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Isolation and characterization of *progesterone receptor-related protein p23* (*Pm-p23*) differentially expressed during ovarian development of the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

A homologue of *progesterone receptor-related protein p23* (*Pm-p23*) was isolated from EST analysis of the cDNA library established from vitellogenic ovaries of the giant tiger shrimp (*Penaeus monodon*). The full length cDNA of *Pm-p23* was further characterized by RACE-PCR and it was 1943 bp, comprising an open reading frame (ORF) of 495 bp corresponding to 164 amino acid residues and the 5′ and 3′ UTRs of 7 and 1441 bp, respectively. *Pm-p23* significantly matched *p23-like protein* of *Nasonia vitripennis* (*E*-value $= 7 \times 10^{-46}$). The predicted molecular mass and p*I* of the deduced Pm-p23 protein was 19.07 kDa and 4.39, respectively. Quantitative real-time PCR analysis revealed that the expression levels of *Pm-p23* in ovaries of both intact and eyestalk-ablated broodstock were significantly greater than that of juveniles (4-month-old shrimp) ($P<0.05$). *Pm-p23* was up-regulated since stage II ovaries of intact and stage III ovaries of eyestalk-ablated *P. monodon* broodstock ($P<0.05$). The mRNA level of *Pm-p23* after spawning was not significantly different from stages II–IV ovaries of intact broodstock ($P>0.05$). *In situ* hybridization indicated that *Pm-p23* was localized in ooplasm of previtellogenic oocytes. Recombinant Pm-p23 protein was successfully expressed *in vitro* and its polyclonal antibody was successfully produced. Western blot analysis indicated that the level of ovarian Pm-p23 protein peaked at the vitellogenic stage and decreased as oogenesis progressed. Taken together, results from this study strongly suggested functionally important roles of *Pm-p23* gene products during vitellogenesis of *P. monodon* oocytes.

1. Introduction

Farming of the giant tiger shrimp (*Penaeus monodon*) in Thailand relies almost entirely on ocean-caught females for farm seed supply. This open reproductive cycle and reliance on wild stocks of *P. monodon* results in heavy exploitation of female broodstock from natural populations (Klinbunga et al., 1999). The lack of high quality wild broodstock and the outbreak of diseases have significantly decreased *P. monodon* production over the last several years (Limsuwan, 2004).

The domestication and selective breeding programs of *P. monodon* would provide a more reliable supply of seed stock and the improvement of their production efficiency (Coman et al., 2006). Genetic improvement of *P. monodon* cannot be achieved without knowledge on the control of its reproductive maturation. However, reduced spawning potential and low degree of maturation of *P. monodon* in captivity crucially prohibit the development of effective domestication and selective breeding programs in this species (Withyachumnarnkul et al., 1998; Leelatanawit et al., 2008). Molecular mechanisms involving gonad development of *P. monodon* have long been of interest by aquaculture industries (Benzie, 1997, 1998; Preechaphol et al., 2007). Mechanisms controlling ovarian maturation of *P. monodon* at the molecular level are important and can be directly applied to the industry.

Different biotechnological approaches, for example; injection of vertebrate steroid hormones, neurotransmitters and ecdysteroids (Benzie, 1998; Okumura, 2004) and the use of specially formulated feed (Harrison, 1990) have been applied to induce ovarian maturation of female penaeid shrimp but results are inconsistent (Meusy and Payen, 1988; Okumura, 2004). The major obstacle in the development of shrimp maturation technology is the limited knowledge of the molecular events of ovarian maturation of shrimp (Benzie, 1998). Over the past few decades, there have been many studies on

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characterization of vitellogenin/vitellin and the elucidation of the process of vitellogenesis in penaeid shrimp (Longyant et al., 2000; Tsutsui et al., 2000; Okumura et al., 2006) as well as molecular endocrinology of shrimp reproduction, particularly on GIH and methylfarnosoate (MF) (Silva Gunawardene et al., 2001; Gu et al., 2002; Yamano et al., 2004). Although these studies begin to reveal a better picture of the endocrine control of ovarian maturation in shrimp, reproductive maturation of penaeid shrimp is still not well understood.

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 et al., 2005). Identification, characterization and expression analysis of genes involving gonad maturation could be directly applied for selection of high quality pond-reared *P. monodon* in the future (Leelatanawit et al., 2008).

The presence of vertebrate-type steroids has been documented in almost all invertebrate groups including crustaceans (Lehoux and Sandor, 1970; Lafont, 1991; Cardosa et al., 1997). Progesterone (P4) and its derivatives are sex steroid hormones that play important roles in gametogenesis (Fingerman et al., 1993; Rodriguez et al., 2002; Miura et al., 2006). P4 and 17α-hydroxyprogesterone administration induced ovarian maturation and spawning in *Metapenaeus ensis* (Yano, 1985, 1987). The conversion of progesterone into estradiol-17β was reported in *Penaeus (Marsupenaeus) japonicus* (Summavielle et al., 2003). Estradiol-17β and progesterone levels in the hemolymph were shown to fluctuate closely with that of the serum vitellogenin level during ovarian maturation stages of *P. monodon* (Quinitio et al., 1994) implying its controlling role in vitellogenesis.

In *P. monodon*, the titers of conjugated pregnenolone and unconjugated and conjugated dehydroepiandrosterone (DHEA) in ovaries were found to be maximal at early (stage II) and late (stage III) vitellogenesis. Unconjugated progesterone was found in ovaries at the late stages (III and IV) of ovarian development whereas conjugated testosterone was only detected in mature (IV) ovaries (Fairs et al., 1990). Molecular mechanisms of vertebrate-like hormones have not been well established in penaeid shrimps at present (Qiu et al., 2005) and knowledge about their targets (steroid receptors) and signaling is still limited.

