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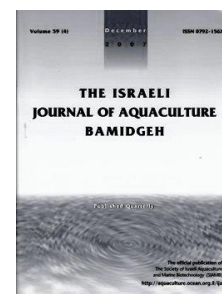
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Molecular Analysis of a Selenoprotein M Gene from *Penaeus monodon* and its Expression at Different Ovarian Stages of Development

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Abstract

In the present study, a Selenoprotein M (SelM) gene was obtained from the ovary and neurosecretory organ in eyestalk cDNA library of *Penaeus monodon*. Its cDNA sequence consisted of 901 nucleotides including an open reading frame (ORF) 396bp. The open reading frame encoded a peptide of 131 amino acids. Putative SECIS element stem-loop structure of PmSelM mRNA was predicted by SECISearch software. Like the SECIS elements in vertebrate selenoprotein mRNAs, PmSelM SECIS element contains several highly conserved sequences, including a quartet containing non-Watson-Crick base pairs (5'-UGAN and NGAN-3'), and an unpaired AA motif in the apical loop that is separated from the quartet by 11 nucleotides. Analysis of the tissue expression pattern of the PmSelM gene showed that the PmSelM mRNA was expressed in all tested tissues however there was a considerable difference in level of expression in the tissues. High expression levels were observed in the ovary and hepatopancreas of both female and male *P. monodon* respectively. The expression of PmSelM increased significantly from stage 1 ovary to stage 2-4 ovaries, which belong to rapid development stage ovaries, followed by a significant decrease in the stage 5 ovary (mature ovary). The results indicated PmSelM might play an important role in ovarian development.

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Introduction

Selenium (Se) is a well-known essential trace element found in all animals (Lobanov et al., 2009; Wang et al. 2009). The biological function of Se is attributed to its presence in selenoproteins as the 21st naturally occurring amino acid, selenocysteine (Sec, U) (Stadtman, 1996). Selenoproteins are Se-containing proteins in which Sec is inserted by the UGA codon that requires the presence of a conserved stem-loop structure known as the Sec insertion sequence (SECIS) element, which is the stem-loop structure present in 3' untranslated regions (UTRs) (Berry et al., 1991). The nucleotide sequences of SECIS elements are not conserved, but the secondary structure of SECIS elements are highly conserved in all identified eukaryotic selenoprotein genes (Berry et al., 1997). The functional part of a eukaryotic SECIS element is composed of a quartet containing non-Watson-Crick base pairs (5'-UGAN and NGAN-3'), and the unpaired invariant adenosines within the apical loop (Korotkov et al., 2002).

Selenoproteins are found in the three domains of life: eukaryota, archaea, and bacteria. They are involved in various biological functions, such as reproduction, endocrine hormones, antioxidation, immunity, peroxide detoxification, selenium transport thyroid function, and cell cycle progression (Hawkes et al., 2009; Wang et al., 2010; Su et al., 2005). So far, more than 25 selenoproteins have been identified; these include selenoproteins K, S, O, I, R, M, T, V, W and Sep15 (Lobanov et al., 2008; Lu et al., 2009). Selenoprotein M (SelM) is a selenocysteine containing protein with redox activity (Ferguson et al., 2006). It has been proved that human SelM plays an important role in protecting against oxidative damage in shrimp (Wang et al.) and in the brain (Reeves et al., 2010). SelM homologues have been identified in crustaceans such as: *Suberites domuncula* (Muller et al., 2005), *Litopenaeus vannamei* (Clavero-Salas et al., 2007), *Eriocheir sinensis* (Lu et al., 2012). Research has demonstrated that SelM in crustaceans play important roles in immune responses and reproduction (Clavero-Salas et al., 2007; Lu et al., 2012; García-Triana et al., 2010).

Penaeus monodon is widely distributed in the Indo-West Pacific Ocean (Mohamed, 1970). In South China, this species has been extensively farmed. In China, in 2011, production of farmed *P. monodon* was about 70,000 metric tons. In the past maturity of the female shrimp was induced by cutting the eyestalk during the process of seeding cultivation. But this method is not feasible for repeated use of broodstock, as it increases seedling cultivation cost and limits the development of *P. monodon* aquaculture. Therefore, seeking a better method of artificially inducing maturation is important to solve these problems. It is necessary to improve the mechanism of propagation and breeding of *P. monodon*, especially the molecular regulatory mechanism of ovary development, and artificially inducing maturation. In *Eriocheir sinensis*, SelM is involved in the regulation of reproduction during the period of rapid ovarian development (Lu et al., 2012). Investigation of SelM function in ovarian development of *P. monodon* is beneficial to understanding the mechanism of ovarian maturation. In the current study, we describe the molecular cloning, characterization, and expression analysis of the *SelM* gene from *P. monodon*. Results from the present study will provide background information on understanding the mechanism of ovary maturation in *P. monodon*.

