluid. Mk = Protein molecular weight markers.

7. Soy bean trypsin inhibitor (SBTI) staining on *P. monodon* and *M. rosenbergii* sperm

Since SBTI has shown the highest inhibition of shrimp sperm trypsin-like enzymes, we used Alexa 488-conjugated SBTI to localize the enzymes on the sperm of *P. monodon* and *M. rosenbergii*. The positive fluorescence staining was shown in the subacrosomal region and base of the anterior spike of P. *monodon* sperm (Fig. 10A). Moreover, in sperm that had undergone acrosome reaction, the positive staining for SBTI was detected specifically at the spherical mass which was formed from the subacrosomal region during the AR (Fig. 10A, arrow head). In *M. rosenbergii* sperm, showed positive staining for SBTI at the acrosome region (Fig. 10B). The results suggested the presence of sperm trypsin-like proteases in the subacrosome region of *P. monodon* and the acrosome region of *M. rosenbergii sperm*

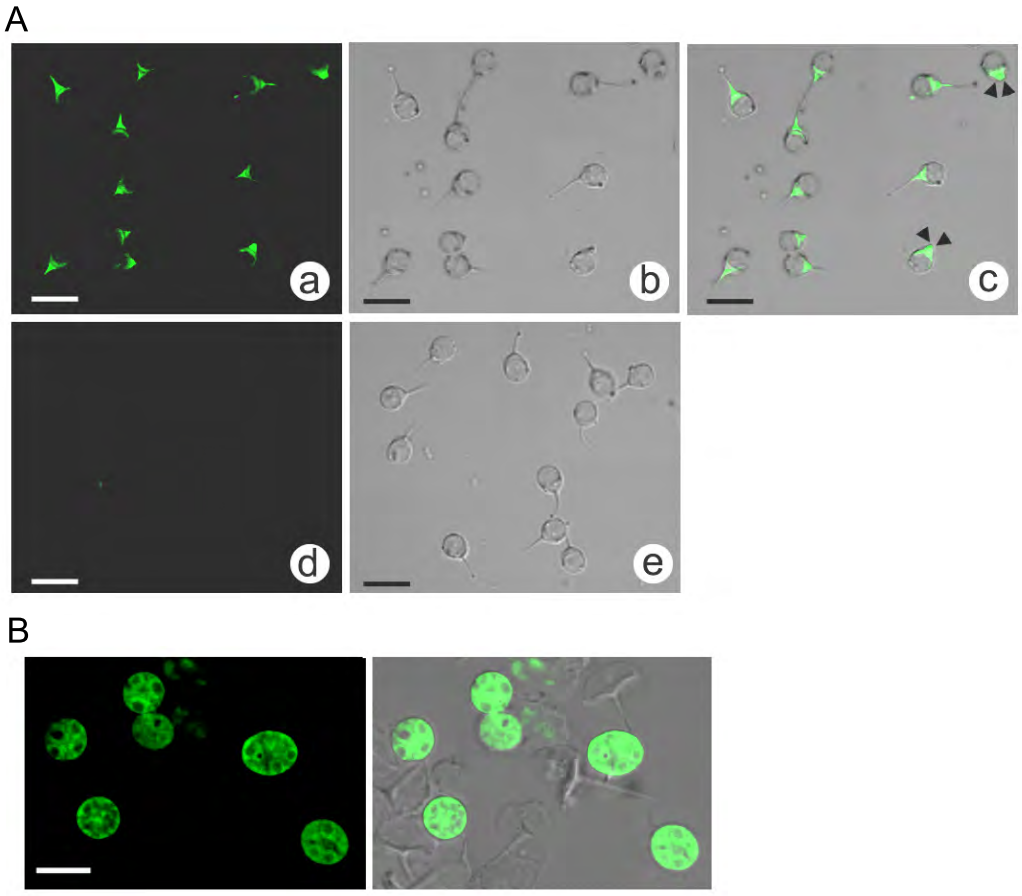

Fig. 10 (A) SBTI staining in sperm retrieved from Tsp (upper panel) and from Ssp (lower panel) of *P. monodon*. The positive fluorescent signal appears only in Tsp at the subacrosomal region and base of the anterior spike. Note that the SBTI positive staining was also shown in acrosome reacted sperm, specifically at the spherical mass formed from the subacrosomal region during AR (c-arrow head). (B) SBTI staining in Ssp of *M. rosenbergii* showing the positive staining in the acrosome region. Bar = 10 μm.

Discussion

In *M. rosenbergii*, spermatogenesis takes place in the testis and the testicular sperm are then emptied into male reproductive tract, i.e., vas deferens, and the terminal ampoule (Poljareon, et al., 2010). In the terminal ampoule, sperm are packaged and surrounded by mucous substance to form the spermatophores which are deposited into female thelycum during mating. In this study we have shown that modification of *M. rosenbergii* sperm proteins occurred during their storage in male reproductive tract and also female thelycum. A post-testicular modification of sperm of close thelycum penaeid shrimp, e.g., *P. monodon*, has long been suggested to be important for fertilization (Clark and Griffin, 1988; Wikramanayake et al., 1992; Vanichviriyakit et al., 2004). Moreover, some open type thelycum species of shrimp, i.e. in sub-genus Litopenaeus, have also been reported on biochemical modifications of sperm (Alfora, 2010). *M. rosenbergii* is one of the open-thelycum species which is shown in this study that the sperm undergo some modification of proteins during their storage in male reproductive tract and female thelycum. However, sperm protein profiles of Tsp at1h- and 6h-post insemination into female thelycum were not different, suggesting that modification of sperm proteins in female thelycum may occur rapidly. These changes may be important for sperm maturation and capacitation which is the process that make the sperm ready for fertilization. Changes of the sperm proteins could occur by the process of removal and adsorption. Studies in mammalian species have shown that the removal of some sperm proteins is to expose the covered egg-binding ligands, or the sperm proteins that serve more purposes during fertilization (Hunter and Nornes, 1969; Oliphant et al., 1985). Moreover, the transfer of proteins from an epididymal source onto the sperm surface during mammalian sperm maturation has been extensively reported, i.e., hamster P26h (Legare et al., 1999), bull P25b (Frenette and Sullivan, 2001), and human P34H (Boue et al., 1996). In this study, we have not identified the proteins that were modified. Identification of those proteins could give more details on functions of each protein.

We also showed the accumulation of actin in *M. rosenbergii* sperm during storage in the male reproductive tract, especially when the sperm were stored in the spermatophore. Mature sperm of *M. rosenbergii* have an everted umbrella-shaped plate with a spike covering the anterior pole of the nucleus and the acrosome appears at the ruffled border of the spike plate as small sac-like structures (Poljareon et al., 2010). In this study, we showed that actin was localized in the spike and the base of spike of

sperm. In *P. monodon*, the sperm also showed different characteristic structural components, i.e., the anterior spike, the subacrosomal region and the spherical mass forming during acrosome reaction. It has been shown that the subacrosomal region of *P. monodon* sperm and the spherical mass of acrosome reacted sperm were made up of actin. In mammalian species, actin has been localized in acrosome and post-acrosome area, neck and tail (Fouquet and Kann, 1992). Moreover, it has been shown in many species that actin play important roles in acrosome reaction และ sperm-egg interaction (Castellani-Ceresa et al., 1993; Brener et al., 2003; Cabello-Agueros et al., 2003). In *M. rosenbergii*, role of sperm actin in fertilization may be studied further.

