



Identification, characterization and expression of *adipose differentiation-related protein (ADRP)* gene and protein in ovaries of the giant tiger shrimp *Penaeus monodon*

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ARTICLE INFO

Article history:

Received 31 July 2009

Received in revised form 11 March 2010

Accepted 12 June 2010

Keywords:

Adipose differentiation-related protein

Ovarian development

Real-time PCR

Penaeus monodon

Recombinant protein

ABSTRACT

In this study, the full length cDNA of *adipose differentiation-related protein*, a gene involved in neutral lipid accumulation that is important in oocyte maturation, of the giant tiger shrimp, *Penaeus monodon* (*PmADRP*) was identified and characterized. *PmADRP* was 1292 bp in length containing an ORF of 1107 bp corresponding to a polypeptide of 368 amino acids. *PmADRP* was abundantly expressed in ovaries but not in testes of *P. monodon* juveniles and broodstock or several tissues (e.g. intestine, eyestalk and gill) of *P. monodon* broodstock. The expression levels of *PmADRP* in ovaries of juvenile *P. monodon* were significantly induced following 5-HT administration at 48 h post treatment ($P < 0.05$). Quantitative real-time PCR illustrated that the *PmADRP* mRNA in ovaries of juveniles was lower than that of broodstock ($P < 0.05$). In intact broodstock, *PmADRP* was significantly up-regulated at early cortical rod (stage III) ovaries and after spawning ($P < 0.05$). Likewise, the expression level of *PmADRP* in early cortical rod and mature (IV) ovaries was significantly greater than that in previtellogenic (I) and vitellogenic (II) ovaries of eyestalk-ablated broodstock ($P < 0.05$). *In situ* hybridization indicated that *PmADRP* was localized in ooplasm of previtellogenic oocytes. The *PmADRP* protein was detected since the vitellogenic stage for intact broodstock and since the previtellogenic stage for eyestalk-ablated broodstock. Results suggested functionally important roles of *PmADRP* on oocyte and ovarian development of *P. monodon*.

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1. Introduction

Ovarian maturation of the giant tiger shrimp (*Penaeus monodon*) results from rapid synthesis and accumulation of a major yolk protein (vitellin) (Meusy and Payen, 1988; Yano and Hoshino, 2006). During vitellogenesis, neutral lipids accumulate and form oil droplets intracellularly, and this process is a prerequisite for oocyte maturation of *P. monodon* (Meusy and Payen, 1988; Yano, 1995; Clark et al., 1990; Tsukimura, 2001).

Lipid droplets are formed by a unique monolayer of amphipatic phospholipids surrounding a central hydrophobic core of neutral lipids, mainly consisting of triacylglycerol (TAG) and sterol esters

(Ravid et al., 1999; Listenberger et al., 2007). Two proteins; perilipin and adipose differentiation-related protein (ADRP also known as adipophilin) have been studied for their properties to specifically localize at the surface of these organelles (Brasaemle et al., 1997; Lu et al., 2001).

Adipogenesis is a complex process controlled by the interplay of intracellular factors and environmental signals. During the differentiation, a large number of genes have to be regulated in a selective, coordinated manner, and dramatic changes occur in both cell morphology and gene expression (MacDougald and Lane, 1995).

Unilateral eyestalk ablation is practically used 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 a long-term goal for the industry (Quackenbush, 2001).

Knowledge of the molecular mechanisms and functional involvement of reproduction-related genes and proteins in ovarian

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development of *P. monodon* is necessary for better understanding of the reproductive maturation of *P. monodon* to resolve the major constraint of this economically important species in captivity (Preechaphol et al., 2007; Klinbunga et al., 2009).

ADRP is functionally related to neutral lipid accumulation. This protein is generally up-regulated in parallel with stored lipid during lipid droplet formation and is present on the surface of lipid droplets from the earliest time of their synthesis (Brasaemle et al., 1997; Imamura et al., 2002; Wang et al., 2003). In addition, ADRP is regarded as a gonadotropin- and prostaglandin-regulated protein in periovulatory follicles of primates (Seachord et al., 2005).

To examine molecular involvement of ADRP on ovarian (and oocyte) development of *P. monodon*, the full length cDNA and recombinant protein of *PmADRP* were characterized and reported for the first time in penaeid shrimp. Expression patterns of *PmADRP* gene and protein during ovarian development of intact and eyestalk-ablated *P. monodon* were examined by quantitative real-time PCR and western blot analysis, respectively.

2. Materials and methods

2.1. Experimental animals and design

Cultured juveniles (4 months old, $n=6$) and domesticated broodstock of *P. monodon* (14 months old, $n=6$) were collected from the Broodstock Multiplication Center, Burapha University (Chanthaburi, Thailand). Female broodstock were wild-caught from the Andaman Sea and acclimated under the farm conditions for 2–3 days. The post-spawning group was immediately collected after shrimp ovulated ($n=6$). Ovaries were dissected out from each shrimp and weighed. For the eyestalk ablation group, shrimp were acclimated for 7 days prior to unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2–7 days after ablation. The gonadosomatic index (GSI, ovarian weight/body weight $\times 100$) of each shrimp was calculated. Ovarian developmental stages were classified by conventional histology (Qiu et al., 2005) and divided to previtellogenic (I, $n=10$ and 4 for intact and eyestalk-ablated broodstock, respectively), vitellogenic (II, $n=7$ and 6), early cortical rod (III, $n=7$ and 9) and mature (IV, $n=9$ and 11) stages, respectively.

In addition, juvenile *P. monodon* (5-month-old) were purchased from a commercial farm in Chonburi (eastern Thailand) and acclimated for 7 days at the laboratory conditions (28–30 °C and 30 ppt seawater) in 150-liter fish tanks. Four groups of female shrimp (approximately 35 g body weight) were injected intramuscularly into the first abdominal segment with 5-HT (50 $\mu\text{g/g}$ body weight, $n=5$ for each group). Specimens were collected at 12, 24, 48 and 72 h post treatment (hpt). Shrimp injected with the 0.85% saline solution (at 0 hpi) were included as the control. For tissue distribution analysis, various tissues of a female and testes of a male broodstock were collected, immediately placed in liquid N_2 and kept at -70 °C until needed.

2.2. Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from ovaries of *P. monodon* using TRI Reagent (Molecular Research Center). The concentration of extracted total RNA was spectrophotometrically measured. One and a half microgram of DNase I-treated total RNA was reverse-transcribed using an Improm-II™ Reverse Transcription System (Promega).