Materials and Methods

Experimental animals. Forty healthy *P. monodon* (fresh weight 10-300g) were purchased from Sanya, Hainan province, P. R. China and maintained at $25\pm 1^{\circ}$ in tanks with recirculated seawater (salinity 30 ppt) for three days before the commencement of the experiment. They were used as material for cDNA library construction and expressed sequence tag (EST) analysis, cDNA cloning, and expression of *PmSelM* mRNA.

cDNA library construction and EST analysis. To obtain ovary-related genes, a cDNA library was constructed from the ovary and neurosecretory organ from the eyestalk of three adult *P. monodon*. Total RNA was extracted using Trizol Reagent. The mRNA was purified using polyA tract mRNA isolation system. The cDNA library was constructed using the ZAP-cDNA synthesis kit and ZAP-Cdna Gigapack[®] III Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 6782 successful sequencing reactions, yielded 1234 expressed sequence tags (ESTs) and clustered into 542 contigs and 692 singletons. Among the 1234 unique genes identified, 53 genes were possible ovary-related genes. BLAST analysis of all the EST sequences revealed that an EST of 770 bp (EST no. Contig613) was homologous to SelM of *Litopenaeus vannamei*.

Cloning the full-length cDNA of PmSelM. Based on the identified EST sequence, the cDNA 3'-end and cDNA 5'-end were obtained by rapid amplification of cDNA ends (RACE) methods, using gene-specific primers. The PmSelM cDNA 3'-end was amplified using following gene-specific primers F1 and adaptor primer for first PCR; F2 and adaptor primer for nested PCR (Table 1).

Table 1. Primers for PmSelM gene amplification and characterization

Primer name	Sequence (5'-3')	Application
F1	ATGTTCCCTTAGAAGCAATG	For 3' RACE PCR
F2	ACTGTTTCGTCATTATTCATCG	
Adaptor	GGCCACGCGTCGACTAGTAC	
R1	AGGGACTTCCTCGTCGGGTG	For 5' RACE PCR
R2	CAAACCGATTGAGAAGAACC	
Oligo-dG	GGGGGGGGGGGGGGG	
F	ACTGTTTCGTCATTATTCATCG	For quantitative PCR
R	TTTGTGCGGTACTTCCATG	
β -actin F	CTTTGCTACATCGCCCTTGACT	
β -actin R	GTTGTGGACGGTTTCCTGAATA	
Adaptor-dT	GGCCACGCGTCGACTAGTACT ₁₇	For reverse transcription
T3	AATTAACCCTCACTAAAGGG	For sequencing

PCR reaction was performed in a Thermocycler (Biometra) and in a 25 μ l of reaction volume, containing 2.5 μ l of 10 \times PCR buffer, 1.5 μ l of MgCl₂ (25 mmol/L), 2.0 μ l of dNTP, (2.5 mmol/L), 1 μ l of each primer (10 mmol/L), 15.8 μ l of double-distilled water, 0.2 μ l (1.0 U) of Ex Taq (TaKaRa, Japan) and 1 μ l cDNA template. The cycle protocol was one initial denaturation cycle of 94 $^{\circ}$ C for 2 min, then 35 PCR cycles of 94 $^{\circ}$ C for 40 s, 59 $^{\circ}$ C for 40 s, 72 $^{\circ}$ C for 50s, and a final extension step at 72 $^{\circ}$ C for 8 min. In 5'RACE-PCR, the first strand cDNA obtained was tailed with poly (C) at the 5' ends using terminal deoxynucleotidyl transferase (TdT), (Takara, Japan). The PmSelM cDNA 5'-end was amplified using the following gene-specific primers, R1 and Oligo-dG for first PCR; R2 and Oligo-dG for nested PCR. The cycle condition was one initial denaturation cycle of 94 $^{\circ}$ C for 2 min, then 35 PCR cycles of 94 $^{\circ}$ C for 40 s, 58 $^{\circ}$ C for 40 s, 72 $^{\circ}$ C for 45s, and a final extension step at 72 $^{\circ}$ C for 8 min. The obtained PCR products were separated by 1.2% agarose gel, and purified by PCR purification kit (KeLi, China). The purified PCR products were cloned into the pMD1-T vector (TakaRa, Japan) and sequenced.