An increase of sperm protein tyrosine has been known for the maker of capacitation process which results from a cAMP-dependent signal transduction pathway (Visconti and Kopf, 1998; Naz and Rajesh, 2004). This conserved signal transduction pathway has been shown in sperm of many species from crustaceans to mammals, e.g., Chum Salmon, *Oncorhynchus keta* (Itoh et al., 2001), starfish (Ishiguro et al., 1982), sea urchins (Yokota et al., 1990), and *P. modondon* (Vanichviriyakit et al., 2004). It has been reported that flagella seem to be the major component that undergoes tyrosine phosphorylation in sperm of most mammal species including human, monkey, hamster, rat and mouse (Leclerc et al., 1997; Lewis and Aitken, 2001; Mahony and Gwathmey, 1999; Naz, 1999; Si and Okuno, 1999; Urner et al., 2001; Liu et al., 2006) and are related to the hypermobility of the sperm in mammal species (Morizawa, 1994; Visconti, 2009). In this study, we aimed to investigate capacitation status of *M. rosenbergii* sperm using protein tyrosine phosphorylation as a marker, Western blot analysis showed that there was a decrease in level of sperm tyrosine phosphorylation in Ssp and Tsp when compared with that of Tes and Vsp. However, Tsp at 6 h-post insemination showed an increase in level of sperm tyrosine phosphorylation. Whether these changes in sperm tyrosine phosphorylation level are related to fertilization ability of the sperm, further study should be performed. Immunofluorescence staining of protein tyrosine phosphorylation in *M. rosenbergii* sperm revealed that Tes showed the positive staining in the body of sperm, while Vsp, Ssp, and Tsp showed the green positive staining at the anterior spike of sperm. These results suggested changes of tyrosine phosphorylation pattern of sperm during translocation from testis into vas deferens and finally into spermatophores, which may be important for sperm maturation process. Moreover, we also performed immunofluorescence staining of tyrosine phosphorylation in *P. monodon* sperm. The result showed that the localization of tyrosine phosphorylation in sperm was changed from spike and base of spike of Vsp

and Ssp to the body of Tsp. Therefore, the localization pattern of *P. monodon* sperm could be used as one of the markers for sperm capacitation in this shrimp species. Change in the pattern of sperm protein tyrosine phosphorylation has also been reported in the shrimp *L. vannamei* (Aungsuchawan et al., 2011).

We also studied sperm proteases using fluorogenic peptidyl-MCA substrates and zymography in *P. monodon*. The results showed that only Tsp highly contained trypsin-like activity compared to Vsp and Ssp, suggesting that the sperm might gain this property during their storage in the female thelycum. Among trypsin specific substrates used, Boc-QAR-MCA was the most preferred trypsin specific substrate for Tsp which is different from other species reported. For example, Ascidian acrosin prefers to hydrolyse Boc-FSR-MCA with the highest specificity, while ascidian spermosin prefers to cleave Boc-VPR-MCA (Sawada et al., 1984a; Sawada and Someno, 1996). In *S. ingentis* sperm, Substrate preference of trypsin-like proteases is Boc-FSR-MCA (Chen et al., 1994). We also showed that using zymography only Tsp showed protease active bands, not Vsp and Ssp. Interestingly, the thelycal fluid also showed protease active bands at 46 and 48 kDa which shared the identical bands of Tsp. Therefore, we hypothesized that thelycal fluid proteases may adsorb onto the sperm surface during their storage in the female thelycum. The existence of sperm surface proteases has been extensively reported in many species. For example, ascidian sperm show several surface enzymes that are important in fertilization, e.g., chymotrypsin-like enzyme (Sawada et al., 1983; Koch et al., 1994), glycosidases (fucosidase and *N*-acetylglucosaminidase) (Hoshi et al., 1994). In mouse sperm, there are many surface enzymes including acrosin (Baba et al., 1994), PC4 (Mbikay et al., 1997), and testicular serine protease (TEST5) (Honda et al., 2002). We also demonstrated the presence of trypsin-like enzymes in sperm by mean of immunofluorescence staining using soybean trypsin inhibitor. The trypsin localization was shown in the subacrosomal region and base of the anterior spike of P*. monodon* sperm. Moreover, in sperm that had undergone acrosome reaction, the positive staining for SBTI was detected specifically at the spherical mass which was formed from the subacrosomal region during the acrosome reaction. In *M. rosenbergii* sperm, trypsin localization was shown at the acrosome region at the ruffled border of the spike plate. The results suggested the presence of sperm trypsin-like proteases in the subacrosome region of *P. monodon* and the acrosome region of *M. rosenbergii* sperm. The sperm proteolytic activities could be necessary for fertilization.

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ข้อเสนอแนะโครงการวิจัยในอนาคต

งานวิจัยนี้ได้ศึกษาถึงกระบวนการพัฒนาอสุจิในกุ้งก้ามกราม และกุ้งกุลาดำ ซึ่งเป็นสัตว์น้ำเศรษฐกิจของประเทศไทย ปัญหาหนึ่งซึ่งทำให้เกิดความไม่แน่นอนขึ้นในการเลี้ยง คือการลดจำนวนลงของกุ้งพ่อแม่พันธุ์ในธรรมชาติที่นำมาใช้ผลิตลูกกุ้ง และการลดลงของประสิทธิภาพการสืบพันธุ์ของกุ้งในโรงเพาะฟัก การศึกษาเพื่อให้เกิดความเข้าใจเกี่ยวกับกระบวนการพัฒนาเซลล์สืบพันธุ์ในกุ้งทั้งพ่อพันธุ์และแม่พันธุ์ และปัจจัยต่างๆที่มีผลต่อการผสมพันธุ์ น่าจะมีส่วนช่วยให้เกิดองค์ความรู้ใหม่ๆเพื่อนำไปประยุกต์ใช้เพื่อเพิ่มประสิทธิภาพการสืบพันธุ์ของกุ้ง และเพิ่มผลผลิตลูกพันธุ์ที่มีคุณภาพต่อไปในอนาคต