2.3. Rapid amplification of cDNA end-polymerase chain reaction (RACE-PCR) and multiple sequence comparisons

Messenger (m) RNA was purified from total RNA using a QuickPrep Micro mRNA Purification Kit (GE Healthcare). The purified mRNA was kept in the absolute ethanol at -70 °C prior to reverse

transcription. A gene specific primer (ADRP-I: R: 5'-CCATCTGTGCTGTAAGGTCG-3') was designed. 5' RACE-PCR was carried out using a SMART RACE cDNA Amplification Kit following the protocol recommended by the manufacturer (BD Bioscience Clontech). The amplified fragment was electrophoretically analyzed, eluted from the gel, cloned into pGEM-T Easy vector and sequenced. Nucleotide sequences of EST and 5' RACE-PCR fragments were assembled and searched against previously deposited sequences in the GenBank using BlastN and BlastX (Altschul et al., 1990; available at <http://ncbi.nlm.nih.gov>). The *pI* value and molecular weight of the deduced *PmADRP* protein were examined using ProtParam (<http://www.expasy.org/tools/prot-param.html>). The protein domain and signal peptide in the deduced *PmADRP* protein were predicted using SMART (<http://smart.embl-heidelberg.de>).

2.4. Semiquantitative RT-PCR and tissue distribution analysis

Expression levels of *PmADRP*₂₈₂ (F: 5'-TCITGCTCTGCCTGTGCTT-3' and R: 5'-CCGTTGGCTTGGTTATGATG-3') in ovaries and testes of wild broodstock ($n=11$ for each sex) and testes of cultured juveniles ($n=5$ for each sex) were analyzed by RT-PCR. *EF-1 α* ₅₀₀ (F: 5'-ATGGTTGCAACTTTGCC-3' and R: 5'-TTGACTCCTTGATCACACC-3') amplified from the same template was included as the positive control. The thermal profiles were 94 °C for 3 min followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s and extension at 72 °C for 45 s. The final extension was carried out at 72 °C for 7 min. Tissue distribution analysis of *PmADRP* in various tissues of female and testes of male broodstock was performed for 30 cycles using the same conditions.

Effects of 5-HT on expression of ovarian *PmADRP*₂₈₂ were analyzed by semiquantitative RT-PCR. Initially, nonquantitative RT-PCR was carried out using 100 ng of the first strand cDNA as the template with varying concentrations of primers (0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.40 μM , respectively). The optimal concentration of MgCl_2 (1.0, 1.5, 2.0, 2.5 and 3.0 mM) was further selected using the optimized primer concentration. Finally, RT-PCR of *PmADRP*₂₈₂ was performed using the optimized primer and MgCl_2 concentrations for 18, 20, 22, 25, 28 30 and 35 cycles. The number of cycles before the product reached amplification plateau was chosen. Semiquantitative RT-PCR was carried out as the following: 94 °C for 3 min, 20 cycles of 94 °C for 30 s, 53 °C for 45 s and 72 °C for 45 s followed by 72 °C for 7 min (with 0.15 μM and 1.5 mM of primer and MgCl_2 concentrations, respectively). The amplicon was electrophoretically analyzed through 1.5% agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001). The intensities of *PmADRP*₂₈₂ and *EF-1 α* ₅₀₀ were quantified from the gel photograph using the Quantity One software (Bio-Rad). Relative expression levels of investigated transcripts (intensity of *PmADRP*₂₈₂/intensity of *EF-1 α* ₅₀₀) in all experimental groups of *P. monodon* were statistically tested using analysis of variance (ANOVA) followed by the Duncan's new multiple range test. Results were considered significant when $P<0.05$.

2.5. Quantitative real-time PCR

Standard curves representing 10^3 – 10^8 copies of recombinant plasmids of *PmADRP*₁₃₇ (ADRP137-F: 5'-ATGGCTCCGAACACGAGTAATGCAC-3' and ADRP137-R: 5'-ACATACTGGTATCGGTCTTGGGTA-3') and the internal control, *EF-1 α* ₂₁₄ (F: 5'-TTCTCTTCCCTTCAGGACGTC-3' and R: 5'-CTTACAGACAGTCTTTCACGTTG-3'), were constructed. *PmADRP*₁₃₈ and *EF-1 α* ₂₁₄ in ovaries of each shrimp were amplified in a 10 μl reaction volume containing 5 μl of 2 \times LightCycler 480 SYBR Green I Master (Roche), 50 ng the first strand cDNA template, 0.3 μM each primer. The thermal profile for quantitative real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Real-time PCR of each specimen was carried out in

duplicate. The relative expression level (copy number of *PmADRP137*/that of *EF-1 α ₂₁₄*) between shrimp possessing different stages of ovarian development were statistically tested ($P < 0.05$).

2.6. In situ hybridization

Ovaries of intact and eyestalk-ablated *P. monodon* broodstock were fixed in 4% paraformaldehyde prepared in 0.1% phosphate-buffered saline (PBS, pH 7.2) overnight at 4 °C. The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at –20 °C until used. Conventional paraffin sections (5 μ m) were carried out. The sense and anti-sense cRNA probes *ADRP-T7/F* (5'-TAATACGACTCACTATAGGGTTACCCAAGACCGATACCAGTATGT-3') and *ADRP-R/SP6* (5'-ATTTAGGTGACACTATA-GAAATTACAGATTCCAGACACCCCTTCAG-3') containing the T7 (underlined) and SP6 (italicized and underlined) promoter sequences, respectively, were synthesized using DIG RNA labeling mix (Roche). Tissue sections were dewaxed with xylene and dehydrated in absolute ethanol. The sections were prehybridized with 2 \times SSC containing 50% deionized formamide, 1 μ g/ μ l yeast tRNA, 1 μ g/ μ l salmon sperm DNA, 1 μ g/ μ l BSA and 10% (w/v) dextran sulfate at 50 °C for 30 min and hybridized with either the sense or antisense probe in the prehybridization solution overnight at 50 °C. After hybridization, the tissue sections were washed twice with 4 \times SSC for 5 min each and once with 2 \times SSC containing 50% formamide for 20 min at 50 °C. The sections were immersed in prewarmed RNase A buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 37 °C for 30 min and treated with RNase A (10 μ g/ml) at 37 °C for 30 min. Tissue sections were washed four times with the RNase A buffer (37 °C, 10 min each) and 2 \times SSC (50 °C, 15 min each). High stringent washing was carried out twice in 0.2 \times SSC at 50 °C for 20 min each. Detection of the positive hybridization signals was carried out with a DIG Wash and Block Buffer kit (Roche) (Qiu and Yamano, 2005).