The actions of P4 are mediated through binding to the nuclear progesterone receptor, a member of the steroid/thyroid hormone receptor superfamily, as the classical pathway (Rao et al., 1974; Evans, 1988). Progesterone receptor-related protein p23 (p23) was first characterized and named as an essential component of the Hsp90 molecular chaperone complex with the progesterone receptor (Johnson and Toft, 1994). p23 binds the ATP-bound form of Hsp90 and blocks its ATPase activity, thereby stabilizing that state and thus client protein binding (Felts and Toft, 2003).

To investigate the functional contribution of progesterone inducing pathway in ovarian development of *P. monodon*, the full length cDNA of *progesterone receptor-related protein p23* (*Pm-p23*) were characterized and reported for the first time in *Penaeus* species. Expression patterns of *Pm-p23* gene products during ovarian development of intact and eyestalk-ablated *P. monodon* broodstock were examined. Localization of the *Pm-p23* mRNA in ovaries of *P. monodon* was determined using *in situ* hybridization.

2. Materials and methods

2.1. Experimental animals

Juvenile shrimp (4-month-old) were purchased from a commercial farm in Chachoengsao (eastern Thailand, $n=6$). Female broodstock were wild-caught from the Andaman Sea (west) and acclimated under the farm conditions for 2–3 days. The post-spawning group was immediately collected after shrimp were ovulated ($n=6$). Ovaries were dissected out from juveniles and intact broodstock 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 the ablation ($n=31$). The ovarian developmental stages of *P. monodon* were classified into four stages according to gonadosomatic indices (GSI, ovarian weight/body weight ×100): <1.5, >2–4, >4–6 and >6% for previtellogenic (stage I, $n=8$ and 6 for intact and eyestalk-ablated broodstock, respectively), vitellogenic (II, $n=7$ and 4), early cortical rod (III, $n=7$ and 10) and mature (IV, $n=9$ and 11) ovaries, respectively. The ovarian stage of each shrimp was further confirmed by a conventional histological technique (Yano, 1988; Qiu et al., 2005). 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. Isolation of total RNA and mRNA

Total RNA was extracted from shrimp tissues using TRI Reagent (Molecular Research Center). Ten micrograms of the extracted total RNA was treated with DNase I (0.2 U/μg of RNA, GE Healthcare) at 37 °C for 30 min and subjected to a phenol–chloroform extraction. For RACE-PCR, mRNA was purified from total RNA using a QuickPrep Micro mRNA Purification Kit (GE Healthcare). RNA was kept in the absolute ethanol at −70 °C prior to reverse transcription.

2.3. Rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) of the 3′ terminus of Pm-p23

A gene-specific primer (3′-RACE; 5′-GCAAGTGATGCGTCGTGGTA-3′) was designed. 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 3′ RACE-PCR were assembled. Sequence similarity was searched against data in the GenBank using Blast*N* and Blast*X* (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). The predicted protein domain was identified using SMART (http://smart.embl-heidelberg.de/). Molecular weight and p*I* of the deduced Pm-p23 protein were examined using Protparam (http://www.expasy.org/tools/protparam.html).

2.4. RT-PCR and tissue distribution analysis

Expression of *Pm-p23* (200 bp) in ovaries and other tissues of wild broodstock was analyzed by RT-PCR. The amplification reaction was performed in a 25 μl volume containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.2 μM each primer (F: 5′-GCACAGGAGGCACTGAAAG-3′ and R: 5′-CCATCGGCTGAAATCTACT-3′) and 1 U DyNazyme™ II DNA Polymerase (Finnzymes). *EF-1α*$_{500}$ (F: 5′-ATGGTTGTCAACTTTGCCCC-3′ and R: 5′-TTGACCTCCTTGATCACACC-3′) was included as the positive control. PCR was performed by predenaturation at 94 °C for 3 min followed by 25 cycles of a 94 °C denaturation for 30 s, a 55 °C annealing for 45 s and a 72 °C extension for 30 s. The final extension was carried out at 72 °C for 7 min. Amplicons were electrophoretically analyzed by 1.5% agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

2.5. Quantitative real-time PCR

One microgram of DNAse I-treated total RNA of each specimen was reverse-transcribed using an Impromt-II Reverse Transcription System (Promega). *Pm-p23* (108 bp) and the internal control (*EF-1α*$_{214}$) transcripts in each shrimp were examined in a 10 μl reaction volume contained 5 μl of 2× LightCycler 480 SYBR Green I Master (Roche), 25 ng

the first strand cDNA template, 0.2 μM each of gene-specific primers for *Pm-p23* (F: 5′-ACGAAGCAGCACTGGCTCAAAG-3′ and R: 5′-CATCTGCCG-CATCATCTCCTC-3′) and *EF-1α*$_{214}$ primers (F: 5′-TCCGTCTTCCCCTTCAG-GACGTC-3′ and R: 5′-CTTTACAGACACGTTCTTCACGTTG-3′). The thermal profile for quantitative real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. Real-time PCR of each specimen was carried out in duplicate. The quantification of *Pm-p23* and *EF-1α* mRNA of each sample well was evaluated from the respective standard curve constructed from 10^3–10^8 copies of recombinant plasmids of the target (501 bp; F: 5′-ATGTCAAC-CACACAGTCTTTGCC-3′ and R: 5′-ACACAGTTACTCCAGGTCAGG-3′) or reference sequences (1183 bp; F: 5′-CGAGCCCAGTACTAAGAGCCTA-3′ and R: 5′-GACCAAGATCGACAGGCGTACT-3′) using the same amplification conditions. The relative expression level (copy number of *Pm-p23* and that of *EF-1α*) between shrimp possessing different stages of ovarian development were statistically analyzed using ANOVA and Duncan's new multiple range tests. Results were considered significant when $P<0.05$.