Sequence analysis of PmSelM. The PmSelM amino acid sequence was deduced using DNASTar software. The protein sequence of the PmSelM was compared to its counterpart sequences currently available in GenBank, retrieved using the BLAST program (Altschul et al., 1997; <http://www.ncbi.nlm.nih.gov>). Multiple alignment of SelM was carried out with the ClustalW program (Thompson et al., 1997). The percentage of similarity and identity of the known SelM sequences was calculated using MatGAT program (Campanella et al., 2003). The phylogenetic analysis was conducted using MEGA version3.0 (Kumar et al., 2004). The phylogenetic tree was constructed with the Neighbor-Joining method based on the SelM amino acid sequences distances. The phylogenetic tree was tested for reliability using 1000 bootstrap replications. The putative ESCIS element stem-loop structure was predicted with SECISearch in <http://genome.unl.edu/SECISearch.html>.

Quantitative Real time PCR (qRT-PCR) analysis of PmSelM gene expression. To investigate tissue expression of PmSelM, total RNA was extracted from hepatopancreas, ovary of female or testis of male, muscle, intestine, neurosecretory organ in eyestalk, neurosecretory organ in brain, stomach, heart of three healthy females (approximately 20g and immature ovary) and three healthy males (approximately 10g and immature testis) using Trizol reagent (Invitrogen) separately. To investigate the level of expression in mature testis, total RNA was extracted from three healthy mature males (approximately 70g). For expression analysis in response to six ovarian different development stages, total RNA was extracted from three shrimp each at a different

ovarian development stage and classified according to two different reports, (Huang et al., 2005, and Zhou et al., 2012). Total RNA was extracted from three shrimp selected in immature and mature testis separately in order to determine expression analysis in response to testis development stages. Single-strand cDNA was synthesized according to manufacturers' instructions of PrimerScript™ 1st Strand cDNA Synthesis Kit (TaKaRa) using the DNase I (Promega)-treated total RNA as template and adaptor-dT primer (Table 1). cDNA mix was diluted to 1:5 and stored at -80 °C for subsequent quantitative RT-PCR.

Two SelM gene-specific primers F and R (table 1), were used in RT-PCR to detect the expression of the SelM gene in *P. monodon*. The primer β-actin F and β-actin R (table 1) were used to amplify a 195 bp fragment of *P. monodon* β-actin gene which was used as the internal control. The qRT-PCR program was 95 °C for 2 min, followed by 40 cycles of 94°C for 15 s, 58°C for 15s, and 72°C for 30s. All analyses were based on the average cycle threshold (CT) values of the PCR products (Qiu et al., 2008).

Statistical Analysis. One-way ANOVA was used to test the significance ($P < 0.05$) of treatment effects on the expression levels of PmSelM in the detected tissues and six developmental stages of the ovary. Duncan's multiple range tests followed for pairing or individual (one-to-one) comparisons.

Results

Cloning and characterization of PmSelM. A 293 bp fragment (EST no. Contig613), which was obtained from the ovary and neurosecretory organ in eyestalk cDNA library of *P. monodon*, showed that it was highly similar to SelM of *L. vannamei*. The PmSelM full-length cDNA sequences coincided with the data achieved from initial 3' and 5' RACE. The PmSelM sequence consisted of 901 nucleotides including an open reading frame (ORF) 396bp, a 5' untranslated region (5'UTR) of 11 bp, and a 3'UTR of 494 bp with a polyadenylation signal sequence "aataaa" and a poly (A) tail (Fig. 1). The PmSelM sequence was deposited in GenBank accession No. JQ713099.