2.7. In vitro expression of recombinant PmADR

Plasmid DNA containing the full length cDNA of *PmADRP* was used as the template for amplification using the forward and reverse primers of *PmADRP* (F: 5'-CCGCATATGCTCCGAACACGAGTAAT-3' and R: 5'-CGGGATCCTCAATGATGATGATGATGCTGCCAACACAGATGTGG-3') containing a *Nde* I site (underlined) and a *Bam* HI site (italicized) and six His encoded nucleotides (boldfaced), respectively. The amplification product was digested with *Nde* I and *Bam* HI and analyzed by agarose gel electrophoresis. The gel-eluted product was ligated into pET15b and transformed into *E. coli* JM109. The recombinant plasmid was subsequently transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL. A bacterial colony carrying recombinant plasmid of *PmADRP* was inoculated into 3 ml of LB medium, containing 50 μ g/ml ampicillin and 50 μ g/ml chloramphenicol at 37 °C and 50 μ l of the overnight culture was transferred to 50 ml of LB medium containing ampicillin and chloramphenicol and further incubated to an OD₆₀₀ of 0.4–0.6. The culture was time-interval taken at 1, 2, 3, 6, 12 and 24 h after IPTG induction (1.0 mM final concentration). One OD₆₀₀ milliliter was centrifuged at 12,000 g for 1 min, resuspended with 1 \times PBS and analyzed by 15% SDS-PAGE (Laemmli, 1970). In addition, 50 ml of the IPTG-induced culture (3 h) were centrifuged, resuspended in 1 \times PBS and sonicated 2–3 times at 15–30% amplitude, pulsed on for 10 s and pulsed off for 10 s in a period of 2–5 min. The protein concentration of both soluble and insoluble fractions was measured (Bradford, 1976). Overexpression of rPmADRP was analyzed by 15% SDS-PAGE. For western blot analysis, the electrophoresed proteins were transferred to a PVDF membrane (Towbin et al., 1979) and analyzed as previously described in Imjongjirak et al. (2005).

2.8. Purification of recombinant proteins

Recombinant protein was purified by using a His GraviTrap kit (GE Healthcare). Initially, 1-l of IPTG-induced culture at the optimal time and appropriate temperature was harvested by centrifugation at 6000 rpm for 15 min. The pellet was resuspended in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4), sonicated and centrifuged at 6000 rpm for 40 min. The soluble and insoluble fractions were separated. The insoluble fraction composed of the recombinant protein was loaded into the column and washed with 10 ml of binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4 and 8 M urea) containing 20 mM imidazole, 5 ml of the binding buffer containing 50 mM imidazole and 5 ml of the binding buffer containing 80 mM imidazole, respectively. The recombinant protein was eluted with 6 ml of the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Each fraction of the washing and eluting step were analyzed by SDS-PAGE and western blotting. The purified proteins were stored at 4 °C for immediate use or –20 °C for long term storage.

2.9. Polyclonal antibody production and western blot analysis

Anti-PmADRP polyclonal antibody was immunologically produced in a rabbit using the purified rPmADRP as an immunogen. For western blot analysis, ovarian tissues of *P. monodon* were homogenized in the extraction buffer (50 mM Tris-HCl; pH 7.5, 0.15 M NaCl) supplemented with the proteinase inhibitors cocktail (EDTA free; Roche). The homogenate was centrifuged at 12,000 g for 30 min at 4 °C. The supernatant was collected. Protein concentrations of the tissue extract were determined by the dye binding method (Bradford, 1976). Thirty micrograms of total ovarian proteins were heated at 100 °C for 5 min and immediately cooled on ice. Proteins were size-fractionated on a 15% SDS-PAGE (Laemmli, 1970). Electrophoretically separated proteins were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) in 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at 100 V for 90 min. The membrane was treated in the DIG blocking solution (Roche) for 1 h and incubated with the primary antibody (1:500 in the blocking solution) for 1 h at room temperature. The membrane was washed 3 times with 1 \times Tris Buffer Saline-Tween20 (TBST; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween20) and incubated with goat anti rabbit IgG (H+L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:3000 for 1 h and washed 3 times with 1 \times TBST. Immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate.

3. Results

3.1. Isolation of the full length cDNA of PmADRP

The full length cDNA of *PmADRP* was 1292 bp in length containing an ORF of 1107 bp corresponding to a polypeptide of 368 amino acids. The 5' and 3' UTRs of *PmADRP* were 33 and 134 bp (excluding the poly A tail), respectively. The poly A additional signal (AATAAA) was located at 13 nucleotides upstream from the poly A tail (GenBank accession no. GU906279; Fig. 1). The closest similarity to *PmADRP* was *adipophilin* of *Canis familiaris* (E -value = $9e-18$). The calculated pI and molecular weight of the deduced *PmADRP* protein was 6.90 and 39.7 kDa, respectively. Four putative glycosylation sites were found at positions 4–6 (NTS), 7–9 (NAT), 141–143 (NDT) and 280–282 (NVT). A perilipin domain functionally involved in adipocyte lipid metabolism (Lu et al., 2001; Teixeira et al., 2003), was found at positions 8–368 (E -value = $3.9e-06$) of the deduced *PmADRP* protein.

