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Identification of reproduction-related proteins and characterization of *proteasome alpha 3* and *proteasome beta 6* cDNAs in testes of the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

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

1. Introduction

Among cultured marine shrimp, the giant tiger shrimp (*Penaeus monodon*) is one of the most economically important species (Klinbunga et al., 2001; Rosenberry, 2003; Clifford and Preston, 2006). The lack of high quality wild and/or pond-reared broodstock and the outbreak of shrimp diseases; e.g. white spot syndrome virus (WSSV), yellow head virus (YHV) and *Vibrio* sp., lead to the significant reduction of its aquacultural production since the last several years (Limsuwan, 2004). Reduced reproductive maturation in cultured *P. monodon* has limited the ability to improve commercially important traits in this species (Withyachumnarnkul et al., 1998; Klinbunga et al., 1999).

Molecular mechanisms of spermatogenesis are not well understood in penaeid shrimp and the information related to testicular development and sperm quality in *P. monodon* is rather limited (Benzie, 1998; Browdy, 1998). Spermatogenesis is a complex cell differentiation process which requires a coordinated series of both mitosis and meiosis cycle events followed by cytoskeleton reorganization for reconstructing flagellated spermatozoa (Abé, 1987). Accordingly, identification and characterization of genes and/or proteins functionally involved in gonad development are the initial step toward understanding molecular mechanisms of testicular development and sperm quality in *P. monodon* (Leelatanawit et al., 2004; 2008).

Previously, reproduction-related genes expressed in testes of *P. monodon* were identified by EST analysis. A total of 896 clones from the testis cDNA library were unidirectional sequenced and 606 ESTs (67.6%) significantly matched sequences in GenBank (*E*-value <1e−04) whereas 290 ESTs (32.4%) were novel transcripts (*E*-value >1e−04). The full-length cDNA of genes functionally involved in testicular development including *cyclophilin A, small ubiquitin-like modifier 1* (*SUMO-1*), *ubiquitin conjugating enzyme E2*, *dynactin subunit 5* and *cell division cycle 2* (*cdc2*) were identified (Leelatanawit et al., 2009).

In addition, suppression subtractive hybridization (SSH) libraries between cDNAs in testes of commercially cultured juveniles and wild broodstock were constructed to isolate differentially expressed transcripts during testicular development of *P. monodon*.

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In total, 37.1% and 53.5% of examined ESTs (178 and 187 clones from the forward and reverse SSH libraries, respectively) were significantly matched known genes. Additional reproduction-related gene homologues including *progestin membrane receptor component 1* (*PGMRC1*) and *Dmc1* were further characterized (Leelatanawit et al., 2008). Nevertheless, cellular proteomic profiles of proteins expressed during testicular development of *P. monodon* have not been reported.

Sperm quality control is an important issue for successful fertilization (Alfaro-Montoya, 2010; Alfaro-Montoya and Vega, 2011). Proteasomes are highly complex proteases responsible for selective degradation of ubiquitinated proteins in eukaryotic cells in an ATP-dependent manner. The 26S proteasome consists of two regulatory 19S cap complexes and 20S proteasome, which acts as the proteolytic or module subunit (Gueckel et al., 1998; Sakai et al., 2004). The extracellular ubiquitin–proteasome system plays a role in fertilization, gametogenesis and sperm quality control in addition to the traditional intracellular ubiquitin–proteasome system (Sakai et al., 2004). Proteasomes are functionally involved in capacitation of human sperm (Kong et al., 2009). Proteasome inhibitors inhibited the motility of salmon spermatozoa (Inaba et al., 1998). Moreover, proteasomes are also involved in the acrosome reaction in sea urchin (Matsumura and Aketa, 1991). In *P. monodon*, sperm capacitation which involves a series of complex biochemical and physiological events occurs while spermatozoa (in spermatophores) are stored in the thelycum of a female shrimp for a period of time (Vanichviriyakit et al., 2004). In penaeid shrimp, the role of proteasome in testicular development, spermatogenesis or sperm capacitation is not known at present.

In female crustaceans, serotonin (5-HT) stimulates the release of several hormones including crustacean hyperglycemic hormone (CHH; Keller et al., 1985), red pigment dispersing hormone (RPDH; Rao and Fingerman, 1970), and molt inhibiting hormone (MIH; Mattson and Spaziani, 1985). 5-HT injection induced ovarian maturation in the crayfish, *Procambarus clarkii* (Kullkarni et al., 1992; Sarojini et al., 1995) and the Pacific white shrimp, *Litopenaeus vannamei* (Vaca and Alfaro, 2000) at rates lower than unilateral eyestalk ablation. Alfaro et al. (2004) reported that injection of combined 5-HT and dopamine antagonist, spiperone stimulated ovarian maturation and spawning in *L. stylirostris* and *L. vannamei*. However, effects of 5-HT on testicular development of penaeid shrimp have not been reported.