The open reading frame encoded a peptide of 131 amino acids. PmSelM contains C×C like motifs (C×U motif), like other SelMs (Fig. 1).

```

1          attcagcaacc
12 atggcgaaaaggagctctccggctctctctcctgctggggtagcg
   M A K R S L R L L L L G V A
57 ttctattataccctcgctgaagaattacgtgagactgatattgcc
   F S Y T L A E E L R E T D I A
102 aaggctcgcgttgagagctgcggtggaatgacgcttgaacaggctc
   K A R V E S C G G U R L N R
147 eccgaggtgaagaattccacagggacatccccctcttccat
   P E V K K F I H E D I P L F H
192 aatgccaaagtcaagcagataggaggtgctctccagaactgtt
   N A K F K Q I G G A P P E L V
237 ettccaatcggctttagcagggttggagcgcctccccctggat
   L L N R F D Q V V E R L P L D
282 aagtgtcccgcgaggagtgcaataaactcatgctgaagaaggga
   K L S R E E C N K L M L K K G
327 ttctataagaagaattcaccgacgaggaagtccctgaagaatac
   F Y K K N S P D E E V P E E Y
372 ettaacgggecttatagagagaggaagaactgtatgaaacct
   L N G P Y R E R E E L *
417 atgaaacctcggcgaaggcaataaaaaacctgttcccttagaag
462 caatgttgtaactgtttctcattatctcatgtaaacctcacc
507 ttagaaaaatcgaagtcgtagacagtgtagtataatagtaa
552 ctatgtaaacatagagacagatagcttagatatttatgtagaaa
597 tatcgagttttatataagtaaacatcacaagaatccccaaaa
642 tgcagcttaccatctcgtctcaaaaacgaacatggaagtacca
687 cacaaaattccgtcagtgacatttattaccaatggagctcgg
732 ggcactcgagggaagacacgattttcgacatgaaaaagatcaga
777 aaagggctcactcaagtgatccttggaagcaagaattatctgg
822 ttgctcttccagtgttttgagacatttgcattcgtttgatctt
867 tagttgttgataaaaatttataaaaaaaaaaaaaaaaaa

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Fig. 1. The nucleotide sequences and deduced amino acid sequences of PmSelM. The asterisk (*) indicates the stop codon. The codon (129TGA131) for selenocysteine (U40) was highlighted with grey shadow. The C×U motif was boxed.

Putative SECIS element stem-loop structure of *P. monodon* SelM mRNA was predicted by SECISearch software. PmSelM SECIS elements contain several highly conserved features, including a quartet containing non-Watson-Crick base pairs (5'-UGAN and

NGAN-3'), and an unpaired AA motif in the apical loop that is separated from the quartet by 11 nucleotides, like the SECIS elements in vertebrate selenoprotein mRNAs, (Fig. 2).

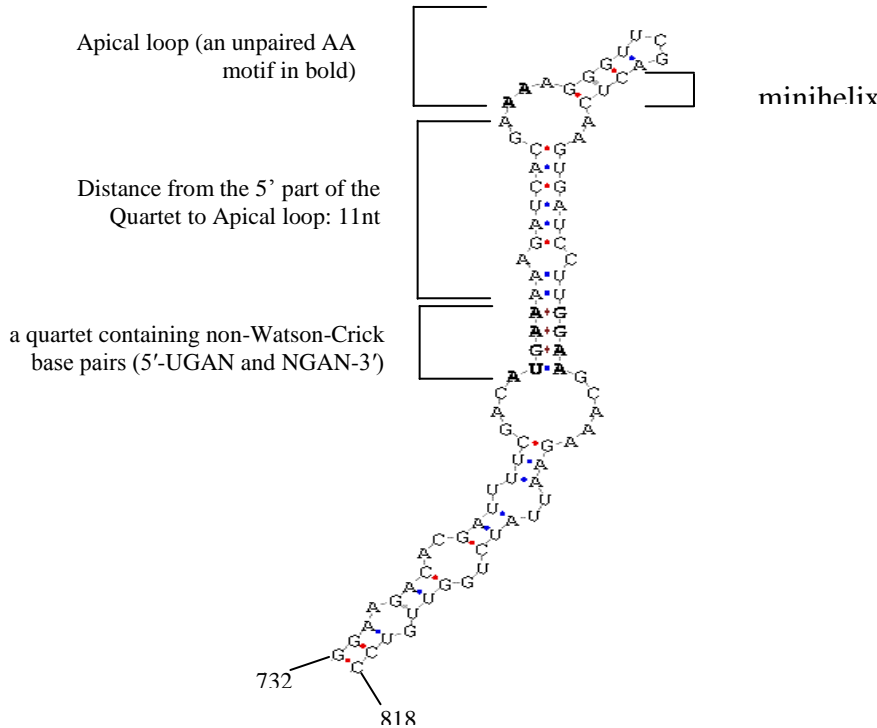


Fig. 2. SECIS element in PmSelM mRNA. PmSelM SECIS element contain several highly conserved features, including a quartet containing non-Watson-Crick base pairs (5'-UGAN and NGAN-3'), and an unpaired AA motif in the apical loop that is separated from the quartet by 11 nucleotides