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TACGCGGGGGTCTCTGCCAGTTTTTCGAACGAAATGGCTCCGAACACGAGTAATGCGACA 60
                                     M A P N T S N A T 9
CCGGCTGCAGAAGGCTTTTTTGGAGAGTCTTGTCTTGGCTGTGCTTAGCGACGCCATC 120
P A A E E G F F E R V L L L P V L S D A I 29
ACCATCGTTTTTCATGCCTACAAAATACCCAAGACCGATAACAGTATGTGGGAAGTGTCT 180
T I V S H A Y K I T Q D R Y Q Y V G T A 49
TTGCGAGTAGCAGAGGCAGGCATCCGGGTGGCCACCGAGGGAGCTCTCCCTCTGGCTATG 240
L R V A E A G I R V A T E G A L P L A M 69
CCCCCTTCTGCACCCCTCGTGGACCGCGTCCGGAGGATGGAGTACCCTGGATGAGTGGGCA 300
P L L H P L V D R V G G W S T L D E W A 89
TGTCGCGGTCTGGATCGCGTTGAGGAGGCAGCGCCCATCATAACCAAGTCAACGGATGAG 360
C R G L D R V E E A A P I I T K S T D E 109
ATCGTGAGTTCAGCGCGCGTCCGGTGTCTCAGCGTCTGGCGGGGAAGGACGCTCTACCC 420
I V S S A R R R V L S V V A G K D A L P 129
CCCTCCCTCTCCGCGCTCACGTCCGGGCAACGACCGTGTGACGTCTGCTCGCTCGCG 480
P S L S A A V T S R A N D T V D V I A A 149
AGTCGGGGCGGGCGAGTCTCGCGGGCGCTGCGGAGCGGGTGTCTCAACACTGCCACACG 540
S R G G R V V A G A A E R V L N T A H T 169
CTGGTGCAGCGCTACTTACCACCCCGGAGGGCGACCTTACGACACAGATGGGCGCGAC 600
L V D A Y L P P R E G D L H D T D G R D 189
GCAACGGTGGGCGTAAAGGTGATAGCGCTGGCAAGCAAGACGAGGCGGGCTTTGGCCCGC 660
A T V G V K V I A L A S K T R R R L A R 209
GCGCTTCATTCGCTCGCGCATCCGACCCACGCCACGCCACACCAACGCCGACGCGCTGT 720
A L H S L A H P H H A H A H T N A D A C 229
GACGCCACGGATGCATCGGGCAGCTTCTGATTGCCTTCAGTCGGGAGTGTGTGTCTGGC 780
D A T D A S G S F L I A F S R E C V S G 249
TGGCACTCTGAAGTCACGCGGCAGCCGGAGCCACGAGGCGGTGCCTGTAGTTTTGAGA 840
W H S E V T R Q P E P H E A V P V V L R 269
ATCGCAGCAACTTCTACAGGTACCTAAGGAACGTTACGGAGAATCTTGGATCACTCGTT 900
I A R T S Y R Y L R N V T E N L G S L V 289
GTTGCTGTTTCGCAATTCCTGGCTCGGAACGCAGTGAGAGAAGCCTTGAGCGGCGCGGC 960
V A V R N S L A R N A V R E A L S G A A 309
GCCAACGGATGTTATGTCGTTTCCGAGATGCAAGCCTGGGGAGAGTACTTTGTGTGTGTC 1020
A N G C Y V V S E M Q A W G E Y F V V V 329
ATGTGATGACGCCAAGTCTGATCGAAGAGGCTTCGCTTCGCTCCAGGGTGTGGGCGTAA 1080
M S M T P S L I E E A S L R S R V W A E 349
GGGTGTCTGGAATCTGTAATGAAAATGAAGTATACAAATCCACATCTGTGTTGGCAGTGA 1140
G C L E S V M K M K Y T N P H L C W Q * 368
GCTGACTGACGCCATCAGTGAAGACCAGAGGAACCTCCCTGAATTGTTTTTCACGGCTGGA 1200
TAATTAATCTTAATCGATGTTAGCTGTTTTGTGGTGGACGTTTGTAAATAAGGGTAAATA 1260
AAATAACTGAAAGCAAAAAAAAAAAAAAAAAA 1292
    
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Fig. 1. The full length cDNA and deduced amino acid sequences of *PmADRP*. Start (ATG) and stop (TGA) codons are illustrated in boldface and underlined (GenBank accession no. GU906279). The poly A additional signal is boldfaced. A perilipin domain is highlighted.

3.2. Tissue expression profiles and effects of 5-HT administration on *PmADRP* transcription

PmADRP was only expressed in ovaries ($n = 10$) but not in testes ($n = 10$) of both juveniles and broodstock of *P. monodon*. Tissue expression analysis further indicated that *PmADRP* was abundantly expressed in ovaries whereas low expression levels were observed in heart, hemocytes, lymphoid organ, thoracic ganglion and stomach of female broodstock (Fig. 2).

The expression level of *PMADRT* in juvenile *P. monodon* was up-regulated at 48 hpt ($0.661 \pm 0.057, P < 0.05$) upon 5-HT administration and returned to the normal levels at 72 hpt ($0.550 \pm 0.019, P > 0.05$) (Fig. 3).

3.3. Expression levels of *PmADRP* mRNA during ovarian development of *P. monodon*

Quantitative real-time PCR illustrated that the mRNA level of *PmADRP* in ovaries of juveniles was lower than that of broodstock ($P < 0.05$). In intact broodstock, *PmADRP* was significantly increased at the early cortical rod (stage III) ovaries and after spawning ($P < 0.05$). Likewise, the expression level of *PmADRP* in early cortical rod and mature (IV) ovaries was significantly greater than that in previtellogenic (I) and vitellogenic (II) ovaries of eyestalk-ablated broodstock ($P < 0.05$) (Fig. 4). The ovarian *PmADRP* mRNA in cultured broodstock

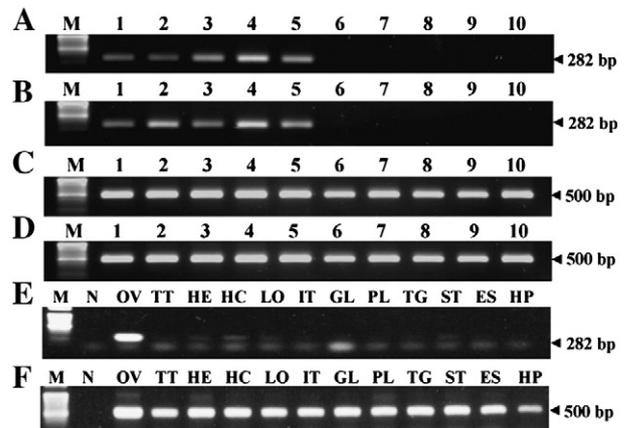


Fig. 2. A 1.5% ethidium bromide-stained agarose gel showing results from RT-PCR (A–D) and tissue distribution analysis (E and F) of *PmADRP* using the first strand cDNA of ovaries (lanes 1–5, A and B), testes (lanes 6–10, A and B) and various tissues (E and F) of broodstock-sized (A and E) and juvenile (B) *P. monodon*. *EF-1α* was successfully amplified from the same template (C, D and F). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively. OV = ovaries, TT = testes, HE = heart, HC = hemocytes, LO = lymphoid organs, IT = intestine, GL = gill, PL = pleopods, TG = thoracic ganglion, ST = stomach, ES = eyestalk and HP = hepatopancreas.