2.6. In situ hybridization

Ovaries of *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 μm) were carried out. The sense and antisense cRNA probes; Pm-p23-T7/F (5′-TAATACGACTCACTA-TAGGGATGTCAACCACACAGTCTTTGC-3′) and Pm-p23-R/SP6 (5′-*ATT-TAGGTGACACTATAGAA*AGTCTTCATCCTCGAGGTCATC-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× SSC containing 50% deionized formamide, 1 μg/μl yeast tRNA, 1 μg/μl salmon sperm DNA, 1 μg/μl 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× SSC for 5 min each and once with 2× 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 μg/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× SSC (50 °C, 15 min each). High stringent washing was carried out twice in 0.2× SSC at 50 °C for 20 min each. Detection of the positive signals was carried out with a DIG Wash and Block Buffer kit (Roche) (Qiu and Yamano, 2005).

2.7. In vitro expression of recombinant Pm-p23

Plasmid DNA containing the full length cDNA of *Pm-p23* was used as the template for amplification using the forward and reverse primers of *Pm-p23* (F: 5′-ATGTCAACCACACAGTCTTTGCC-3′ and R: 5′-ACACAGTTACTCCAGGTCAGG-3′). The resulting product was used as the template and amplified by the other pair of primers (F: 5′-CCGCATATGTCAACCACACAGTCTTTGCC-3′ and R: 5′-GGC*GGATTC*TTA**ATGATGATGATGATGATG**CTCCAGGTCAGGGA-GATCGT-3′ containing a *Nde* I site (underlined) and an *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 pET32a 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 *Pm-p23* was inoculated into 3 ml of LB medium, containing 50 μg/ml ampicillin and 50 μg/ml chloramphenicol at 37 °C and 50 μl of the overnight culture was transferred to 50 ml of LB medium containing ampicillin and chloramphenicol and further incubated to an OD_{600} of 0.4–0.6. One OD_{600} milliliter was time-interval taken at 1, 2, 3, 4, 6 h and overnight after IPTG induction (1.0 mM final concentration). The culture was centrifuged at 12,000 *g* for 1 min, resuspended with 1× PBS and analyzed by 15% SDS-PAGE (Laemmli, 1970). In addition, 20 ml of the IPTG-induced culture (6 h) were centrifuged, resuspended in the lysis buffer (0.05 M Tris–HCl; pH 7.5, 0.5 M Urea, 0.05 M NaCl, 0.05 M EDTA; pH 8.0 and 1 mg/ml lysozyme) 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 rPm-p23 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 using a His GraviTrap kit (GE Healthcare). Initially, 1 l of IPTG-induced cultured overnight at 25 °C was harvested by centrifugation at 5000 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 14,000 rpm for 30 min. The soluble fraction was loaded into the column and washed with 10 ml of the binding buffer 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 Pm-p23 protein was eluted with 6 ml of the elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Fractions from the washing and eluting steps were analyzed by SDS-PAGE and western blotting. The purified rPm-p23 was stored at 4 °C or −20 °C for long-term storage.

2.9. Anti-Pm-p23 polyclonal antibody production and western blot analysis

Polyclonal antibody against rPm-p23 was immunologically produced in rabbit. For western blot analysis, ovarian tissues of *P. monodon* were homogenized in the sample 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 and the supernatant was collected. Protein concentrations of the tissues extract were determined by the dye binding method (Bradford, 1976). Thirty micrograms of ovarian proteins were heated at 100 °C for 5 min, immediately cooled on ice and size-fractionated by 15% SDS-PAGE (Laemmli, 1970). Electrophoretically separated proteins were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) in the 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at a constant current of 350 mA for 1 h. The membrane was treated with the DIG blocking solution (Roche) for 1 h and incubated with the primary antibody (1:300 in the blocking solution) for 1 h at room temperature. The membrane was washed 3 times with 1× Tris Buffer Saline-Tween-20 (TBST; 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween-20) 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× TBST. Immunoreactional signals were visualized using NBT/BCIP (Roche) as the substrate. The intensity of Pm-p23 bands was quantified from the scanned photographs using the Quantity One software (Bio-Rad). The expression level of Pm-p23 in each ovarian stage was normalized by that of stage I ovaries.