Homology and phylogenetic analysis of PmSelM. The PmSelM shared 60.4–95.4% similarity and 38.8–92.3% identity to the other known SelM homologs (Table 2). The PmSelM showed high homology with that of *L. vannamei* (95.4% similarity, 92.3% identity) (Table 2).

Table 2 Amino acid sequence homology of PmSelM with other animal SelMs sequences determined by MatGat program

Species	Protein name	Accession number	Similarity%	Identity%
<i>Litopenaeus vannamei</i>	selenoprotein M	ABI93178.1	95.4	92.3
<i>Danio rerio</i>	selenoprotein M	NP_840071	62.0	38.8
<i>Bos taurus</i>	selenoprotein M	NP_001156643	63.8	46.5
<i>Ixodes scapularis</i>	selenoprotein M	AAY66722.1	66.9	49.6
<i>Macaca mulatta</i>	selenoprotein M	NP_001152871	60.4	43.8
<i>Pan troglodytes</i>	selenoprotein M	NP_001140172	62.5	44.4
<i>Mus musculus</i>	selenoprotein M	NP_444497	63.9	44.4
<i>Homo sapiens</i>	selenoprotein M	NP_536355	60.4	43.1
<i>Rattus norvegicus</i>	selenoprotein M	NP_001108485	63.2	43.1
<i>Xenopus laevis</i>	selenoprotein M	NP_001085717	66.7	45.0
<i>Sus scrofa</i>	selenoprotein M	NM_001161648	66.7	46.5

Multiple sequence alignment analyses were performed to compare the animal SelM protein sequences. The result showed the C×U motif conserved with the other animal SelMs (Fig. 3).

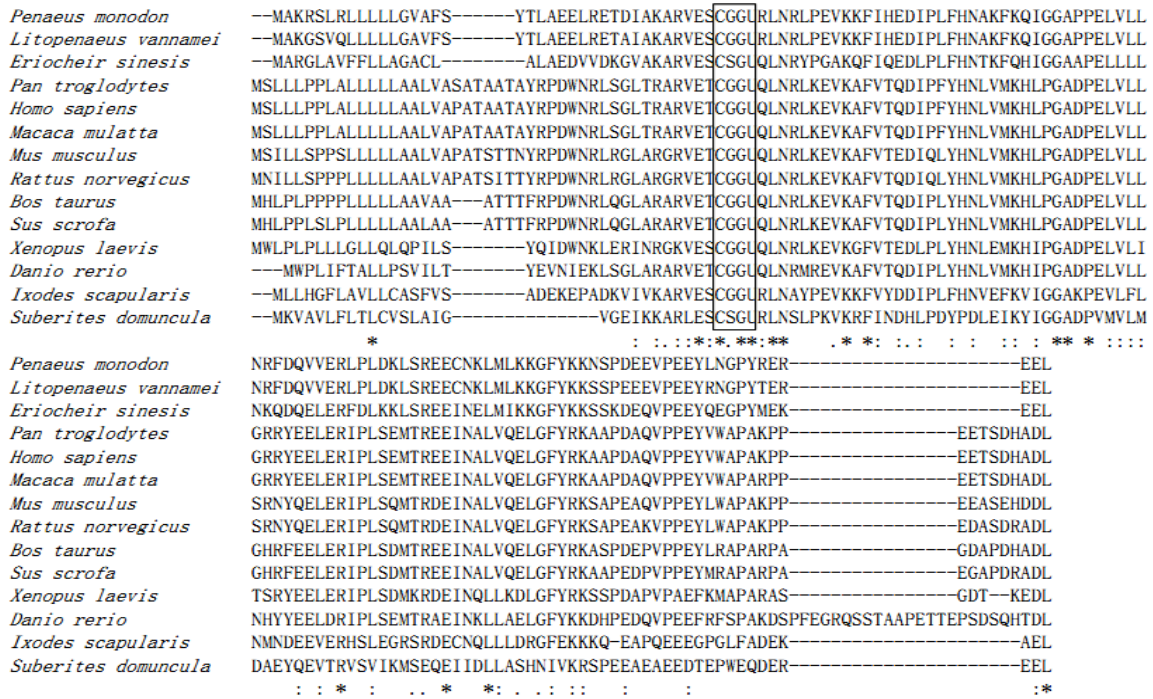


Fig. 3. Multiple alignments of PmSelM with other known Ran proteins. Identical and similar sites are shown with an asterisk (*) and dots (.) or (:). * indicates identical residues; : indicates residues with more similar properties; . indicates residues with some similar properties. The Cx×C like motifs (Cx×U motif) are marked in the rectangle.

The phylogenetic tree was constructed using the UPGMA method based on the distances between the SelM amino acid sequences (Fig. 4). Apart from the Suberites domuncula SelM, the UPGMA tree of the 13 species SelM genes showed two distinct clusters: the first cluster included vertebrate SelM genes; the second cluster included Arthropoda SelM genes. The results indicated the closest relationship was between PmSelM and the SelM of *L. vannamei*.

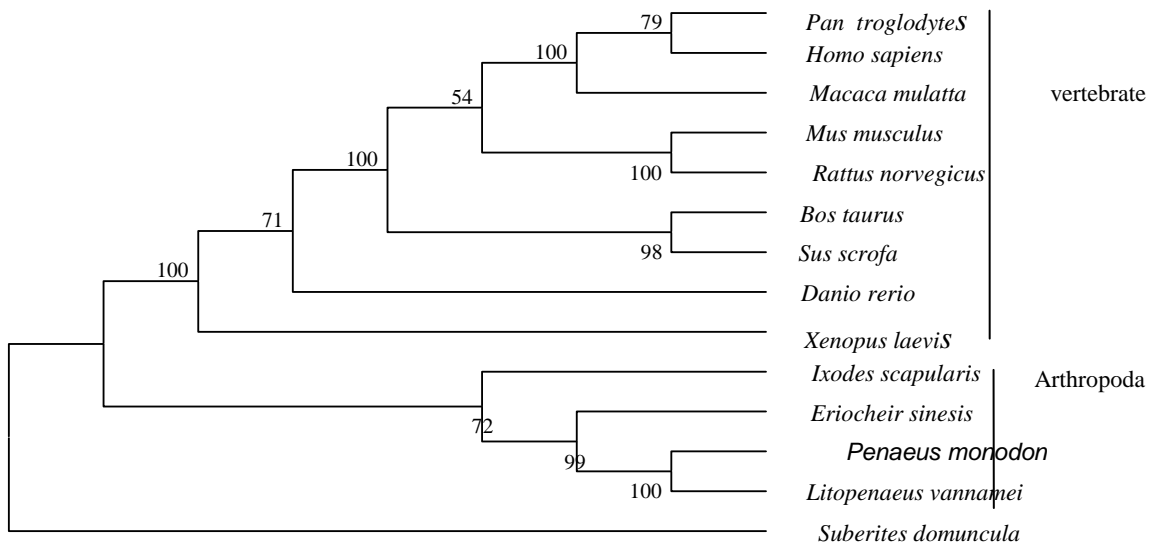


Fig. 4. Phylogenetic tree showing the evolutionary relationship of PmSelM with other animal SelMs. The tree was constructed using the UPGMA method. Numbers at branch nodes indicate percent bootstrap confidence values derived from 1000 replications.