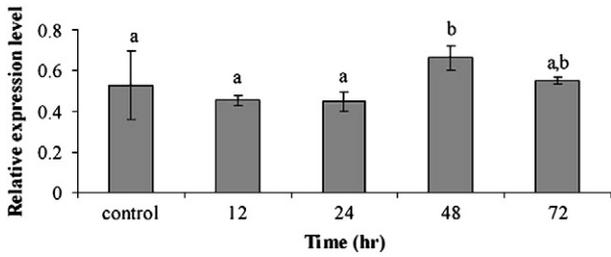


Fig. 3. Histograms showing time-course relative expression levels of *PmADRP* at 12, 24, 48 and 72 h post treatment (hpt) of 5-HT (50 µg/g BW). Shrimp injected with 0.85% NaCl was included as the control. The same letters above histograms indicate non-significant differences between different treatments ($P>0.05$).

(14-month-old) was not significantly different from that in stages I and II ovaries of both intact and eyestalk-ablated wild broodstock ($P>0.05$).

3.4. In situ hybridization

The antisense *PmADRP* probe gave clear signals in the ooplasm of previtellogenic oocytes in various ovarian stages of both intact (Fig. 5) and eyestalk-ablated (Fig. 6) broodstock. Generally, oocytes at the late previtellogenic stage showed weaker signals than early previtellogenic oocytes. Positive signals were not detected in the germinative zone, follicular cells, oogonia, and vitellogenic, early cortical rod and mature oocytes.

3.5. In vitro expression of PmADRP, polyclonal antibody production and western blot analysis

Overexpression of recombinant (r) *PmADRP* was observed after induction by 1 mM IPTG at 37 °C for 3 h. The rADRP was majorly expressed as the insoluble protein (Fig. 7). Anti-*PmADRP* polyclonal antibody was successfully produced with the relatively high titer (1:32,000 with OD₄₅₀ = 0.557 against 1 µg of purified r*PmADRP*).

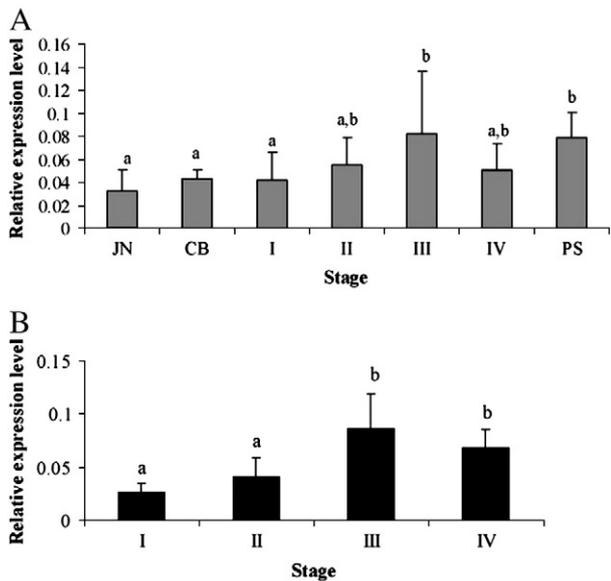


Fig. 4. Histograms showing relative expression profiles of *PmADRP* in ovaries of cultured juveniles (JN, A) and different stages of ovarian development (stages I, previtellogenic; II, vitellogenic; III, early cortical rod; and IV, mature ovaries) of intact (A) and unilateral eyestalk-ablated (B) broodstock and intact post-spawning (PS, A). Captive broodstock-sized shrimp (14 months old; CB, A) was also included. The same letters above histograms reveal non-significant differences between groups of samples ($P>0.05$).

The molecular size of ovarian *PmADRP* (39 KDa) was identical to that of r*PmADRP* suggesting that it was not posttranslationally modified by glycosylation. The immunological signals of ovarian *PmADRP* were observed in vitellogenic, early cortical rod and mature ovaries but not in premature (juveniles) and previtellogenic ovaries. Eyestalk ablation clearly promoted earlier expression of *PmADRP* in previtellogenic. *PmADRP* was also found in vitellogenic and cortical rod ovaries but not in mature ovaries of eyestalk-ablated *P. monodon* broodstock (Fig. 8).

4. Discussion

Ovaries are functionally important in reproduction and secretion of hormones for growth and development regulation (Amparyup et al., 2004; Preechaphol et al., 2007; Wu et al., 2009). The development of oocytes consists of a series of complex cellular events, in which different genes express to ensure the proper development of oocytes and to store transcripts and proteins as maternal factors for early embryogenesis (Qiu and Yamano, 2005). Understanding the role of lipid metabolism during ovarian and oocyte development of *P. monodon* requires identification of genes governing the biogenesis, trafficking and turnover of lipid droplets (Ravid et al., 1999; Teixeira et al., 2003).

Perilipin and *ADRP* genes were originally identified according to their abundant expression in adipose tissue (Greenberg et al., 1991; Jiang and Serrero, 1992). The expression of *perilipin* or *ADRP* increases the capacity of cells to take up long fatty acids from the medium and to accumulate neutral lipids of cultured cells. Reciprocally, supplementation of fatty acids to the culture medium stimulates neutral lipid accumulation in cells and increases intracellular levels of *Perilipin* or *ADRP*. (Brasaemle et al., 1997; Gao et al., 2000; Imamura et al., 2002; Souza et al., 1998; Teixeira et al., 2003). The reciprocal regulation of *perilipin/ADRP* and neutral lipid levels suggests their functional involvement in lipid metabolism regulation (Teixeira et al., 2003).

In this study, the full length cDNA of *PmADRP* was characterized and reported for the first time in penaeid shrimp. Tissue-specific transcription is important during development and during maturation of specific cell types from stem cells in the adults (Grimes, 2004). *PmADRP* was only expressed in ovaries but not in testes of *P. monodon* juveniles and broodstock. Tissue expression profiles of the *PmADRP* transcript suggested its functional importance during ovarian but not testicular development and maturation of *P. monodon*.

