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MnHSP90 cDNA characterization and its expression during the ovary development in oriental river prawn, *Macrobrachium nipponense*

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Abstract Heat shock protein 90 (HSP90) is not only involved in environmental stress but also plays roles in the ovary development in some vertebrates. To understand its role in crustacean, we examined the HSP90 cDNA for the first time in the ovary and hepatopancreas of the oriental river prawn, *Macrobrachium nipponense* and designated this protein as MnHSP90 in this study. The MnHSP90 was cloned by the methods of degenerated oligonucleotide primers and rapid amplification of the cDNA ends (RACE). Bioinformatics analysis showed that the MnHSP90 cDNA was 2,684 bp in length, containing a 126 bp 5′ untranslated region (UTR), a 359 bp 3′ UTR, and an open reading frame (ORF) of 2,199 bp encoding a 732-amino acid polypeptide with predicted molecular mass of 84.3 KDa. Sequence alignment showed that the MnHSP90 shared 72–79% identity with other animals. Real-time quantitative PCR (qPCR) analysis demonstrated that the MnHSP90 mRNA was ubiquitously detected in all tested tissues, with the highest expression in the thoracic ganglia, the mediate in heart, muscle and intestine, and the lowest in haemocytes and gills. The MnHSP90 mRNA levels in the hepatopancreas and ovary of *M. nipponense* reached a maximum at the stage III (early vitellogenic stage) and stage IV (later vitellogenic stage) ovaries, respectively, and then decreased significantly in both tissues as the ovarian development proceeded. The level of MnHSP90 expression in the hepatopancreas was higher than that in the ovary when compared with in the same ovarian developmental stage. Our results indicate that MnHSP90 is involved in ovarian development in oriental river prawn and may play a regulatory role in ovary maturation.

Keywords MnHSP90 · *Macrobrachium nipponense* · Ovarian maturation · Ovary · Hepatopancreas

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Introduction

Heat shock protein 90 (HSP90) is a major molecular chaperone in cells and has particular significance to the process of cellular regulation. In most cells, 1–2% of cellular proteins are HSP90, making it one of the most abundant proteins in eukaryotes [1, 2]. HSP90 plays crucial roles in protein degradation, protein folding, protein assembly, signal transduction, and myofibril organization in skeletal muscles of embryos under increasing temperature or other stressing conditions [1, 3–10].

Recent studies have shown that HSP90 is also involved in regulating ovarian development in vertebrates. HSP90 binds to estrogen receptor (ER) in the absence of estrogen (E) [11–14] to increase the activity of estrogen hormone-receptor complex to transcribe target genes [15, 16]. In vertebrates, estrogen hormones play a key role in regulating the synthesis of vitellogenin (VTG) [17, 18], the main

nutrition for ovary development. During vitellogenesis, estrogen hormones are synthesized in gonad and transported to liver where estrogen (E) and its receptors form a hormone-receptor complex to bind estrogen responsive elements (ERE) located at the upstream of the VTG DNA. This leads to the activation or enhancement of the VTG gene transcription and a subsequent increase and stabilization of VTG mRNA [19]. There is a HSP90-ER-E-VTG regulation channel widely existed in vertebrates [15–19], but it is unclear if the similar mechanism exists in crustacean.

Estrogen hormone has not been found in crustacean for a long time. It is thus believed that there was no HSP90-ER-E-VTG regulation channel in crustacean. However, recent studies have showed that exogenous estrogen hormones can promote ovarian maturation in greasy back shrimp *Metapenaeus ensis* [20] and kuruma shrimp *Marsupenaeus japonicus* [21], and accumulation of VTG in *M. ensis* [22]. Likewise, the level of HSP90 expression in the ovary of crustacean has been reported to be related to the stage of ovarian development [23]. Furthermore, HSP90 was found to be an important regulator participating in VTG synthesis in *M. ensis* [24]. These findings seem to suggest that the regulatory function of HSP90 in vertebrates may also exist in crustacean. However, little is known on the regulatory function of HSP90 genes in the ovary development of freshwater crustacean. Based the existing information, we hypothesize that HSP90 participates in the regulation of maturation and reproduction in freshwater crustacean.