เอกสารแนบหมายเลข 3

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ
   - Rapeepun Vanichviriyakit, Hathairat Kruevaisayawan, Charoonroj Chotwiwatthanakun, Wattana Weerachtyanukul, Boonsirm Withyachumnarnkul, Ajoy Basak, Nongnuj Tanphaichitr, Prasert Sobhon. Acquisition of Trypsin-like Proteases from Thelycal Fluid by Sperm of *Penaeus monodon* (ผลงานวิจัยกำลังอยู่ในระหว่างการตรวจแก้ไขความถูกต้องของ manuscript เพื่อตีพิมพ์ในวารสาร Molecular Reproduction and Development, โปรดดูภาคผนวก)
   - Tongrak Yimpak, Rapeepun Vanichviriyakit, Charoonroj Chotwiwatthanakun, Wattana Weerachtyanukul, Prasert Sobhon. Post-testicular Modifications of Sperm of the Fresh Water Prawn, *Macrobrachium rosenbergii* (ผลงานวิจัยกำลังอยู่ในระหว่างรวบรวมเพื่อเขียน manuscript เพื่อตีพิมพ์ในวารสาร Cell and Tissue Research)

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
     ยังไม่มี
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
     ยังไม่มี
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
     ยังไม่มี
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
     ผลงานวิจัยเรื่องนี้บางส่วนใช้สำหรับวิทยานิพนธ์ของนักศึกษาปริญญาโท หลักสูตร กายวิภาคศาสตร์และชีววิทยาโครงสร้างจำนวน 1 คน (น.ส. ต้องรัก ยิ้มพักตร์) ซึ่งจะเป็นการสร้างนักวิจัยใหม่

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
   ผลงานวิจัยเรื่องนี้ถูกนำไปเสนอในงานประชุมวิชาการดังนี้

- Vanichviriyakit, R., Chotwiwatthanakun, C, Weerachatyanukul, W., Withyachumnarnkul, B., Sobhon, P. Study of modifications that are related to maturation and capacitation processes of sperm in *Macrabrachium rosenbergii* and *Penaeus monodon* งานประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส ครั้งที่ 11 19-21 ตุลาคม 2554 ที่ โรงแรมฮอริเดย์อินน์ รีสอร์ท บีช ชะอำ จ.เพชรบุรี (poster presentation) (โปรดดูภาคผนวก)

ภาคผนวก

1. Abstract: งานประชุมนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส ครั้งที่ 11 19-21 ตุลาคม 2554 ที่ โรงแรมฮอริเดย์อินน์ รีสอร์ท บีช ชะอำ จ.เพชรบุรี

Study of modifications that are related to maturation and capacitation processes of sperm in *Macrabrachium rosenbergii* and *Penaeus monodon*

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Abstract

In this study, we aimed at investigating sperm modifications during their transport into spermatic ducts and a female thelycum of two species of shrimps, i.e., *Macrobrachium rosenbergii* and *Penaeus monodon*. The fresh water prawn, *M. rosenbergii*, is of opened-type thelycum species, whereas the black tiger shrimp, *P. monodon*, is of close-type thelycum species. We are interested in how sperm of these two species are changed in order to make the sperm capable in fertilization. To investigate protein markers that could relate to the capacitation status of *M. rosenbergii* sperm, SDS-PAGE and Western blotting using anti-protein tyrosine phosphorylation and anti-actin antibodies were performed in sperm taken from testis (Tes), vas deferens (Vsp), spermatophore (Ssp) and female thelycum (Tsp). Moreover, sperm protease activity was determined in sperm of *P. monodon* and *M. rosenbergii* by enzyme assay using fluorogenic substrates. Localization of protein tyrosine phosphorylation and proteases in sperm of both species was also performed using anti-protein tyrosine phosphorylation and Alexa-488 conjugated soy bean trypsin inhibitor (SBTI), respectively. We showed in *P. monodon* that only Tsp contained high trypsin-like activities, which were effectively inhibited by SBTI. Staining of SBTI in sperm suggested that the proteases could be localized at the subacrosome region and base of spike of *P. monodon* sperm and the acrosome region of *M. rosenbergii* sperm. We also showed the accumulation of actin in the sperm of two species during their traveled along the male reproductive tracts. Moreover, changes of tyrosine phosphorylation pattern of sperm during translocation from testis into spermatic duct and finally into thelycum were observed. The staining pattern for tyrosine phosphorylation could also be used to detect the final maturation or capacitation status of *M. rosenbergii* and *P. monodon* sperm. These biochemical changes on the sperm of *M. rosenbergii* and *P. monodon* during storage in the male reproductive tract and the female thelycum could be part of the shrimp sperm maturation and capacitation processes.

Keywords: *Penaeus monodon*, *Macrobrachium rosenbergii*, sperm, protease, capacitation, tyrosine phosphorylation

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2. Manuscript:

Acquisition of Trypsin-like Proteases from Thelycal Fluid by Sperm of *Penaeus monodon*

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Keywords: *Penaeus monodon*, sperm, thelycum, serine proteases, capacitation

ABSTRACT

In penaeoid shrimp, sperm packaged in spermatophores of the male are inserted into and stored in the thelycum of the female during their mating. The thelycum plays an active role in imparting fertilizing ability to the stored sperm (Vanichviriyakit et al., 2004). At the spawning time, the female releases both stored sperm and eggs for external fertilization. In a number of marine animals, sperm serine proteases are important for fertilization. Therefore, in this study we asked whether serine proteases are secreted by the thelycum, and whether these enzymesbecame associated with penaeoid sperm during their residence in this organ, in order to confer them the fertilizing ability. We first compared serine protease activities of sperm retrieved from the male reproductive tract with those of thelycal sperm. Using fluorogenic peptidyl-4-methylcoumaryl-7-amides (MCA) substrates, we showed that only thelycal sperm contained high trypsin-like activities, which were effectively inhibited by serine protease inhibitors, soybean trypsin inhibitor (SBTI), amidino phenylmethanesulfonyl fluoride (AMPSF), and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). Positive staining of Alexa 488 conjugated SBTI was shown only in the base of anterior spike and the subacrosome region of thelycal sperm. Gelatin zymography revealed that only thelycal sperm produced two major translucent bands of 46 and 48 kDa molecular masses. These protease bands were also found in thelycal fluid together with another protease band at 35 kDa, suggesting that the two serine protease bands present on thelycal sperm were originated from the thelycal fluid. This postulation was confirmed by the presence of both 46 and 48 kDa serine protease bands in spermatophoric sperm after their incubation with the thelycal fluid. These biochemical changes on *P. monodon* sperm during storage in the female thelycum could be part of the shrimp sperm capacitation process.