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AAACACCATGTCAACCACACAGTCTTTGCCACCCCCAGTGACATGGGCACAGCGAAAAAA 60
       M  S  T  T  Q  S  L  P  P  P  V  T  W  A  Q  R  K  N  18
TCTCATCTTCCTAACTATCTGTGTTGAAGACTGCAAATCTCCCACCATTAACATAGAAGC 120
 L  I  F  L  T  I  C  V  E  D  C  K  S  P  T  I  N  I  E  A  38
AGATAAGGTGTACTTCAAGGGCACAGGAGGCACTGAAAGAAAAGATTATGAATATACTTA 180
 D  K  V  Y  F  K  G  T  G  G  T  E  R  K  D  Y  E  Y  T  Y  58
CAACTTGTTTAAAGATATTGATACAGATAAAAGCCGTAGTTTTGTACGAGATCGTAACAT 240
 N  L  F  K  D  I  D  T  D  K  S  R  S  F  V  R  D  R  N  I  78
AGAGCTGATACTGGTCAAAAAAGAGGAGGGACCTTACTGGCCACACCTGCTTAAGGAGAA 300
 E  L  I  L  V  K  K  E  E  G  P  Y  W  P  H  L  L  K  E  K  98
AACGAAGCAGCACTGGCTCAAAGTAGATTTCAGCCGATGGAAGGACGAAGACGACAGTGA 360
 T  K  Q  H  W  L  K  V  D  F  S  R  W  K  D  E  D  D  S  D  118
CGATGAGGAGGGCCAGAACCAAGACTTGGAGGAGATGATGCGGCAGATGGGAGGTCTAGG 420
 D  E  E  G  Q  N  Q  D  L  E  E  M  M  R  Q  M  G  G  L  G  138
TGGTGGCGGAGATGACAGGCCATCCCTCGATGACCTCGAGGATGAAGACTCCGACGACGA 480
 G  G  G  D  D  R  P  S  L  D  D  L  E  D  E  D  S  D  D  D  158
CGATCTCCCTGACCTGGAGTAACTGTGTCAGTAGCCCAAAGAGGCCTTCGCAGACCACTT 540
 D  L  P  D  L  E  *                                          164
CTTGCAAGTGGGTGCAAGTGATGCGTCGTGGTAAGCTGTCCTGTACCTAAGTCAGTGTGT 600
GCGAATTCCAGTTACCTCCAGATGGACAACTTTGCTATGTGGGATCCACTGCAGTTGGAA 660
ACAATACAAAGAATATTGTCCCGCGGAGTTTTTTTGTACACACAGTGTGACTAGGACTTG 720
ACTGCCCTCCTTGTGCAGGGTTCCAGTGAGCGTGGATTAGTGGCTCTTTTCTTTTTCTCA 780
TTTTCTTTTCTTTTCCAACCAACCTGCCATAAAAGAAAAACTAAATGAAATTTTCCATTT 840
TCGTTTCCATATATACATAGCATACAAGAACCAAGCTTGTTCCAGAACAGTCCTTCCAAA 900
CTTAGTTAGAATTTTTTTTTTTTTTACTTTTGTTTTCTCAACGTCTTGGCCTGGCAGCT 960
CTGGAGAGAAATGTGTCCCACCTTTCTGCCCCTGCTAAAGAAAAGTGAATTAAAATGGAA 1020
AGTGCTGTCTTTTATCATTTAAACTTCGCAATTTATCAGATAAGCAGATTTTTTGTTTAA 1080
GAAACTACATGTTTAGCTTGCAAATATTCTCATCTTTACATGTCTGTTATATGGCAAAAC 1140
CAAAACCAAAAAAATTCCACAATCCTGTGTAGGCCTTACATTATTTATTTGTCAAGGTAC 1200
ATGGAGTTGGGTTTGATGAGAAAATCATATTGATTGCCCTCAGCAGACCAATTTCTTGTG 1260
TTTTTGATGAGTCGTGATGTAATGAGTGAGTCTAGGCTATTCCCACGGCTTAGCACATGA 1320
AAACTTTGTAAGGTTATTTCTTATGTTTATATTTATTATTGTTAATTATAGATGTTTCAT 1380
TTTTCATTTTTTTAGAGGGAGGAAAATTGAATGTAACTTGGTAGTGTGCATGTGAAGAGT 1440
AAAAAACTAAAAGTTTATTATTCCTTGTATTAAATGGGGACGTGAGTTCTTGGTCTTACA 1500
AAGAGATCTCATAGAACCAGTGGATAGGGTACATCAGATGTTTGCTGCCTTAGTGTCAAA 1560
ATTTTATATACATGTATTATGTCATATCATTATGGAAACTAGAAATCAAAGTGTTTTTAA 1620
GACATTGTGATTTTGTACCACCTGGGGTAAGTCTTTATATATATAATATATACTCTTGGC 1680
AGAACAGCATACATACCTGAAGGTTTTCTTCCAATACACAAGTCATGATTCAAAGTGTTT 1740
TATAATCCTGTTTGTATAAGTTAATGAAAACTAGATATTTCTTTTTTCATCGCAGAAATT 1800
ATTTCCGATTAATTAATAATCTGTCTCTGTATATGATTAAAGTCTTTTTGAGTTGAAAAT 1860
TTAGTTTTGTATTTCTTTCTGCATGTAAGGGAGATGAAGGCAAAGAAACAATTTGAAAAA 1920
AAAAAAAAAAAAAAAAAAAAAAA                                       1943
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Fig. 1. The full length cDNA and deduced protein sequences of *Pm-p23* (1943 bp, ORF of 495 bp corresponding to a deduced polypeptide of 164 amino acids; Genbank accession no. GU906280). The putative start (ATG) and stop (TAA) codons are illustrated in boldface and underlined. The predicted CS domain (positions 9–84) is highlighted. A p23 signature (WPHLLKE; position 91–97) is double-underlined.

3. Results

3.1. Isolation of the full length cDNA of Pm-p23

An approximately 1400 bp fragment of 3′ RACE-PCR of *Pm-p23* was successfully amplified. After assembly, the full length cDNA of *Pm-p23* was 1943 bp in length composing of an ORF of 495 bp corresponding to a polypeptide of 164 amino acids with 5′ and 3′ UTRs of 7 and 1441 bp, respectively (GenBank accession no. GU906280; Fig. 1). The closest similar sequence to *Pm-p23* was *p23-like protein* of *Nasonia vitripennis* (*E*-value = 7e-46). The deduced Pm-p23 protein contained a p23 signature (WPHLLKE; position 91–97) and an unknown function, CS, domain (positions 9–84, *E*-value = 1.3e−10). The predicted molecular mass and p*I* of the deduced Pm-p23 protein was 19.07 kDa and 4.39, respectively.