Expression analysis of PmSelM mRNA. The PmSelM mRNA was expressed in all detected tissues of female and male (Fig. 5). In female shrimp, the expression level of the PmSelM mRNA was highest in the hepatopancreas, and lowest in muscle. The expression level of the PmSelM mRNA was also very high in the ovaries and low in the neurosecretory organs in the brain and in the eyestalk (Fig. 5). In male shrimp, the expression level of the PmSelM mRNA was highest in the hepatopancreas. The expression level of the

PmSelM mRNA was also very low in the muscle and neurosecretory organ in the brain as in female shrimp (Fig. 5).

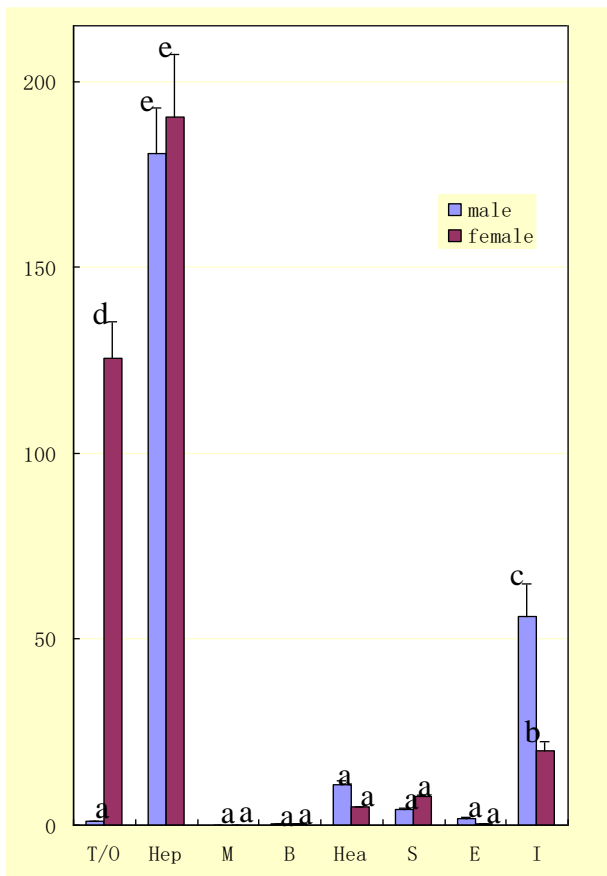


Fig. 5. qRT-PCR analysis of PmSelM expression in various tissues of male and female P.monodon. Vertical bars represented the mean \pm S.E. (N=3). T: testis, O: ovary, M: muscle, Hep: hepatopancreas, B: neurosecretory organ in brain, Hea: heart, S: stomach, E: neurosecretory organ in eyestalk, I: intestine. Vertical bars are means \pm SEM of three replicates, and significant differences were indicated with different letters above vertical bars ($P < 0.05$).

The expression level of PmSelM mRNA was much higher in mature testis than immature testis (Fig. 6). A one-way ANOVA analysis showed a significant difference in mRNA expression of PmSelM in the detected tissues. (Figs. 5, 6).

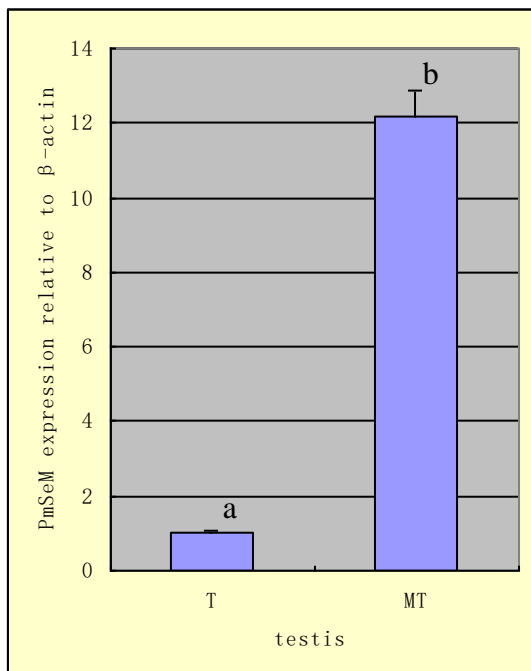


Fig. 6. qRT-PCR analysis of PmSelM expression in testis different development stages. Vertical bars represented the mean \pm S.E. (N=3). T: immature testis, MT: mature testis. Vertical bars are means \pm SEM of three replicates, and significant differences were indicated with different letters above vertical bars ($P < 0.05$).

The expression level of the PmSelM mRNA was high in the six different ovarian developmental stages (Fig. 7). A one-way ANOVA analysis of the six development stages

of ovary showed a significant difference in the mRNA expression of PmSelM (Fig.6). The expression level in the chromatin nucleolus stage (stage 2) was the highest among the six stages, and the expression level in primordial germ cell stage (stage 1) was the lowest (Fig. 7).

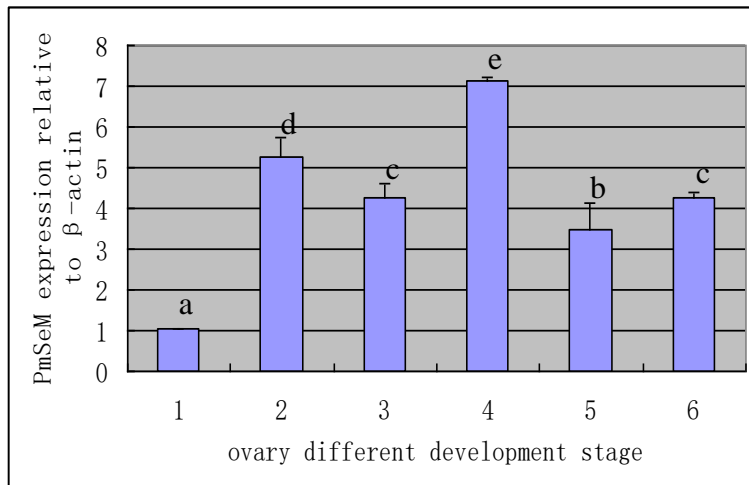


Fig. 7. qRT-PCR analysis of PmSelM expression in ovarian different development stages. Vertical bars represented the mean \pm S.E. (N=3). 1: primordial germ cell stage (stage 1), 2: chromatin nucleolus stage (stage 2), 3: perinucleolus stage (stage 3), 4: yolky stage (stage 4), 5: cortical rod stage (stage 5), 6: spent stage (stage 6). Vertical bars are means \pm SEM of three replicates, and significant differences were indicated with different letters above vertical bars ($P < 0.05$).