INTRODUCTION

Serine proteases have long been known to be involved in regulating fertilization in many invertebrates. In particular, trypsin-like and chymotrysin-like enzymes are the first two serine proteases that are shown to be necessary for sperm penetration of the egg investments in an ascidian, *Halocynthia roretzi* (Hoshi et al., 1981). Moreover, the existence of sperm proteasomes, a cluster of trypsin, chymotrypsin and peptidyl-glutamyl peptide hydrolyzing proteases, and their involvement in egg jelly-induced acrosome reaction and sperm-zona pellucida interaction have also been demonstrated in sea urchin (Matsumura and Aketa, 1991; Takizawa et al., 1993; Yokota and Sawada, 2007). Among these three sperm proteases, trypsin-like enzymes are the most extensively studied, thus information on their characteristics and physiological functions has already been well established. In sea urchin, *Strongylocentrotus purpuratus*, spermatozoa contains a 20 kDa protease that retains its activity toward the trypsin substrate (Resing et al., 1985). In ascidian, *H. roretzi*, two types of sperm trypsin-like enzymes, 35-kDa acrosin and 33-/40-kDa spermosin, have been characterized (Sawasa and Someno, 1996). These two enzymes, together with a chymotrypsin-like enzyme, have been shown to be essential in sperm penetration through the eggs' coat (Sawada et al., 1984a; Kodama et al, 2002). However, in another ascidian species, *Ciona intestinalis*, only a chymotrypsin-like enzyme is involved in sperm penetration (Pinto et al., 1990; Marino et al., 1992). In shrimp, *Sicyonia ingentis*, trypsin-like and aminopeptidase-like activities have been reported in spermatozoa; nevertheless, only the trypsin-like protease is involved in sperm penetration (Chen et al., 1994). The presence of trypsin-like enzymes in the sperm has also been shown in another shrimp, *Rhynchocinetes typus*, and was shown to be involved in sperm penetration through the egg-coat (Rios and Barros, 1997). The significance of the trypsin-like enzymes in the acrosome reaction has also recently been documented in the shrimp, *Penaeus monodon*, (Kreuvaisayawan et al., 2008). From this finding, it is indicative that trypsin-like enzymes, are most likely derived from the sperm rather than egg substances, in contrast to the previous report in *S. ingentis* (Griffin and Clark, 1990; Lindsay and Clark, 1992), and that they are the key modulators for sperm acrosome reaction (Kreuvaisayawan et al., 2008). In this context, our group has recently reported that activation of sperm protease activities appears to be closely related with fertilizing capacity of the sperm in an abalone, *Haliotis asinina* , as it was shown that the higher enzyme activity that sperm acquired,

the higher fertilizing ability they gained (Suphamungmee et al., 2008).
Generally, it is believed that acquisition of sperm fertilizing ability is associated with a complicate molecular event known as sperm "capacitation". The hallmark characteristics of this event are well-established in mammals, which include the modifications of sperm membrane proteins and the enhancement of PKA-dependent tyrosine phosphorylation (Visconti et al., 1999; 2002). In marine invertebrates, it has also been reported that the capacitation-like characteristics are also detectable in some marine shrimp and abalone, and are considered to be prerequisites for successful fertilization in these species (Alfaro et al., 2003; Vanichviriyakit et al., 2004; Suphamungmee et al., 2008). However, unlike other species being studied so far, in the closed-thelycum penaoied shrimp sperm have to be translocated into female thelycum (sperm-storing sac) in order for them to be capacitated, and after the storage in thelycum the sperm could undergo acrosome reaction, and acquiring their fertilizing ability (Vanichviriyakit et al., 2004). As mentioned above that the serine protease activity (particularly trypsin-like) is indispensable for modulating sperm acrosome reaction in *P. monodon* (Kreuvaisayawan et al., 2008), we postulated that the acquisition of sperm trypsin-like proteases in this shrimp species may take place in the female thelycum. In this study, we found that *P. monodon* sperm acquired trypsin-like activity (TLA) during their storage in the female thelycum, and this could be a part of capacitation-like process which enables the shrimp sperm to gain their full fertilizing ability.

MATERIALS AND METHODS

Collections of Sperm and Thelycal Fluid

Male and female broodstocks were caught from the Andaman Sea and maintained at the Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi, Thailand. Vas deferens and spermatophores were dissected out. To obtain vas deferens (V-), and spermatophoric (S-) sperm, the vas deferens and spermatophores were physically torn apart by pairs of fine forceps to release the gelatinous masses containing sperm into calcium-free artificial seawater (CFASW; 423 mM NaCl, 9 mM KCl, 23 mM $MgCl_2$, 9.3 mM $MgSO_4$, 2.1 mM $NaHCO_3$, pH 7.8). These masses were gently pipetted and agitated to segregate individual sperm. Thereafter, the sperm suspension was filtered through a 212-µm metal sieve (Endecotts, London, UK) to remove cell clumps, washed (500 ×g, 5 min) and resuspended in CFASW at the final concentration of 1.0×10^7 sperm/ml.

Collections of thelycal (T-) sperm and T-fluid followed the recently described protocol (Kreuvaisayawan et al., 2008). Briefly, naturally inseminated females were anesthetized under ice, and the thelyca located between the 5th pairs of walking legs were carefully dissected out. Subsequently, the gelatinous masses containing sperm and fluid were collected into CFASW. T-sperm were segregated and cleaned in the same manner as described above for S -sperm. The final sperm pellet was resuspended in CFASW at the concentration of 1×10^7 sperm/ml before being used further. T-fluid was also collected from the female shrimp, which had not been inseminated to avoid factors released from the sperm. This fluid was centrifuged (12,000 ×g, 10 min, 4°C) to remove any debris, and the protein concentration was quantified by Bradford's protein assay using Sigma reagent kit (Sigma Co., St. Louis, MO, USA).

Fluorogenic Enzyme Assay

The assay using fluorogenic-4-methylcoumarin-7-amide (MCA) substrates was performed to quantify enzyme activities according to the method described by Zimmerman et al. (1977) with some modifications. Substrates used in this experiment included 1) trypsin substrates: Boc-QAR-MCA, Boc-FSR-MCA, Boc-VPR-MCA, Boc-LRT-MCA and Boc-LSTR-MCA (Boc = *t*-butyloxycarbonyl; Peptides International, Louisville, KY, USA); 2) chymotrypsin substrate, Suc-AAPF-MCA (Suc = succinyl; Bachem, Torrance, CA, USA); 3) proprotein convertase (PC) substrates; pERTKR-MCA (Peptides International) and Boc-RVRR-MCA (Bachem). MCA substrates were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and diluted to 0.2 mM with artificial seawater (ASW) just prior to use. The enzyme assay was performed in a final volume of 100 µl reaction in a black 96-well flat bottom plate (Corning Incorporated, Corning, NY, USA) at room temperature. The reaction mixture comprised of 5-10 µl the tested samples (live S- and T-sperm), 10 µl of 0.2 mM substrate solution and 80-85 µl of substrate buffer (10 mM $CaCl_2$, 0.001% Triton X-100 in 50 mM Tris-HCl, pH 7.5 for trypsin assay; 10 mM $CaCl_2$ in 50 mM Tris-HCl, pH 8.0 for chymotrypsin assay; and 1 mM $CaCl_2$, 0.5% Triton X-100 in 100 mM HEPES, pH 7.5 for PC assay). The free AMC being released was measured at various time points using a Spectra Max Gemini XS (Molecular Dynamics, Sunnydale, CA, USA) with the excitation and emission wavelengths of 360 and 470 nm, respectively. The obtained fluorescent values were converted to the amount of free AMC released by comparing with the AMC standard curve. One unit of the enzyme activity was

defined as picomoles of AMC released per hour at 25ºC. Specific activity of the enzyme was given as unit of activity per microgram protein.