Transcription in germ cells during oogenesis follows carefully regulated programs corresponding to a series of developmental events in oocytes (Grimes, 2004; Qiu and Yamano, 2005; Qiu et al., 2005). Khamnamtong et al. (2006) identified sex-specific expression markers in ovaries and testes of *P. monodon* by RAP-PCR. Five (*Fl-4*, *Fl-44*, *FIII-4*, *FIII-39* and *FIII-58*) differential display-derived unknown transcripts revealed female-specific expression patterns in ovaries of 3-month-old juveniles and broodstock implying that these unknown genes should contribute to gonadal development and/or sex differentiation of *P. monodon*.

Recently, *vasa*, a member of gene the DEAD-box protein family which encodes an ATP-dependent RNA helicase was identified in the Pacific white shrimp, *Penaeus (Litopenaeus) vannamei* (Aflalo et al., 2007) and the giant freshwater prawn, *Macrobrachium rosenbergii* (Nakkrasae and Damrongphol, 2007). *Vasa* of both species was only expressed in gonads of adults and regarded as a potential biomarker for their germ cell development.

In addition, the partial cDNA sequence (5051 bp in length containing a coding region of 5031 bp corresponding to 1677 amino acids) of a transcript specifically expressed in ovaries but not in testes and other tissues of *P. monodon* broodstock was isolated and termed *Ovary-Specific Transcript 1 (Pm-OST1)*. *Pm-OST1* was comparably expressed throughout ovarian development in intact broodstock ($P>0.05$). Nevertheless, eyestalk ablation caused a significant increase

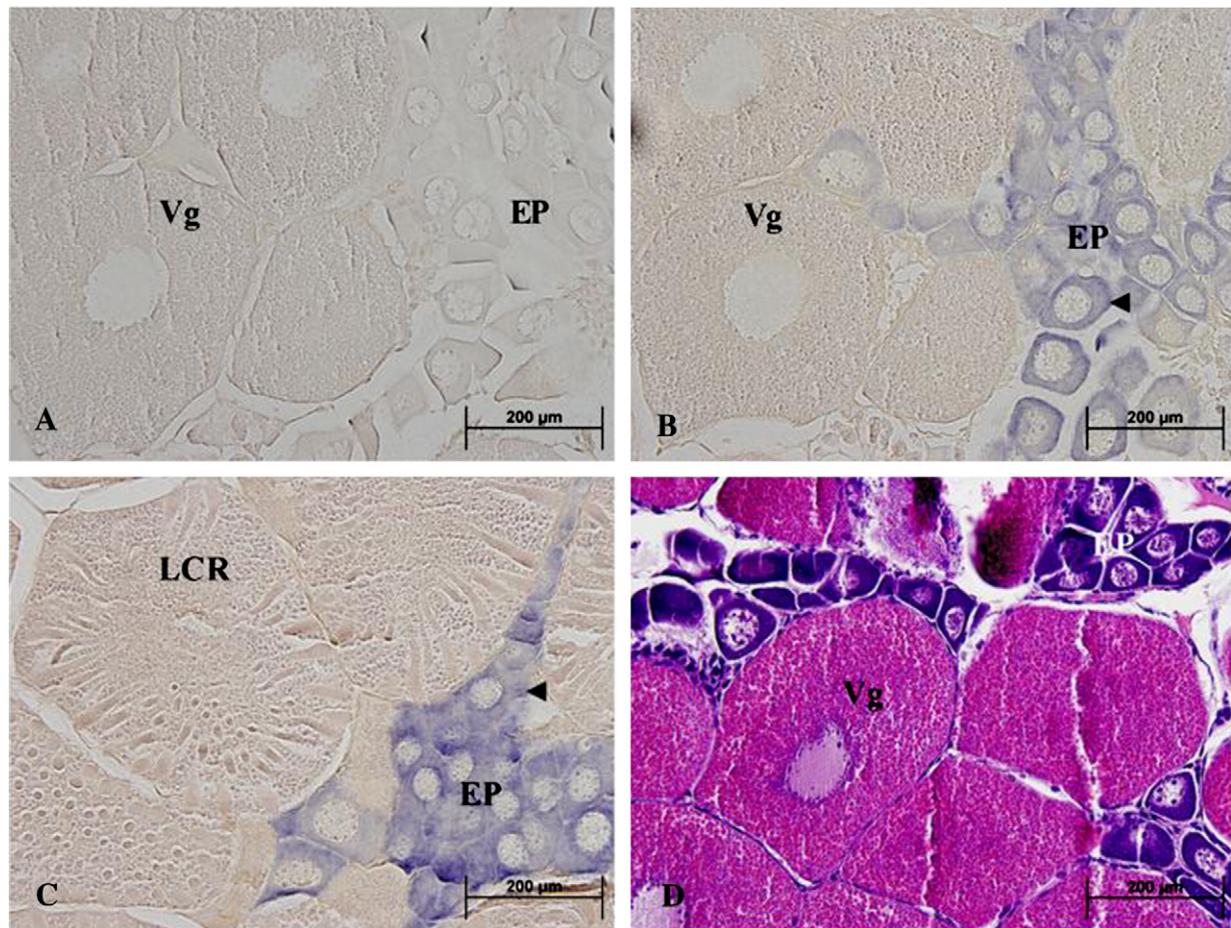


Fig. 5. Localization of the *PmADRP* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the sense *PmADRP* (A) and antisense *PmADRP* probes (B and C). A conventional HE staining was carried out for classification of oocyte stages (D). EP = early previtellogenic oocytes; Vg = vitellogenic oocytes; LCR = late cortical rod oocytes. Arrowheads indicated examples of the positively hybridized signals of *PmADRP*.

of *Pm-OST1* mRNA at the early cortical rod (III) stage. *Pm-OST1* was localized in ooplasm of previtellogenic oocytes. The results indicated that *Pm-OST1* should play the functional important role in development of germ cells and oocytes of *P. monodon* (Klinbunga et al., 2009).

A particular gene product may play multifunctions in physiological processes. Multifunctions of cathepsin C (dipeptidyl peptidase I), an active peptidase playing a major role on intracellular protein degradation, gene product during oogenesis of the kuruma prawn, *Penaeus (Marsupenaeus) japonicus* was reported (Qiu et al., 2005). In this study, tissue distribution analysis indicated that *PmADRP* should also contribute to female germ cell development and can potentially be used as a biomarker to enhance an understanding of developmental and reproductive processes in the female germ line of *P. monodon*.