3.2. Tissue distribution and expression of Pm-p23 during ovarian development of P. monodon

Pm-p23 was comparably expressed in ovaries, testes, and various tissues (hemocytes, gill, lymphoid organ, intestine, hepatopancreas, stomach and thoracic ganglion). A low expression level of *p23* was observed in eyestalk. This transcript was not expressed in a heart and a pleopod of wild *P. monodon* broodstock (Fig. 2).

The expression levels of *Pm-p23* in ovaries of both intact and eyestalk-ablated broodstock were significantly greater than that of juveniles (4-month-old shrimp) ($P<0.05$). *Pm-p23* was up-regulated at stage II (vitellogenic) ovaries of intact and at stage III (early cortical rod) ovaries of eyestalk-ablated *P. monodon* broodstock ($P<0.05$). The expression levels of this gene were comparable at the subsequent stages within each sample group. The mRNA level of *Pm-p23* after

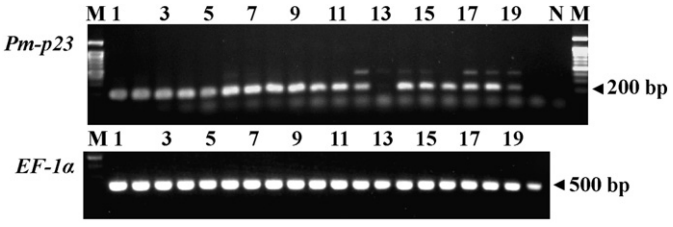

Fig. 2. Tissue distribution analysis of *Pm-p23* using the first strand cDNA from ovaries of juvenile (lane 1), and intact and eyestalk-ablated broodstock (previtellogenic, lanes 2 and 6; vitelligenic, lanes 3 and 7; early cortical rod, lanes 4 and 8 and late cortical rod ovaries, lanes 5 and 9) and various tissues of broodstock-sized *P. monodon*; testes (lane 10), hemocytes (lane 11), gills (lane 12), heart (lane 13), lymphoid organs (lane 14), intestine (lane 15), hepatopancreas (lane 16), stomach (lane 17), thoracic ganglion (lane 18), eyestalks (lane 19) and pleopods (lane 20). *EF-1α* was successfully amplified from the same template. Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

spawning was not significantly different from stages II–IV ovaries of the intact broodstock ($P>0.05$) (Fig. 3).

3.3. Localization of Pm-p23 in ovaries of intact P. monodon broodstock

The antisense *Pm-p23* probes gave a clear signal in ooplasm of previtellogenic oocytes (Fig. 4). Generally, oocytes at the late previtellogenic stage showed weaker positive signals than early previtellogenic oocytes. Positive signals were not detected in the germinative zone and oogonia. Surprisingly, no signals were observed in vitellogenic, early cortical rod and mature oocytes.

3.4. In vitro expression of recombinant Pm-p23 protein and production of anti-Pm-p23 polyclonal antibody

Recombinant Pm-p23 protein was successfully expressed in *E. coli* (Fig. 5A and B) as the soluble protein when the recombinant clone was cultured at 25 °C overnight (Fig. 5C and D). Anti-Pm-p23 polyclonal antibody was successfully produced in rabbit with a relatively high titer ($OD_{450}=0.828$ for 1:32,000 of serum against 1 μg of purified rPm-p23).

3.5. Expression profiles of Pm-p23 protein during ovarian development of P. monodon

The discrete bands corresponding to a monomeric form (approximately 19 kDa) of Pm-p23 were detected. The immunological signals of Pm-p23 in ovaries of 4-month-old juveniles were greater than those in stage IV (mature) ovaries of wild *P. monodon* broodstock. In adults, the Pm-p23 protein was obviously increased in stage II (vitellogenic) ovaries and decreased afterward. The lowest level of Pm-p23 was observed in mature ovaries of *P. monodon* (Fig. 6).

4. Discussion

4.1. Isolation and characterization of Pm-p23

Both nuclear and membrane progesterone receptor have been identified and characterized in various organisms (Peluso et al., 2006; Thomas, 2008). Two different classes of membrane-bound progesterone receptors; membrane progestin receptors (mPR; subtypes α, β, γ) which contain seven transmembrane domains and progestin membrane receptor components (PGMRC; subtypes 1 and 2) which contain one transmembrane domain (Mourot et al., 2006) have been reported.

In crustaceans, nuclear progesterone receptor was firstly identified in ovaries and hepatopancreas of the female crayfish (*Austropotamobius pallipes*) by immunohistochemistry and Western blotting analysis using antibodies against human and chicken progesterone receptor (Paolucci et al., 2002). The full length cDNAs and genes of nuclear progesterone receptor and mPR have not been reported in any crustacean at present. Recently, three (short, medium and long) isoforms of progestin membrane receptor component 1; *Pgmrc1-s* (1980 bp), *Pgmrc1-m* (2848 bp), and *Pgmrc1-l* (2971 bp), with an identical ORF of 573 bp but length polymorphism at the 3′ UTR (1380, 2248 and 2366 bp excluding the poly A; GenBank accession no EU440763–EU440765, respectively), were successfully identified in testes of *P. monodon* broodstock (Leelatanawit et al., 2008).