Discussion

The PmSelM cDNA has an opal terminator codon at 129TGA131 encoding selenocysteine (Sec) as U40, which is the main characteristic feature of the selenoprotein family (Allmang and Krol 2006), and its mRNA contains an SECIS element. This suggests that PmSelM belongs to the family of selenocysteine-containing proteins (Stadtman 1996; Berry et al. 1991; Korotkov et al. 2002). Multiple sequence alignment showed that C \times C like motifs (C \times U motif) were highly conserved (Fig. 3). The C \times C like motifs (C \times U motif) were active-site redox motifs and the activities of these enzymes was dependent upon active-site cysteine residues in the motif (Korotkov et al. 2002; Fomenko et al. 2003; Ferguson et al. 2006). It suggested PmSelM might function as redox protein.

The putative secondary structure of SECIS element in PmSelM by SECISearch software indicated that it is a type 2 SECIS element-, which differs from the type 1 SECIS element in that it contains an additional minihelix in the apical loop. The PmSelM SECIS is different from mammalian SelM SECIS, which contains a CC motif in the apical loop, and also is different from Zebra fish SelM SECIS, which is a type 1 SECIS. It indicated that the SECIS elements in all known eukaryotic SelMs are not conserved while they are conserved in mammals (Korotkov et al. 2002).

The SelM gene is expressed in a wide range of tissues (Korotkov et al. 2002; Lu et al. 2012). In the present study, the mRNA expression of PmSelM also was detected in all studied tissues. But there was a great difference in the detected tissues. High expression levels were observed in ovaries, hepatopancreas, and intestine of female shrimp, and the hepatopancreas, heart, and intestine of male shrimp. PmSelM also showed tissue-specific differential expression as in the SelMs of other crustaceans (Clavero-Salas et al., 2007; Lu et al., 2012; García-Triana et al., 2010). In addition, the tissue expression pattern of the PmSelM differed between females and males (Fig. 5). The mRNA expression of PmSelM in gonads and intestines of female and male shrimp were significantly different. It is possible that gender differences resulted in different patterns of tissue expression. However it is also possible that the female and male shrimp used for expression analysis of SelM mRNA, were at different stages of development. The female shrimp used for expression analysis were bigger than the male shrimp, even though they were all immature. PmSelM possibly plays different functions at different development stages of *P. monodon*.