Effects of exogenous 5-HT on the reproductive performance of shrimp were reported (Vaca and Alfaro, 2000). 5-HT induced ovarian development of *P. monodon* (Wongprasert et al., 2006) and *M. rosenbergii* (Meeratana et al., 2006) dose dependently. The 5-HT injection clearly promoted expression levels of *PmADRP* in ovaries of *P. monodon* subadults at 48 hpt. Molecular effects of 5-HT on expression of *PmADRP* should be further examined in both wild and domesticated broodstock to evaluate the use of 5-HT to enhance ovarian/oocyte development of *P. monodon* in place of eyestalk ablation.

In *P. monodon*, effects of dopamine on ovarian development have not been reported. Nevertheless, simultaneous injections of 5-HT (25 µg/g body weight) and the dopamine antagonist spiperone (1.5 or 5 µg/g body weight) induced ovarian maturation and spawning in wild *P. stylirostris* and pond-reared *P. vannamei* (Alfaro et al., 2004).

In monkey, administration of human chorionic gonadotropin (hCG) elevated ADRP mRNA and protein in granulosa cells, with the peak levels shortly before the expected time of ovulation. Administration with hCG and the prostaglandin (PG) synthesis inhibitor, celecoxib resulted in a lower level of ADRP mRNA levels in granulosa cells compared with those of animals treated with hCG alone suggesting that ADRP is a gonadotropin- and PGE2-regulated protein in granulosa cells of primate periovulatory follicles. Typically, ADRP facilitates arachidonic acid uptake in non-ovarian cells. Accordingly, ADRP-associated lipid droplets may enhance arachidonic acid uptake by granulosa cells to provide a precursor for periovulatory prostaglandin production (Seachord et al., 2005).

In *P. semisulcatus*, approximately equal amounts (16 mg lipid g⁻¹ protein) of phospholipids and triacylglycerols were accumulated by the end of oocyte development. Approximately 30% of these were made up of polyunsaturated fatty acids (PUFAs) indicating the occurrence of lipid transport to ovaries during oocyte maturation. The gradual decrease in the relative abundance of PUFAs following more mature ovarian developmental stages suggested intra-ovarian synthesis of saturated and monounsaturated fatty acids (Ravid et al., 1999).

In wild *P. monodon*, the ovarian lipid was increased significantly at stage II (early maturing ovaries) and remained high through stage IV (late maturing ovaries). The highest levels was found at stage III (late maturing ovaries) and dramatically decreased in spawned ovaries. The phospholipids were mainly responsible for increases in ovarian lipids of *P. monodon*. This information suggested the essential of

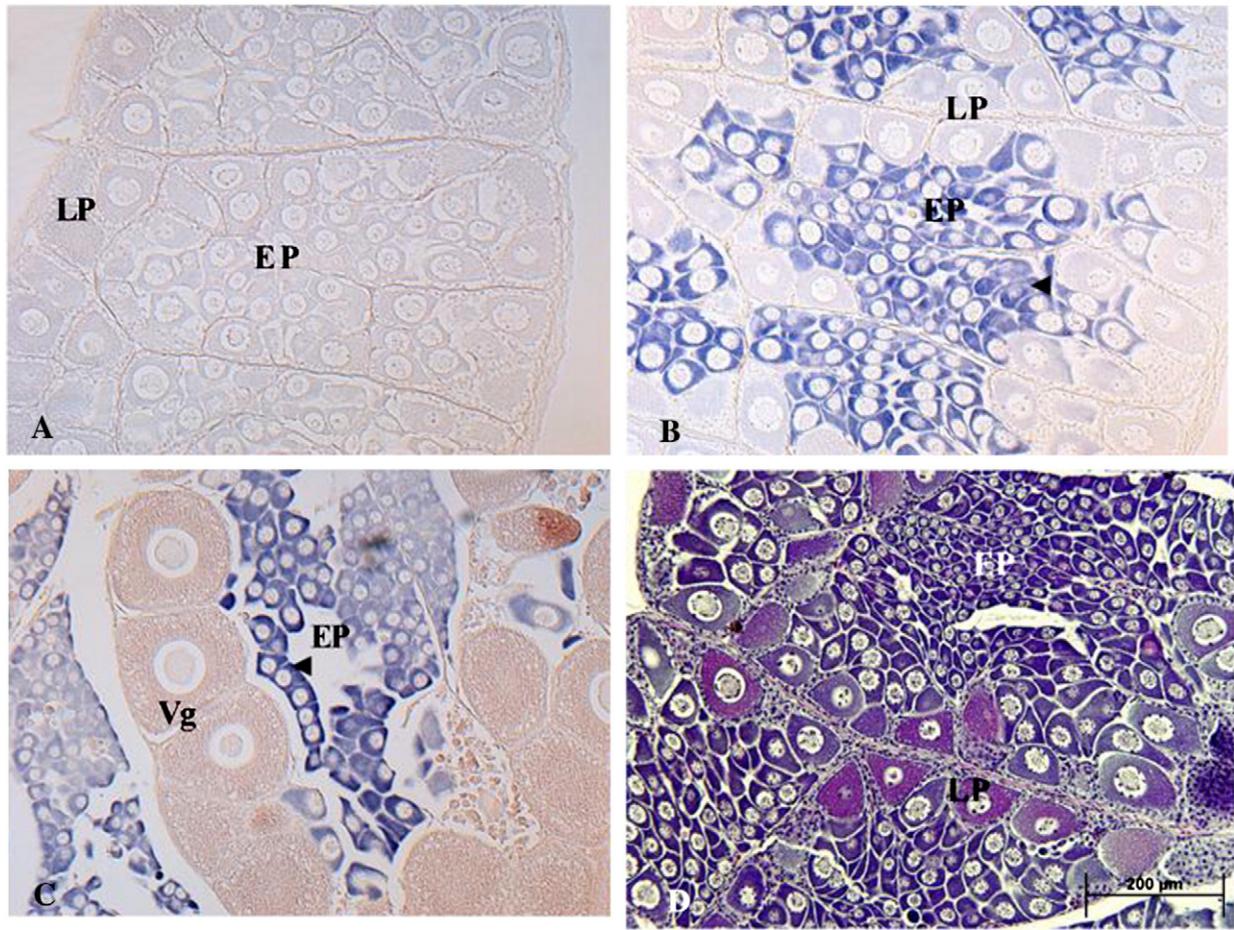


Fig. 6. Localization of the *PmADRP* transcript during ovarian development of eyestalk-ablated *P. monodon* broodstock visualized by *in situ* hybridization using the sense *PmADRP* (A) and antisense *PmADRP* probes (B and C). A conventional HE staining was carried out for classification of oocyte stages (D). EP = early previtellogenic oocytes, LP = late previtellogenic oocytes, Vg = vitellogenic oocytes. Arrowheads indicated examples of the positively hybridized signals of *PmADRP*.

storage and utilization of lipids for maturation and spawning processes (Millamena and Pascual, 1990).