Subsequently, we successfully identified the full length cDNA of the single form of ovarian *P. monodon Pgmrc1* (2015 bp in length with an ORF of 573 bp deducing to 190 amino acids; GenBank accession no. GQ505293). A predicted transmembrane domain (IFTSPLNVFLLGVCTVLIY) was found in the deduced *P. monodon* Pgmrc1 protein. Immunohistochemistry revealed that the ovarian Pgmrc1 protein was localized in cell membrane of various oocyte stages and follicular cells, and in the follicular layers (Preechaphol et al., 2010). *Pgmrc1* was up-regulated in stage IV (mature) ovaries of intact broodstock ($P<0.05$). Unilateral eyestalk ablation resulted in an earlier up-regulation of *Pgmrc1* since the stage II (vitellogenic) ovaries. Moreover, the expression level of *Pgmrc1* in stages II–IV (vitellogenic, early cortical rod and mature) ovaries of eyestalk-ablated broodstock was significantly greater than that of the same ovarian stages in intact broodstock ($P<0.05$) (Preechaphol et al., 2010). Results indicated that *Pgmrc1* gene products play functional important roles during ovarian/oocyte development of *P. monodon*.

Recently, *p23* was identified from the gut cDNA library of amphioxus (*Branchiostoma belcheri*). It contains the ORF of 513 bp corresponding to a deduced protein of 170 amino acids. Northern blot analysis reveals that *B. belcheri p23* was constitutively expressed in all examined tissues (gill, muscle, testis, ovary, hepatic caecum, hind-gut and notochord) of adults (Zhao et al., 2006). In mice, *p23* was found to be expressed in all tissues examined except a heart and skeletal muscles (Freeman et al., 2000).

In this study, the full length cDNA of a *progesterone receptor-related protein p23* homologue is successfully isolated in *P. monodon* and reported for the first time in crustaceans. The predicted transmembrane domain was not found in the deduced Pm-p23 protein suggesting that it is a nuclear rather than a membrane-bound protein. Recently, p23 has been claimed to possess a prostaglandin synthase activity (Tanioka et al., 2000). Tissue distribution analysis shows that *Pm-p23* was observed in a variety of tissues of *P. monodon* broodstock suggesting that it is a multifunctional protein not only acting as one of the chaperone complex for the progesterone receptor, but also exhibiting nuclear chaperone activity in the folding and processing of proteins (Smith, 2000; Felts and Toft, 2003).

4.2. Eyestalk ablation has no direct effect on Pm-p23 transcription

Understanding the roles of steroid hormones on vitellogenesis may lead to the development of ways to induce ovarian maturation in decapod crustaceans (Yano and Hoshino, 2006). The *Pm-p23* mRNA was up-regulated in vitellogenic (stage II) ovaries suggesting its important role (s) during ovarian development of *P. monodon* oocytes. Unilateral eyestalk ablation had no effect on the transcription of *Pm-p23*. Relatively stable levels of the *Pm-p23* transcript in subsequent stages suggested that the steady state amounts of this mRNA should be sufficient to maintain its translational product during late stages (early cortical rod and mature ovaries) of ovarian development. Nonetheless, a significantly greater level of the *Pm-p23* mRNA in vitellogenic ovaries than that in other stages clearly suggested that more translated Pm-p23 protein was required during yolk accumulation and rapid oocyte development in *P. monodon*. The fluctuation of *Pm-p23* gene products indicated its important role in the control of reproduction and suggested that progesterones (progestins) are required for ovarian/oocyte development of *P. monodon*.

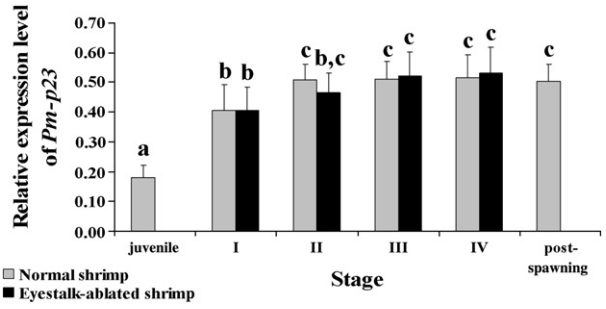

Fig. 3. Histograms showing the relative expression profiles of *Pm-p23* during ovarian maturation of intact ($n=6$ for 4-month-old juveniles and $n=8, 7, 7, 9$ and 6 for stages I, II, III, IV and post-spawning ovaries of broodstock, respectively) and unilateral eyestalk-ablated ($n=6$, 4, 10 and 11 for stages I–IV, respectively) *P. monodon*. Each bar corresponds to a particular ovarian stage. The same letters indicate that the expression levels were not significantly different ($P>0.05$).