The oriental river prawn, *Macrobrachium nipponense*, is a commercially important freshwater prawn in China, Japan, Korea and Vietnam because of its significant contribution to rural economy. The natural populations of *M. nipponense* in China have declined rapidly due to overfishing, degradation and fragmentation of its natural habitats. Therefore, there is a need to spawn this prawn species under captivity. To achieve a year round seed supply, it is essential to understand the controlling mechanism of ovary maturation. In this study, we aimed to examine the expression of HSP90 in various tissues of this freshwater crustacean and particularly focused on its expression in the ovary and hepatopancreas at different ovarian developmental stages.

Materials and methods

Animals

A total of 170 female oriental river prawns, *M. nipponense* (1.3–2.1 g) at various ovarian developmental stages were collected from a local seafood market in Shanghai, P. R. China. The developmental stage of ovaries were classified by the criteria described by Gao et al. [25] and Wu et al. [26] (Table 1). A variety of tissues, including heart (HE), gills (GI), muscles (MU), thoracic ganglia (TG), intestine (IN), haemocytes (HA), hepatopancreas and ovary were ablated and stored in liquid nitrogen at −80°C until RNA extraction. The number of animals used for RNA extractions varied among tissues in this study. There were four to six animals used for RNA extractions in ovaries at the ovarian stages I, II and VI, two animals at stage III, and one animal at stages IV and V. One animal was used for the muscle and hepatopancreas, four animals for gills, and 10 animals for the thoracic ganglia, intestine, haemocytes and heart at ovarian stage II. When more than one individual was used, all tissues were pooled for analysis. The husbandry condition throughout the 10-day period was at ambient temperature, under water aeration, and a photoperiod of 12 h light and 12 h dark. The experimental prawns were fed with minced mussels twice daily. Feeding was stopped 12 h before tissue sampling.

Total RNA extraction and reverse transcription

All tissues were homogenized in the Unizol Reagent (Biostar, Shanghai, China), and total RNA was prepared according to the manufacturer's instruction. Total RNA was quantified on a Genova UV/visible spectrophotometer at 260 nm. The cDNA was synthesized from 5 μg of total RNA by the Takara PrimerScript™ First Strand cDNA

Table 1 Developmental stages of the oriental river prawn (*M. nipponense*) ovaries

Ovarian development	Stage	Ovary color	Histological characteristics
Previtellogenesis			
Early stage	I	Colorless transparency	Nucleoli arranged peripherally in nucleus
Later stage	II	White	Fusion between peripheral nucleoli
Vitellogenesis			
Early stage	III	Khaki	Appearance of oil globules
Later stage	IV	Viridans	Accumulation of yolk granules
Maturation	V	Dark green	Occurrence of GVBD
Paracmasis	VI	Dark gray	Emergence of cavities enclosed by follicular cells

Synthesis kit (TaKaRa, Dalian, China) according to the manufacturer's instruction.

Characterization of MnHSP90 cDNA

Initially, PCR was performed using the cDNA prepared above as a template, with the degenerated primers of dHSPF and dHSPR (Table 2) based on the highly conserved regions of HSP90 from the alignment of the sequences of *Homo sapiens* (NM_001017963), *Caenorhabditis elegans* (NM_074225), *Danio rerio* (BC065359), *Xenopus laevis* (AY785160), and *Drosophila melanogaster* (NM_079175) to obtain the partial fragment of MnHSP90 cDNA.