Similarly, the ovarian *PmADRP* transcript was significantly increased at the early cortical rod stage onwards in both intact and

eyestalk-ablated broodstock and after spawning in the former group. The expression profile of *PmADRP* in ovaries of broodstock suggested that the *PmADRP* gene products should be functionally involved in oogenesis of *P. monodon*. Results also clearly indicated that eyestalk ablation did not have direct effects on transcription of *PmADRP*. The *PmADRP* level in ovaries of 14-month-old shrimp was not significantly different from that in juveniles and stages I and II ovaries of wild

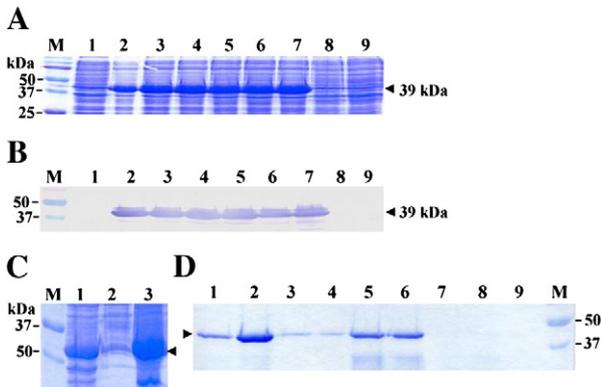


Fig. 7. A 15% SDS-PAGE (A) and western blot analysis (B) showing *in vitro* expression of recombinant *PmADRP* protein at 0, 1, 2, 3, 6, 12 and 24 h (lanes 1, 2, 3, 4, 5, 6 and 7, A and B) after induction with 1 mM IPTG. *E. coli* BL21-CodonPlus(DE3)-RIPL containing pET 15b vector and *E. coli* BL21-CodonPlus(DE3)-RIPL (lanes 8 and 9, A) were included as the control. Recombinant *PmADRP* was majorly expressed in the insoluble form; C, lanes 1–3 are whole cells ($OD_{600}=1.0$), a soluble protein fraction (30 µg total proteins), and an insoluble protein fraction (30 µg total proteins), respectively. The rPmADRP protein was purified and size-fractionated in a 15% SDS-PAGE; D, lanes 1–6 = purified rPmADRP, Lanes 7–9 = fractions after washed. Arrowheads indicated the target recombinant *PmADRP* protein (39 kDa).

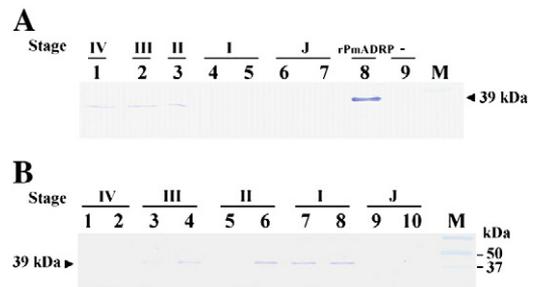


Fig. 8. Western blot analysis of anti-ADRP PcAb (1:500) against total proteins extracted from ovaries of cultured juveniles and intact broodstock (A) and eyestalk-ablated broodstock (B) of *P. monodon*. Arrowheads indicated the positive signal of approximately 39 kDa. Lanes 1 (A) and 1–2 (B) = stage IV, lanes 2 (A) and 3–4 (B) = stage III, lanes 3 (A) and 5–6 (B) = stage II, lanes 4–5 (A) and 7–8 (B) = stage I ovaries, lanes 6–7 (A) and 9–10 (B) = ovaries of juveniles, lane 8 (A) = purified rPmADRP (positive control), lane 9 (A) = *E. coli* BL21-CodonPlus(DE3)-RIPL containing pET15b (negative control) and lanes M = protein standard.

broodstock suggesting that the domesticated females are still immature. Therefore, the mRNA levels of *PmADRP* may be used as the biomarker to reveal degrees of reproductive maturation of *P. monodon*.

The *PmADRP* transcript localized in cytoplasm of early previtellogenic oocytes was more intense than that of late previtellogenic oocytes. *In situ* hybridization signals of *PmADRP* was not observed in follicular cells, oogonia and more mature (vitellogenic cortical rod and mature) stages of oocytes. This further indicated cell-type specific expression of *PmADRP* in ovaries of *P. monodon* broodstock. Contradictory expression patterns based on quantitative real-time PCR and *in situ* hybridization on the disappearance of hybridized signals of *PmADRP* in ooplasm of more mature stages of oocytes may be due to significantly increasing oocytes sizes as oogenesis proceeded. In addition, the sensitivity of real-time PCR on detecting gene expression is much greater than that of *in situ* hybridization.

The expression profiles of *PmADRP* mRNA and protein were different. Unlike results at the transcriptional level, the *PmADRP* protein was not observed in premature (juveniles) and previtellogenic (broodstock) ovaries of intact shrimp. This implied that *PmADRP* was transcribed and stored as mRNA pools in the early stages of oocytes (Nishimura et al., 2009). A greater level of the *PmADRP* mRNA in cortical rod ovaries suggested that the accumulated *PmADRP* mRNA in oocytes was not sufficient for translation of the *PmADRP* protein during the late stages of ovarian development of *P. monodon*.

The disappearance of this protein in mature ovaries of eyestalk-ablated broodstock illustrated that this technique causes more rapid expression of the *PmADRP* protein during previtellogenic, vitellogenic and early cortical rod stages of *P. monodon*. Accordingly, the protein profile of *PmADRP* may be used to follow reproductive maturation of *P. monodon* as a consequence of maturation inducing feed and/or hormonal treatment (Yano and Hoshino, 2006).

Taking the information together, *PmADRP* gene products appear to be functionally involved in both development of female germ cells and lipid accumulation during oocyte and ovarian maturation of *P. monodon*. The basic knowledge and tools derived from this study allows further investigation of the effects of intra-ovarian lipid synthesis, accumulation and turnover on reproductive maturation of this economically important species.

Acknowledgment

This research is supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand awarded to SK.

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