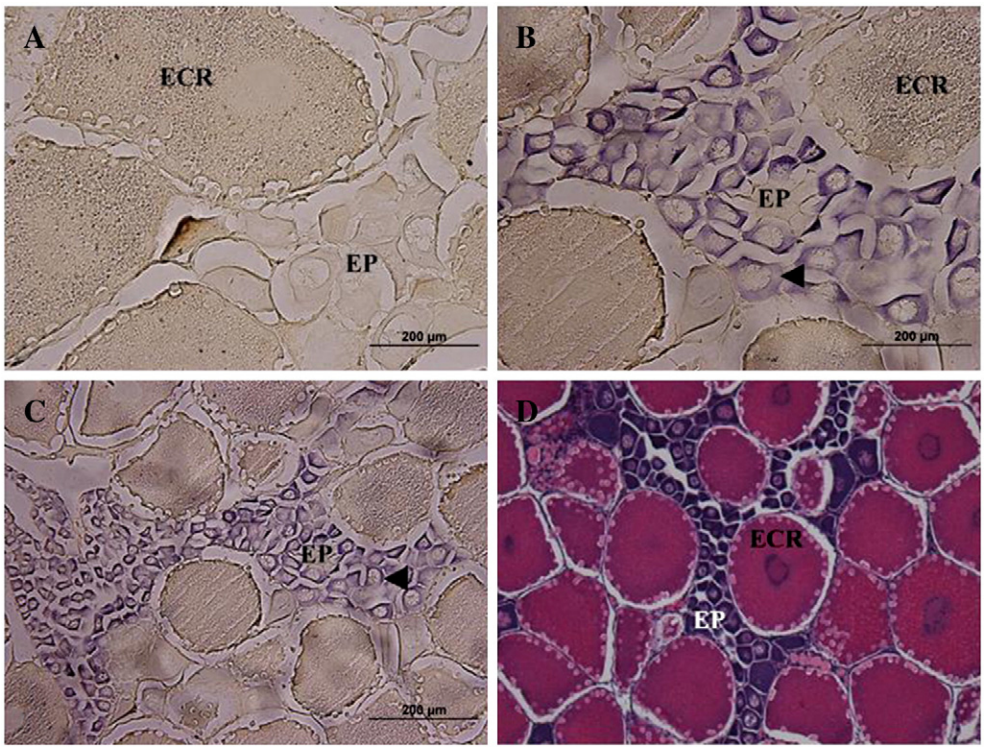

Fig. 4. Localization of *Pm-p23* transcript during ovarian development of intact *P. monodon* broodstock visualized by *in situ* hybridization using the sense (A) and antisense *Pm-p23* (B and C) probes. Conventional HE staining was carried out for identification of oocyte stages (D). Arrowheads indicated examples of the positively hybridized signals of *Pm-p23*. EP = early previtellogenic oocytes; ECR = early cortical rod oocytes.

4.3. Cell type-specific expression of Pm-p23

Localization of *Pm-p23* was not observed in advanced (vitellogenic, early cortical rod and mature) stages of oocytes, the germinal tissues and follicular cells suggesting cell type-specific expression of this transcript in ovaries of *P. monodon* broodstock. Contradictory expression patterns based on quantitative real-time PCR, where gene expression in more mature gonads was detected, can be explained by the lower sensitivity of the *in situ* hybridization method and that *Pm-p23* in the ooplasm of the later stage oocytes may have

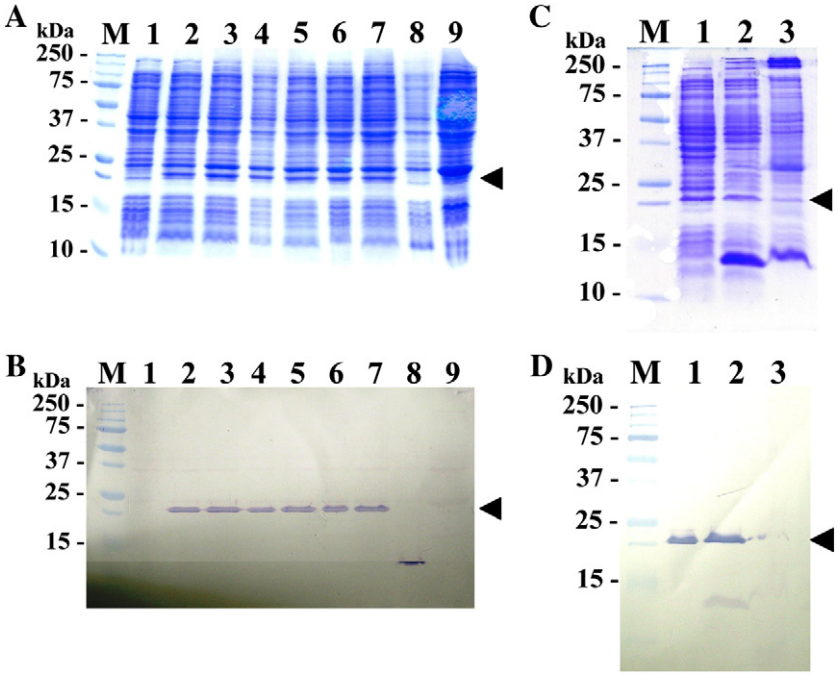

Fig. 5. *In vitro* expression of rPm-p23 at 0, 1, 2, 3, 4, 6 h and overnight after induction with 1 mM IPTG (lanes 1–7) analyzed by 15% SDS-PAGE (A) and western blot analysis (B). *E. coli* BL21-CodonPlus(DE3)-RIPL containing pET-15b vector (lanes 8; A and B) and *E. coli* BL21-CodonPlus (DE3)-RIPL (lanes 9; A and B) were included as the control. (C) A 15% SDS-PAGE and (D) western blot analysis showing expression of rPm-p23 when a positive clone was cultured at 25 °C overnight after IPTG induction (1 mM). Arrowheads indicated the target protein product. Lanes 1–3 are whole cells (OD_{600} = 1.0), a soluble protein fraction (30 µg protein), and an insoluble protein fraction (30 µg protein), respectively.