SelM expression was abundant in gills and hepatopancreas of healthy shrimp *L. vannamei*. SelM of *L. vannamei* affected peroxidase activity and hydrogen peroxide concentration in gills and hepatopancreas (García-Triana et al, 2010). This suggests that PmSelM mRNA tissue-specific differential expression may offer useful cues about its possible functions. The PmSelM mRNA expression in the hepatopancreas was greatest among all detected tissues. It indicated that PmSelM may be involved in the antioxidant response, as SelM of *L. vannamei*.

In *E. sinensis* the high expression transcript of SelM in both the testicular and ovarian tissues suggests that the relationship between SelM and gonad function is pronounced (Lu et al, 2012). The expression of PmSelM in ovaries was very high (Fig. 5), and the expression of PmSelM in mature testis was much higher than in immature testis (Fig. 6). These results suggest that PmSelM could be involved in gonadal development. To better understand the possible function of PmSelM, its mRNA expressions at the six different ovarian development stages were studied (Fig. 7). The expression of PmSelM increased significantly from stage I ovaries, to stage 2-4 ovaries, which belong to rapid development stage ovaries, followed by a significant decrease in stage 5 (mature) ovaries. The expression pattern of the PmSelM mRNA in ovaries was similar to that of *E. sinensis* SelM. The expression pattern of *E. sinensis* SelM showed that the Chinese mitten crab ovary must consume a large amounts of SelM to meet the nutritional requirements for the development of the reproductive organs and the late stages of reproductive development; SelM was selectively reserved to meet the energetic needs of embryonic development (Lu et al, 2012). Therefore, PmSelM in *P. monodon* may be applied to ovarian development as suggested for *E. sinensis* SelM. According to present results, we hope that recombinant protein of PmSelM will be applied to *P. monodon* to promote ovarian development and maturation.

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