The trypsin inhibition assay was also performed using various trypsin inhibitors including soybean trypsin inhibitor (SBTI), amidino phenylmethanesulfonyl fluoride (APMSF), and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma Co.) at various concentrations (1, 10 and 100 μM). The inhibitors were pre-incubated (30 min, room temperature) with the samples before performing the enzyme assay under the same conditions described above. The remaining enzyme activity was expressed as mean ± S.D. of percent inhibition calculated from three replicates.

Staining Thelycal Sperm with Alexa 488 Conjugated Soybean Trypsin Inhibitor in

T-sperm were fixed with 4% paraformaldehyde for 1 h and washed several times with ASW by centrifugation (500 ×g, 5 min). Subsequently, the sperm (~5×10^6 sperm/ml) were incubated with 4% BSA for 1 h followed by 2 μg/ml Alexa 488-conjugated SBTI (Molecular probe, Eugene, OR, USA) for 2 h at room temperature. The sperm were then washed 3 times with ASW containing 0.01% tween 20 by centrifugation (500 ×g, 5 min), mounted onto a glass slide and viewed under a confocal laser scanning microscope using sets of filters of excitation/emission wavelength for 488/520 nm. Since S-sperm exhibited very little TLA , S-sperm stained with Alexa 488 conjugated SBTI was used as a negative control.

Gelatin Zymography

Proteolytic activities in the sperm and T-fluid were visualized by zymography according to the method described by Caballero et al. (2001) with some modifications. Protein extracts of S- and T-sperm as well as T-fluid were separated electrophoretically under a non-reducing condition in 10% polyacrylamide gel containing 0.3% (w/v) gelatin. The gel was immersed twice, for 30 min each, in 50 mM Tris-HCl, pH 8.0 containing 3% (w/v) Triton X-100, washed and incubated at 37°C, overnight in the incubation buffer (50 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, 50 mM $CaCl_2$, pH 7.6) with gentle shaking. The gel was then stained with 0.025% Coomassie Brilliant Blue R-250. Protein bands containing protease activity appeared translucent against intensely blue background. Purified bovine pancreatic trypsin (Sigma Co.) (0.05-0.1 μg) was also co-electrophoresed as a positive control. Alternatively, trypsin inhibitors including SBTI, APMSF and TLCK were also included into the sperm proteins (10 μM for SBTI and 100 μM for APMSF and

TLCK) before being loaded into the gel. Visualization of the remaining protease activity in the protein bands was followed by the same protocol as described above.

***In vitro* incubation of spermatophoric sperm in thelycal (T-) fluid**

T-fluid used in this experiment was prepared from sperm-free thelyca as mentioned earlier. The enzyme activity in T-fluid was determined and only those T-fluids with high total enzyme activity (~4,000-6,000 pmol AMC released per one hour) were used in the following experiments. An *in vitro* incubation assay of S-sperm in T-fluid was performed as followed. Approximately 3×10^6 S-sperm were incubated (2 h, room temperature) with T-fluid in a presence or an absence of 1 mM APMSF. Artificial seawater (ASW), hemolymph (HL) and spermatophore fluid (S-fluid) were also used for incubating with S-sperm as a control. After washing, the sperm were stained with propidium iodine to check the sperm viability, and then used in the assay for the TLA with BOC-QAR-MCA substrate. Two samples were prepared for the assay from 6×10^5 sperm: 1) live sperm representing sperm surface activity, and 2) sperm lysate representing the total sperm protease activity. Furthermore, total sperm proteolytic activity was also visualized by zymography as described earlier.

RESULTS

Serine Protease Activities in V-, S- and T-Sperm

Four fluorogenic peptidyl-MCA substrates were used to demonstrate the activities of trypsin-like, chymotrypsin-like and PC-like enzymes in sperm collected from the vas deferens, spermatophores and thelycums. Results in Table 1 revealed that T-sperm extracts had the highest hydrolytic activity (589.19 ± 253.42 units/μg protein) toward trypsin substrate (Boc-QAR-MCA). A lower hydrolytic activity (31.57 ± 24.29 units/μg protein) was observed for one PC substrate, pERTKR-MCA, whereas a lowest activity of 3.51 ± 3.41 and 5.40 ± 4.77 units/μg protein for another PC substrate (Boc-RVRR-MCA) and chymotrypsin substrate (Suc-AAPF-MCA), respectively. In contrast to T-sperm, V- and S-sperm exhibited very low proteolytic activities (less than ~0.6 units/μg protein) for all four fluorogenic peptidyl-substrates used in this study. These results suggested that T-sperm, but not V- or S-sperm, possessed very high trypsin-like activities and moderate to low PC and chymotrypsin-like activities.

Substrate Preference and Inhibitor Study of Trypsin-like Enzymes in T-sperm

It has been shown in ascidian sperm that various trypsin-like enzymes (e.g., acrosin and spermosin) have a different degree of interaction with the peptide sequences upstream to their R/K cleavage site (Sawada et al., 1984b; Sawada and Someno, 1996). We thus used various trypsin specific substrates with different peptidyl sequences to investigate the substrate preference for the trypsin-like enzymes in T-sperm. Tryspin specific substrates used in this experiment included Boc-QAR-MCA, Boc-FSR-MCA, Boc-VPR-MCA, Boc-LRT-MCA and Boc-LSTR-MCA. The results revealed that Boc-QAR-MCA was the most preferred trypsin-specific substrate for T-sperm, and was thereby set as 100% substrate preference for the comparison purpose (Fig. 1). The substrate preference for the other 4 substrates were ~39%, 38%, 22%, and 18% for Boc-VPR-MCA, Boc-FSR-MCA, Boc-LTR-MCA, and Boc-LSTR-MCA, respectively (Fig.1).

Inhibitions of trypsin-like enzymes of T-sperm by specific trypsin inhibitors were tested using SBTI, APMSF and TLCK. The hydrolytic activity of T-sperm against Boc-QAR-MCA was dramatically inhibited when SBTI was included (Fig. 2). At 1 μM SBTI, the inhibition was as high as 91.0 ± 0.7% while SBTI at 10 and 100 μM caused inhibition at 92.8 ± 1.7% and 96.0 ± 0.4%, respectively. The inhibition by APMSF was less effective and concentration dependent, as 1, 10 and 100 μM APMSF caused inhibition at 8.3 ± 1.7%, 50.8 ± 8.9%, and 76.9 ± 1.4%, respectively. TLCK was the least effective inhibitor as 100, 10, 1 μM of TLCK causedinhibition at 32.0 ± 2.2%, 11.3 ± 5.4%, and 0.0 ± 10.8%, respectively).

Staining of T-sperm with Alexa 488-conjugated SBTI in

Since SBTI has shown the highest inhibition of T-sperm trypsin-like enzymes (Fig. 2), we further investigated the presence of these enzymes in T-sperm by staining with Alexa 488-conjugated SBTI. The positive fluorescence staining was shown exclusively in the subacrosomal region and base of the anterior spike of T-sperm (Fig. 3, a and c). Moreover, in the sperm that underwent acrosome reaction (AR), the positive staining for SBTI was detected specifically at the spherical mass which was formed from the subacrosomal region during the AR (Fig. 3c, arrow head). In contrast, S-sperm, having a very little TLA, did not show any positive staining for SBTI (Fig. 3d).