To provide the further insight concerning molecular mechanisms of reproductive development and maturation of male *P. monodon*, proteins expressed in testes of wild and domesticated shrimp were examined by two-dimensional gel electrophoresis (2-DE). A large number of protein spots were further characterized by nanoESI-LC–MS/MS. The full-length cDNAs of *P. monodon proteasome alpha 3 subunit* (*PmPsma3*) were isolated. Expression profiles of testicular *PmPsma3* and *proteasome beta 6* (*PmPsmb6*) in different groups of wild *P. monodon* males and in 18-month-old domesticated shrimp upon 5-HT administration were further examined by quantitative real-time PCR analysis.

2. Materials and Methods

2.1. Sampling

Specimens for proteomic studies were wild male broodstock ($N = 3$, average body weight, BW = 138.88 ± 17.10 g; gonadosomatic index, GSI = 1.08 ± 0.18% and spermatophore weight = 0.37 ± 0.04 g; group A) live-caught from the Andaman Sea and domesticated F1 broodstock (cultivated in the earth pond for 14 months; hereafter called 14-month-old shrimp) collected from the Broodstock Multiplication Center (BMC), Burapha University, Chanthaburi Campus. The domesticated *P. monodon* broodstock were divided to two groups according to spermatophore weight: B ($N = 3$, average BW = 61.70 ± 2.1 g, GSI = 0.37 ± 0.05% and spermatophore weight = 0.05 ± 0.01 g) and C ($N = 3$, average BW = 67.37 ± 2.14 g, GSI = 0.31 ± 0.05% and spermatophore weight = 0.11 ± 0.01 g).

For quantitative real-time PCR analysis of *PmPsma3* and *PmPsmb6*, male domesticated *P. monodon* juveniles ($N = 3$, average BW = 37.80 ± 1.85 g, 6-month-old) and broodstock: 10-month-old ($N = 3$, average BW = 51.24 ± 3.27 g), 14-month-old ($N = 4$, average BW = 62.40 ± 3.87 g), and 18-month-old ($N = 5$, average BW = 74.10 ± 4.28 g) and wild broodstock ($N = 5$, average BW = 126.16 ± 10.75 g), were analyzed. Testes were dissected out from each shrimp, immediately placed in liquid N_2 and kept at −80 °C until needed.

To determine effects of 5-HT on expression of testicular *PmPsma3* and *PmPsmb6*, domesticated shrimp (18-month-old, $N = 44$, average BW = 74.18 ± 1.85 g) were collected and acclimated at the laboratory conditions (28-30 °C and 15 ppt seawater) in fish tanks (1000 l) for 7 days. Nine groups of male shrimp were injected intramuscularly into the first abdominal segment with 5-HT (50 μg/g body weight, $N = 4$ for each group). Specimens were collected at 0, 0.5, 1, 3, 6, 12, 24, 48 and 72 h post injection (hpi). Testes of each shrimp were dissected out, immediately placed in liquid N_2 and kept at −80 °C until needed. Shrimp without any treatment and those injected with the saline solution (0.85% at 0 hpi) were included as the negative (NC) and vehicle (VC) controls, respectively.

2.2. Total protein extraction

Approximately 500 mg of frozen testes of *P. monodon* was ground to the fine powder in the presence of liquid N_2, suspended in 300 μl of 10% (w/v) trichloroacetic acid in acetone (TCA) containing 0.1% dithiothreitol (DTT) and homogenized. Three volumes of the homogenization buffer were added and left at 20 °C for 1 h. The mixture was centrifuged at 10000 *g* for 30 min at 4 °C. The supernatant was discarded and the pellet was washed three times in acetone. The sample was centrifuged at 10000 *g* for 30 min at 4 °C. The pellet was air-dried and dissolved in the lysis buffer (30 mM Tris–HCl, 2 M thiourea, 7 M urea, 4% CHAPS, w/v). The amount of extracted protein was spectrophotometrically measured (Peterson, 1977).

2.3. Two-dimensional gel electrophoresis (2-DE)

One hundred micrograms of total testicular proteins were added to 360 μl of the rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS and 0.002% bromophenol blue) containing 2.4 mg DTT and 1% IPG buffer. Isoelectric focusing (IEF) was performed using a 18 cm Immobiline Drystrip linear pH 3–10 gradient gel strip (GE Healthcare) in an Ettan IPGphor III IEF using the step voltage focusing protocol; 500 V for 500 Vh, 1000 V for 800 Vh, 8000 V for 13500 Vh and 8000 V for 12200 Vh. All the above processes were carried out at 20 °C. After the first dimension, the IPG strip was equilibrated in the equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue; 200 ml) containing 1% DTT for 15 min and in the equilibration buffer containing 2.5% iodoacetamide for 15 min. SDS–PAGE (12.5%) was carried out initially at 2.5 W per gel for 30 min followed by 20 W per gel at 20 °C for 3–4 h. The electrophoresed proteins were visualized by silver staining.

2.4. Mass spectrometry analysis

The protein spot was individually excised from the silver-stained 2-DE gel and digested by trypsin (10 ng/μl of trypsin in