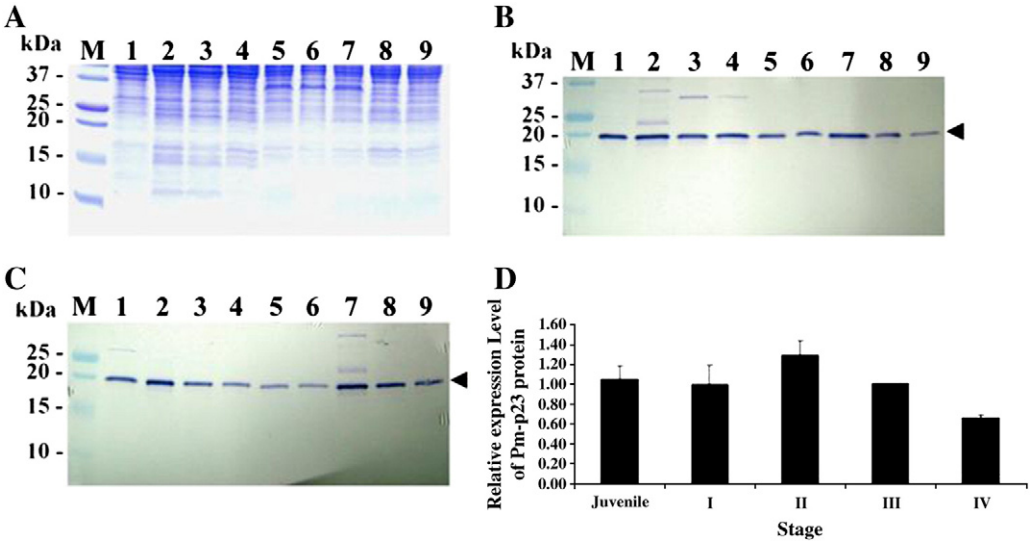

Fig. 6. SDS-PAGE (A) and western blotting analysis (B and C) of anti-Pm-p23 PcAb (dilution 1:300, expected MW of 19.04 kDa; B and C) using total proteins extracted from ovaries of *P. monodon* ($n = 2$ for 4-month-old juveniles and $n = 2, 4, 1$ and 3 for intact broodstock with stages I–IV ovaries, respectively). Ovarian proteins (30 μg) were size-fractionated by 15% SDS-PAGE and visualized by Coomassie brilliant blue staining. The average intensity of positive immunological signals in different ovarian developmental stages of *P. monodon* is illustrated (D). Lanes 1, B and C = ovaries of 4-month-old shrimp; lanes 2, B and C = stage I ovaries (GSI = 0.89 and 1.08%, respectively); lanes 3–4, B and C = stage II ovaries (GSI = 2.24, 2.15, 3.31 and 2.95%, respectively); lanes 5, B = stage III ovaries (GSI = 5.91%); lane 5, C and lanes 6, B and C = stage IV ovaries (GSI = 10.41, 11.19 and 11.21%, respectively); Lanes 7–9, B and C = positive controls of each membrane including stage I, II and IV ovaries (GSI = 1.08 and 0.89%, 2.95 and 3.31% and 10.41 and 11.19%, respectively). Lanes M = protein standard.

been significantly diluted in the increasingly large oocytes. This would decrease of the hybridization signal even though the number of mRNA copies per oocyte remained unchanged (Mourot et al., 2006). Similar circumstances were also observed in *P. monodon Pgmrc1* (Preechaphol et al., 2010) and *Ovary-Specific-Transcript 1* (*Pm-OST1*; Klinbunga et al., 2009).

4.4. Sufficient Pm-p23 protein is necessary for vitellogenesis of P. monodon

Exogenous injection of 17α-hydroxyprogesterone resulted in approximately 9.6 times increase of yolk protein in vitellogenic females of *M. japonicus* (Yano, 1987). The information reflected that progesterone may directly enhance maturation through vitellogenesis stimulation or act as a precursor of the ovarian vitellogenesis stimulating hormone (VSOH) (Fingerman et al., 1993; Yano and Hoshino, 2006).

A relatively high level of Pm-p23 in premature ovaries of juvenile *P. monodon* indicated another role of this protein in intracellular signaling pathways (Smith, 2000). In adults, the Pm-p23 protein was found in all ovarian stages but the expression reached the maximal level in vitellogenic ovaries. This illustrated that Pm-p23 plays the functionally important role during vitellogenesis in female *P. monodon*.

Yamano et al. (2004) pointed out that ovaries of *P. japonicus*, in most cases, start to develop in the reproductive season but fail to reach the necessary stage required by the formation of CRs. Accordingly, ovaries degenerate without spawning. Ovarian development of penaeid shrimps may not pass through to achieving the accumulation of yolk substances or, in some cases, to cortical rod and mature stages. Further studies about hormonal mechanisms controlling ovarian (and oocyte) maturation is, therefore, required to control maturation of *P. monodon* in captivity successfully. Unilateral eyestalk ablation is practiced to induce ovarian maturation of penaeid shrimp in commercial hatcheries, but the technique leads to an eventual loss in egg quality and death of the spawners (Benzie, 1998). 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). Bilateral eyestalk ablation caused an increase of the mRNA level of *vitellogenin*, a major yolk protein in ovaries of *P. japonicus* (Tsutsui et al., 2000). In contrast, unilateral eyestalk ablation did not alter expression patterns of *Pm-p23*. This strongly suggested that the signal transduction of progesterone through the nuclear progesterone receptor and its chaperone complex (i.e. Pm-p23, heat shock proteins and immunoplilins; Johnson and Toft, 1994) is not relevant to gonad inhibiting hormone (GIH) (Meusy and Payen, 1988).

In the present study, the full length cDNA of *Pm-p23* and expression of its mRNA and protein in ovaries of intact and eyestalk-ablated *P. monodon* broodstock were examined and revealed that *Pm-p23* gene products should play the main function during vitellogenesis rather than final maturation of *P. monodon* oocytes. The basic knowledge obtained allows functional characterization of *Pm-p23* gene products on ovarian and oocyte development for better understanding of the reproductive maturation of female *P. monodon* in captivity.

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