Zymography of T-sperm Trypsin-like Enzymes and the Presence of Proteases in Thelycal Fluid

We further identified the trypsin-like enzymes in T-sperm by gelatin zymography with or without trypsin inhibitors. Zymogram in Fig. 4 revealed that T-sperm exhibited a broad proteolytically active bands, ranging from 33-48 kDa (lane 1). The bands with the most intense protease activity were positioned at 46 and 48 kDa. The sperm protease activities were diminished upon treating the T-sperm extracts with trypsin inhibitors. SBTI and APMSF inhibited most of T-sperm proteases, with only weak enzyme activities remaining at the 36 and 46 kDa bands (for SBTI treatment) and at the 48 kDa (for APMSF treatment) (Fig. 4; lanes 2 and 3, respectively). On the other hand, TLCK showed little inhibition of T-sperm proteases on the zymogram and the two major bands of 46 and 48 kDa were still present (Fig. 4; lane 4).

Since the S-sperm showed very little proteolytic activity (Table 1), it is indicative that proteases were transferred to the sperm only when they were deposited in the female thelycum. This was supported by the zymogram of T-fluid which demonstrated that there were at least three major active protease bands at the molecular masses of 35, 46 and 48 kDa (Fig. 5A). Based on their mobilities, the upper two active protease bands (46 and 48 kDa) in T-fluid were identical to the two major active bands in T-sperm (Fig. 5A). This result suggested that the two active protease bands present in T-sperm could be derived from T-fluid during sperm storage in the thelycum. It should be noted that T-sperm also showed some minor active protease bands at the molecular masses of 28, 30, 33, and 36 kDa, while S-sperm possessed no proteolytically active bands in their protein extracts (Fig. 5A). The corresponding Coomassie blue-stained gels showed two major protein bands at molecular masses of 83 and 72 kDa in T-fluid, while these two bands were shown in less amount in S- and T-sperm (Fig. 5B). Moreover, in T-fluid, there were some moderate protein bands at molecular mass of 34, 35, 37, 38, 46, 55, and 68 kDa. T-sperm showed some moderate bands at molecular mass of 28, 30, 33, 36, 44, and 54, and only faint bands at 46 and 48 kDa (Fig. 5B).

In vitro Acquisition of Thelycal Enzymes onto S-sperm

We further performed the *in vitro* co-incubation of S-sperm (having very little enzymatic activity) with T-fluid to test whether trypsin-like enzymes residing in the sperm could be transferred from the T-fluid. Upon incubation with T-fluid for 2 h, the TLA detected in S-sperm increased in both live sperm sample (representing TLA on the sperm surface) and sperm lysate (representing total sperm TLA) which were

estimated to be 20,589 ± 12,707 and 24,671 ± 8,249 fluorescent units, respectively (Fig. 6A). In contrast, S-sperm incubated with T-fluid that was pretreated with 1 mM APMSF showed relatively low enzyme activity in both the live sperm and the sperm lysate (1,092 ± 1,117 and 1,529 ± 1,641 fluorescent units, respectively) (Fig. 6A). In the control experiment where ASW, HL, or S-fluid was used for incubation, S-sperm exhibited no trypsin-like enzyme activity (Fig. 6A).

The sperm treated under the three conditions above were further subjected to zymography to illustrate active protease bands in T-fluid-treated S-sperm. The result revealed that two major active protease bands with molecular masses of 46 and 48 kDa were visible exclusively when S-sperm were treated with T-fluid (Fig. 6B; lane 2). On the other hand, S-sperm treated with APMSF pre-treated T-fluid or with ASW showed only background intensity of these two protease bands (Fig. 6B; lanes 3 and 4, respectively). Taken together, our results suggested that the active-trypsin-like enzymes present in T-fluid were translocated onto S-sperm, presumably during the sperm storage in the thelycum.

DISCUSSION

We provided evidence herein that while being stored in the female thelycum, the sperm of *P. monodon* acquired trypsin-like enzyme activity in addition to the other capacitation-like characteristics previously reported by Vanichviriyakit et al. (2004). These events could be considered as a part of the shrimp sperm "capacitation", as they enhanced sperm fertilizing capacity after being stored in this organ. Among the three sperm population collected from different regions of reproductive tracts, (V-, S-, and T-sperm), TLA was detected exclusively in T-sperm (Table 1). Although trypsin-like enzymes preferentially cleave R/ K residue at the carboxyl end of the peptide sequence, previous studies have reported that these enzymes have different preferences for substrates with different peptide sequences upstream to their cleavage site at R/K residue. For example, Ascidian acrosin prefers to hydrolyse Boc-FSR-MCA and Boc-LSTR-MCA with the highest and second highest specificity, whereas ascidian spermosin prefers to cleave Boc-VPR-MCA (Sawada et al., 1984a; Sawada and Someno, 1996). Substrate preferences of trypsin-like proteases have also been reported in *S. ingentis* sperm, which exhibited the highest specificity to Boc-FSR-MCA (Chen et al., 1994). In this study, we showed that Boc-QAR-MCA was the most preferred trypsin specific substrate for T-sperm (Fig. 1), suggesting the unique feature of *P. monodon* sperm trypsin in contrast to other species. This substrate

preference of *P. monodon* sperm trypsin-like enzymes may be determined by the presence of specific substrates present on the egg vestment of this species. We also showed that SBTI, which is a reversible serine protease inhibitor, gave the strongest inhibition to *P. monodon* sperm TLA. The staining of Alexa 488 conjugated SBTI in the base of anterior spike and the subacrosome region of T-sperm (Fig. 3 a and c) indicated that these enzymes are stored in the specific regions of the sperm. In many species of peneoid shrimp, including *P. monodon*, the anterior spike serves as a primary binding structure to the egg coat (Clark et al., 1981; Pongtippatee et al., 2007; Rios and Barros, 1997; Yudin et al., 1980), which could also be involved in digestion of the egg jelly coats. After the binding, the sperm of *P. monodon* undergo acrosome reaction which includes spike disappearance, exocytosis of acrosomal cap, and formation of a spherical mass which is formed from the subacrosome region (Kruevaisayawan et al., 2008; Pongtippatee et al, 2007). Our group has shown that the sperm-born trypsin-like proteases play an important role in modulating acrosome reaction (Kruevaisayawan et al., 2008). Apart from acrosome reaction, the sperm trypsin-like enzymes in *P. monodon* may also be involved in others steps of sperm-egg interactions. Since we also found the SBTI labeling in the spherical mass formed after the acrosome reaction, the trypsin-like enzymes in this subacrosome-derived mass could also be involved in penetration of the sperm through the egg plasma membrane.

