Comparative Biochemistry and Physiology, Part D 7 (2012) 180–190

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

Identification of reproduction-related proteins and characterization of the *protein disulfide isomerase A6* cDNA in ovaries of the giant tiger shrimp *Penaeus monodon*

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ARTICLE INFO

Article history:
Received 14 November 2011
Received in revised form 14 February 2012
Accepted 14 February 2012
Available online 21 February 2012

Keywords:
Proteomics
2-dimensional gel electrophoresis
Real-time PCR
Penaeus monodon
Ovarian development

ABSTRACT

Proteomic analysis was carried out for identification of proteins functionally involved in ovarian development of the giant tiger shrimp (*Penaeus monodon*). A total of 335 protein spots including 183 spots from vitellogenic (stage II) and 152 spots from mature (stage IV) ovaries of intact *P. monodon* broodstock were examined. Of these, 75 (40.98%) and 59 (38.82%) spots significantly matched known proteins in the databases, respectively. In addition, 270 protein spots including 167 and 103 spots from respective ovarian stages of eyestalk-ablated broodstock were also characterized. A total of 95 (56.89%) and 62 (60.19%) spots matched known proteins, respectively. Among differentially expressed reproduction-related proteins, the full-length cDNA of *protein disulfide isomerase A6* (*PmPDIA6*) was further characterized by RACE-PCR. *PmPDIA6* was 1946 bp in length containing an open reading frame (ORF) of 1293 bp corresponding to a polypeptide of 430 amino acids. *PmPDIA6* was up-regulated at stage III ovaries in intact shrimp ($P<0.05$). Interestingly, eyestalk ablation resulted in a lower expression level of *PmPDIA6* in each stage of ovarian development compared to that of intact broodstock ($P<0.05$). Results in this study clearly indicated the potential of cellular proteomic studies and gene expression analysis for identification of proteins/genes differentially expressed during ovarian development of *P. monodon*.

1. Introduction

The giant tiger shrimp, *P. monodon* has dominated production of farmed shrimp along with the Pacific white shrimp (*Litopenaeus vannamei*) and is one of the most economically important penaeid species in South East Asia (Rosenberry, 2003). Reduced reproductive maturation of captive *P. monodon* females is found (Kenway et al., 2006; Preechaphol et al., 2007). Accordingly, breeding of pond-reared *P. monodon* is extremely difficult and rarely produced enough quality of larvae required by the industry. In Thailand, farming of *P. monodon* relies almost entirely on wild-caught broodstock for supply of juveniles (Withyachumnarnkul et al., 1998; Klinbunga et al., 2001). The lack of high quality wild and domesticated broodstock has probably caused the reduction of aquacultural production of *P. monodon* since the last several years (Limsuwan, 2004).

Unilateral eyestalk ablation is used commercially to induce ovarian maturation of penaeid shrimp but the technique leads to an eventual loss in egg quality and death of the spawners (Benzie, 1998; Okumura, 2004; Okumura et al., 2006). Therefore, predictable maturation and spawning of captive penaeid shrimp without the use of eyestalk ablation is an ultimate goal for the industry (Quackenbush, 2001).

The domestication and selective breeding programs of penaeid shrimp would provide a more reliable supply of seed stock and the improvement of their production efficiency. The use of selectively bred stocks having improved culture performance on commercially desired traits rather than the reliance on wild-caught stocks is a major mean of sustainability of the shrimp industry (Makinouchi and Hirata, 1995; Browdy, 1998; Clifford and Preston, 2006; Coman et al., 2006).

Nevertheless, genetic improvement of *P. monodon* is slow owing to the lack of the basic information related with ovarian development and maturation in penaeid shrimp. An initial step toward understanding molecular mechanisms of ovarian and oocyte development in *P.*

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doi:10.1016/j.cbd.2012.02.003

monodon is the identification and characterization of genes/proteins differentially expressed in different stages of ovaries in this economically important species (Preechaphol et al., 2007).

Discovery of genes expressed in ovaries of *P. monodon* has been reported based on EST (single-pass sequencing of randomly selected clones from cDNA libraries, Preechaphol et al., 2007) and suppression subtractive hybridization (SSH) (Leelatanawit et al., 2004; Preechaphol et al., 2010a) analyses. Several reproduction-related genes (e.g. *anaphase promoting complex subunit 11*, *selenoprotein M precursor*, *chromobox protein homologue 1*, *ovarian lipoprotein receptor*, *progestin membrane receptor component 1* and *ubiquitin-specific proteinase 9*) were isolated. Nevertheless, cellular proteomic profiles of proteins expressed during ovarian development of *P. monodon* have not been reported.

To provide the further insight governing molecular mechanisms of reproductive development and maturation of *P. monodon*, proteins expressed in vitellogenic (stage II) and mature (IV) of intact and eyestalk-ablated broodstock of wild *P. monodon* 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 cDNA of *protein disulfide isomerase A6* (*PmPDIA6*) was further isolated. Expression profiles of the *PmPDIA6* transcript during ovarian development of *P. monodon* broodstock were further examined using quantitative real-time PCR.