We further characterized trypsin-like enzymes in sperm samples from male reproductive tract and female thelycum, using gelatin zymography, and the results generally confirmed enzyme activity assay. Only T-sperm exhibited protease activity with the major bands appearing at molecular weights of 46 and 48 kDa and some minor active bands at 28, 30, 33 and 36 kDa (Fig. 5A). Inhibition profiles of T-sperm protease by inhibitors indicated that the protease bands at 33, 36, 46 and 48 kDa belong to the serine protease family. However, these protease bands in T-sperm exhibited different susceptibilities to various trypsin inhibitors (SBTI, APMSF and TLCK) indicating their subtle structural differnces(Fig. 4).

Recently, our group has demonstrated that T-fluid also exhibits a high level of TLA (Kruevaisayawan et al., 2008). We further demonstrated here that the T-sperm and T-fluid shared identical 46 and 48 kDa gelatin-hydrolyzing protease bands, base on their electrophoretic motilities (Fig. 5A). The presence of protease activity on T-sperm could, therefore, be interpreted as: 1) the proteases from T-fluid were adsorbed onto

the sperm surface during their storage in the female thelycum, or 2) the conversion of inactive proenzymes in S-sperm to active enzymes in T-sperm. Evidence from our *in vitro* incubation assay of S-sperm (having minimal enzyme activity) with T-fluid (known to possess high enzyme activity) supported the first possibility. In addition, the appearance of the 46 and 48 kDa protease bands in S-sperm post-incubated with T-fluid also supported this transfer. Since trypsin substrate used in the enzyme assay (Boc-QAR-MCA) was a short peptide which was unable to pass through live cell membrane, the protease activity measured from the live sperm should represent trypsin-like enzymes that were absorbed onto the sperm surface. Furthermore, the 46 and 48 kDa gelatin-hydrolysing bands adsorbed onto the S-sperm were not easily removed by ASW washings (Fig. 6B, lane 2), suggesting a reasonably strong binding of these enzymes onto S-sperm surface. The existence of sperm surface proteases has been extensively reported from ascidians to mammals. Ascidian sperm express several surface enzymes that are important in fertilization, e.g., chymotrypsin-like enzyme (Koch et al., 1994; Sawada et al., 1983), glycosidases (fucosidase and *N*-acetylglucosaminidase) (Hoshi et al., 1994). Mouse sperm also possess many surface enzymes including acrosin (Baba et al., 1994), PC4 (Mbikay et al., 1997), and testicular serine protease (TEST5) (Honda et al., 2002). Moreover, it has been reported that an aminopeptidase activity is transferred from prostasome (membranous vesicles secreted by the prostate gland) onto human sperm whose surface is initially devoid of the enzyme activity (Arienti et al., 1997a, 1997b). Fusion of prostasome to sperm plasma membrane is believed to be modulated through both lipids and coating proteins of the prostasomes. Whether this mechanism is also true in the case of protease adsorption onto the surface of *P. monodon* sperm requires further investigation.

While the sperm protease bands at 46 and 48 kDa in T-sperm are presumably derived from the adsorption of the T-fluid proteases, the other bands detected in T-sperm, namely 28, 30, 33 and 36 kDa bands, could be the intrinsic proteases that were activated after the sperm are stored in the thelycum for a certain period. These biochemical changes in the sperm during their storage in the female thelycum could be a part of the shrimp sperm capacitation. Similar event has also been reported in sperm of *S. ingentis* taken from the female receptacle, in which several proteases at molecular weights of 30 and 46 kDa were observed in the gelatin gel (Chen et al., 1994). The study in a shrimp, *R. typus*, has also indicated the presence of 18-kDa

trypsin-like enzyme in the sperm extract (Rios and Barros, 1997). However, none of these shrimp sperm proteases have been characterized in term of amino acid compositions and nucleotide sequences, which could be used to compare their identity with the other sperm trypsin-like enzymes already discovered and documented in the bioinformatics data base. The molecular characterization and specific physiological roles of the trypsin-like proteases found in both sperm and T-fluid of *P. monodon* should be further investigated.

In conclusion, this study has shown that *P. monodon* T-sperm possessed remarkably high level of TLA, which could be due to the presences of several members of the trypsin-like enzymes that were strongly and specifically inhibited by SBTI. The proteases were likely acquired by sperm during their storage in the female thelycum. These enzymes were localized principally on the sperm surface as well as the base of anterior spike and the subacrosome region. Their acquisition and activation could be a part of capacitation process which enables the shrimp sperm to gain their full fertilizing ability.

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FIGURE LEGENDS

Fig. 1 Substrate preference for T-sperm trypsin-like enzymes. Fluorogenic trypsin-specific substrates used were Boc-QAR-MCA, Boc-VPR-MCA, Boc-FSR-MCA, Boc-LRT-MCA and Boc-LSTR-MCA. Since the highest activity was obtained with

Boc-QAR-MCA at 545.4 ± 88.5 pmol/h/μg, the hydrolase activity for this substrate is set at 100%, while the activities for other substrates were expressed as percent of substrate preference. Data are expressed as means ± standard deviations of three experiments.

Fig. 2 Inhibition of T-sperm trypsin-like proteolytic activity by SBTI, APMSF and TLCK. T-sperm were incubated with Boc-QAR-MCA in the presence and absence of trypsin inhibitors. Hydrolase activity without trypsin inhibitor was considered as 0% inhibition (or 100% activity) and used as baseline for the expression of percent inhibition by each inhibitor. Note that SBTI shows the highest inhibition compared with APMSF and TLCK. Data are expressed as means ± standard deviations of three experiments.

Fig. 3 Sperm retrieved from the thelycum of female (T-sperm, upper panel) and from spermatophore of male (S-sperm, lower panel) are stained with Alexa 488-conjugated SBTI. The positive fluorescent signal appears only in T-sperm at the subacrosomal region and base of the anterior spike (a), but not in the S-sperm (d). The corresponding phase contrast micrographs of a and d are shown in b and e, respectively. Panel c showed the merged image of a and b. Note that the SBTI positive staining was also shown in acrosome reacted sperm, specifically at the spherical mass formed from the subacrosomal region during AR (c-arrow head). Bar = 10 μm.

Fig. 4 Gelatin zymograms of T-sperm. Lane 1: untreated T-sperm extract; Lanes 2-4, T-sperm extracts treated with SBTI, APMSF and TLCK, respectively. The zymograms shown are representatives of 3 replicated experiments.

Fig. 5 Gelatin zymograms (A) and Coomassie blue-stained gels (B) of T-sperm, S-sperm and T-fluid. Mk = Protein molecular weight markers. Note similar proteolytically active bands at 46 and 48 kDa in both T-sperm and T-fluid. The results shown are representatives of 3 replicated experiments.

Fig. 6 Experiments which indicate trypsin-like proteases from T-fluid are adsorbed onto S-sperm. A) Trypsin-like activities of live sperm and sperm lysate after S-sperm incubated with T-fluid for 2 h (S-sperm + T-fluid), S-sperm incubated with APMSF treated T-fluid (S-sperm + APMSF treated T-fluid), and S-sperm incubated with ASW (S-sperm + ASW). Data are expressed as means ± standard deviations of three experiments. B) Zymograms of T-fluid (lane 1), S-sperm after 2 h incubated with the T-fluid (lane2), with APMSF treated T-fluid (lane 3), and with ASW (lane 4). Note

the presence of proteolytically active bands at 46 and 48 kDa in S-sperm after incubated with T-fluid. RFU: relative fluorescent unit.

Table 1 Proteolytic activities in live sperm collected from the vas deferens (V-), spermatophore (S-), and thelycum (T-)

Substrates	Specific Activity (Units/µg Proteins)		
	V-Sperm	S-Sperm	T-Sperm
Boc-QAR-MCA (trypsin)	0.07 ± 0.06	0.28 ± 0.17	589.19 ± 253.42
Suc-AAPF-MCA (chymotrypsin)	0.01 ± 0.01	0.05 ± 0.09	5.40 ± 4.77
pERTKR-MCA (PC)	0.00 ± 0.00	0.61 ± 0.79	31.57 ± 24.29
Boc-RVRR-MCA (PC)	0.03 ± 0.06	0.10 ± 0.08	3.51 ± 3.41

Figure 1

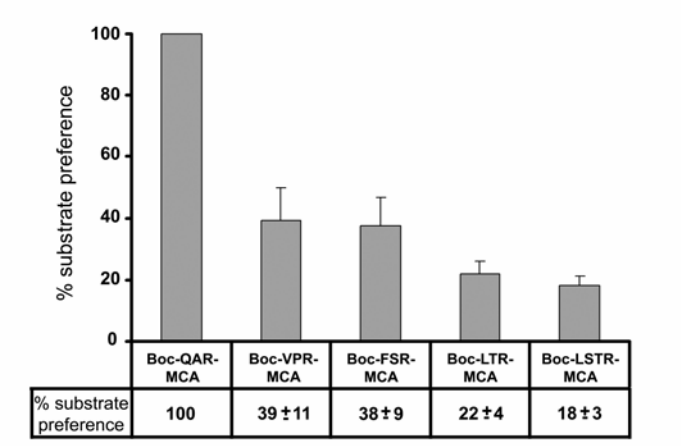

Figure 2

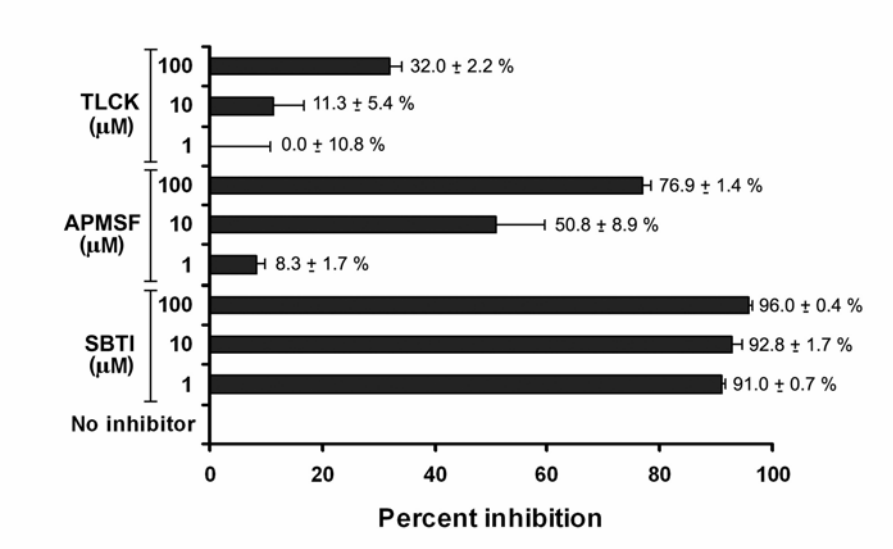

Figure 3

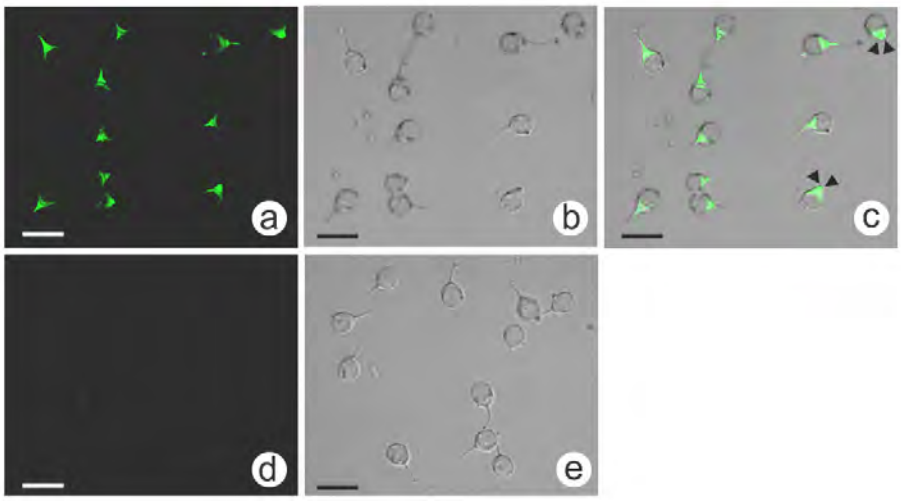

Figure 4

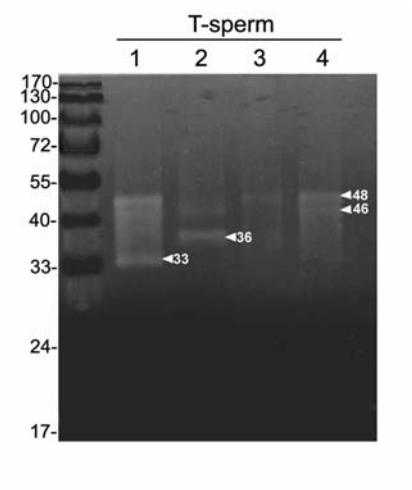

Figure 5

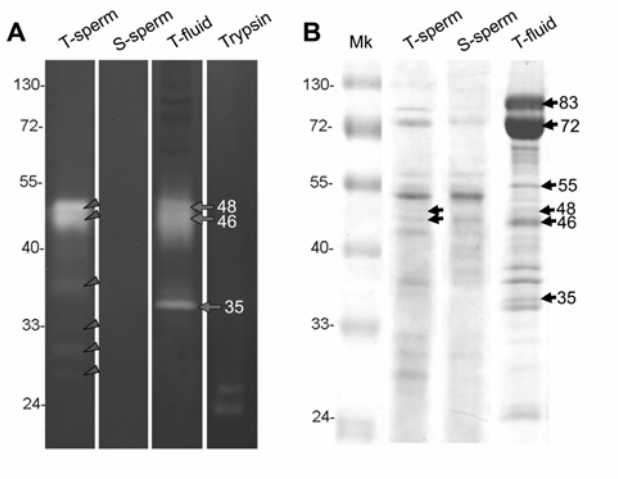

Figure 6