2. Materials and methods

2.1. Sampling

Female broodstock of *Penaeus monodon* were wild-caught from the Andaman Sea (average body mass of 142.98 ± 28.37 g) and acclimated under the farm conditions (28–30 °C, natural daylight and 30 ppt seawater) for 2–3 days. The post-spawning group was immediately collected after shrimp were ovulated ($N=6$). Ovaries were dissected out from cultured juveniles ($N=5$) and intact broodstock and weighed ($N=34$). For the eyestalk ablation group, shrimp were acclimated for 7 days before unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2–7 days after ablation ($N=32$). The gonadosomatic index (GSI, ovarian weight/body weight × 100) of each shrimp was calculated. Ovarian developmental stages were classified by the GSI values and further confirmed by conventional histology (Qiu et al., 2005) and divided to previtellogenic (GSI < 1.5% for stage I, $N=10$ and 4 for normal and eyestalk-ablated broodstock, respectively), vitellogenic (> 2–4% for stage II, $N=7$ and 7), early cortical rod (> 4–6% for stage III, $N=7$ and 10) and mature (> 6% for stage IV, $N=10$ and 11) stages, respectively.

2.2. Total protein extraction

Initially, proteins from stages I–IV ovaries of intact and eyestalk-ablated broodstock of *P. monodon* ($N=3$ for each group) were further extracted from the organic phase after total RNA isolation by using TRI Reagent following the protocol recommended by the manufacturer (Molecular Research Center).

Subsequently, 0.5 g of frozen ovaries of *P. monodon* broodstock were ground to fine powder in the presence of liquid N_2 and suspended in a three fold-diluted PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) containing protease inhibitor cocktail (Roche) and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was collected and trichloroacetic acid in acetone (TCA; 10% w/v) was added and left at −20 °C overnight. The mixture was centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was discarded and the pellet was washed in acetone containing 0.1% dithiothreitol (DTT). The protein sample was centrifuged as above. 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 total proteins was measured by a dye binding assay (Bradford, 1976).

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

One hundred micrograms of TRI Reagent-extracted proteins or total 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 Immobiline Drystrip linear pH gradient gel strips (GE Healthacre) initially at pH 3–10 (TRI Reagent-extracted proteins) and subsequently at pH 4–7 (total proteins) in an Ettan IPGphor III. IEF was performed using the following step voltage focusing protocol: pH 3–10; 500 V for 500 Vh, 1000 V for 800 Vh, 8000 V for 13,500 Vh, 8000 V for 12,200 Vh and pH 4–7; 500 V for 500 Vh, 1000 V for 800 Vh, 8000 V for 13,500 Vh, 8000 V for 21,200 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 0.02% bromophenol blue) containing 1% DTT for 15 min and in the equilibration buffer containing 2.5% iodoacetamide and equilibrated for 15 min. SDS-PAGE was carried out using 12.5% polyacrylamide gels 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

Protein profiles from different ovarian stages of *P. monodon* were matched by Imagemaster 2D Platinum™ (GE Healthcare). Comparison of p*I* and MW between protein spots from different shrimp ($N=3$ for each stage) was performed. Only a single spot of a protein representing identical p*I* and MW was further characterized by mass spectrometry. The protein spot was individually excised from the silver-stained 2-DE gel and digested by trypsin (10 ng/µL of trypsin in 10 mM ammonium bicarbonate) at 37 °C overnight. The digested proteins were dried at 40 °C for 3–4 h and stored in −80 °C. The digested proteins were analyzed by nano-electrospay liquid chromatography ionization tandem mass spectrometry (nanoESI-LC-MS/MS). Briefly, selected protein spots were submitted to an integrated HCTultra ETD II system™ operated under a HyStar™ (Bruker Daltonics). Injected samples were first trapped and desalted on an AccLaim PepMap C18 µ Precolumn Cartridge (5 µm, 300-µm inside diameter by 5 mm) for 3 min with 0.1% formic acid delivered by a loading pump at 20 µL/min, after which the peptides were eluted from the pre-column and separated on a nano column, AccLaim PepMap 100 C18 (15 cm × 3 µm) in-line connected to the mass spectrometer: 30 min fast gradient of 4 to 96% of solvent B (80% acetonitrile in 0.1% formic acid) : solvent A (0.1% formic acid) at 300 nL/min.

2.5. Database searches

After data acquisition, MS/MS ions from nanoESI-LC-MS/MS were identified using Mascot (http://www.matrixscience.com) searched against data in the *P. monodon* database (http://pmonodon.biotec.or.th) and those in the non-redundant protein sequence (nr) database (http://www.ncbi.nlm.nih.gov) and SWISSPROT (http://expaxy.org/). The peptide charge was 1+, 2+ and 3+ with MS/MS ion mass tolerance of ±1 Da, fragment mass tolerance of ±0.5 Da, and allowance for 1 miss cleavage. Variable modification was methionine oxidation and cysteine carbamidomethylation. The significant hit proteins were considered when the Mascot score ≥ 45 ($P<0.05$) and regarded as the positive identification if the characterized results conformed with molecular weight (MW)/ isoelectric point (p*I*) values. The biological and molecular functions of the matched proteins were further searched