The cDNA for 5′-RACE and 3′-RACE was synthesized using the Smart Race kit (Clontech, Palo Alto, CA, USA) and the 3′-RACE system (Invitrogen, Carlsbad, CA, USA) respectively, according to the manufacturer's protocol. A total of 5 μg RNA was reversely transcribed using the MMLV reverse transcriptase (TaKaRa, Dalian, China) with the adapter primer (AP:5′-GGCCACGCG-TCGACTAG-TACTTTTTTTTTTTTTTTT-3′) for 3′-RACE and the 5′-CDS primer A (5′-(T)25 VN-3′, N = A, C, G, or T; V = A, G, or C) for 5′-RACE to obtain the first-strand cDNA. The 5′ and 3′ regions of MnHSP90 were amplified by nested PCR using two 5′-RACE primers (5′ HSPR1, 5′ HSPR2, Table 2) and two 3′-RACE primers (3′ HSPF1, 3′ HSPF2, Table 2) which were based on the known nucleotide sequence of the MnHSP90 cDNA fragment.

The homology search for the nucleotide and protein sequences was performed using BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). The full-length multiple alignment of the MnHSP90 amino acid sequence was compared with the HSP90s of other organisms.

Table 2 Oligonucleotide primers used in polymerase chain reactions

Primer	Sequence (5′–3′)
dHSPF	ATGATCGGNCAATTYGGTGT
dHSPR	TTRTARAAYTCSCCRTAITC
5′HSPR1	GTCAGGTCGCACTGTGAAGGAGC
5′HSPR2	AGGGGTTCTGGTCCATAAAGGCT
3′HSPF1	GCGACCTGACCACGGAGAACCTA
3′HSPF2	CAAGCCTTTATGGACCAGAACCC
qHSP90F	GTTGGCAGAATTACTCCGCTAC
qHSP90R	AGACCACCTCAAATCCACGTT
β-actinF	AATGTGTGACGACGAAGTAG
β-actinR	GCCTCATCACCGACATAA

Real time quantitative PCR (qPCR) analysis

The expression of the MnHSP90 mRNA was demonstrated by a SYBR Green real-time quantitative RT-PCR (qPCR) analysis in an ABI StepOne Sequence Detection System. The total RNA for the cDNA synthesis was first digested with RNase-free DNase I to eliminate possible genomic DNA contamination.

The qPCR amplifications were carried out in a total volume of 20 μl, containing 10.0 μl 2× SYBR Premix Ex Taq (TaKaRa, Dalian, China), 2.0 μl diluted cDNA, 0.4 μl 50× ROX reference dye, and 0.5 μl of each primer. The gene-specific primer pairs of HSP90 qHSP90F and qHSP90R (Table 2) were used to amplify the HSP90 transcript. β-actin has been successfully used as an internal gene for the mRNA expression and characterization of the HSP90 gene in *M. nipponense* [27]. Therefore, the β-actin sequence was used as the internal gene in all qPCR assays, which was amplified with the primers, β-actin F and β-actin R (Table 2) based on the EST sequence (GenBank accession N0: FL589653). The PCR temperature profile was 95°C for 10 s, followed by 40 cycles of 95°C for 10 s, and 60°C for 1 min. DEPC-water for the replacement of template was used as a negative control. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected.

Data analysis

The MnHSP90 and β-actin standard curves were developed with serially diluted cDNA templates from ovaries of known concentrations (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} of the original cDNA solution). The concentration of cDNA in each sample was calculated from the standard curve. The relative expression level of MnHSP90 was calculated by the ratio of the MnHSP90 concentration to the β-actin concentration. The values were imported to Microsoft Excel for subsequent data analyses. All data were presented as means ± SE. The results were subjected to one-way ANOVA test, and the level of significant difference was set at $P < 0.05$.

Results

Characterization of the MnHSP90 cDNA of *M. nipponense*

The nucleotide sequence and the deduced amino acid sequence of MnHSP90 are shown in Fig. 1. The sequences included a 126 bp 5′-terminal untranslated region (UTR), a 359 bp 3′ UTR and a 2,199 bp open reading frame (ORF)

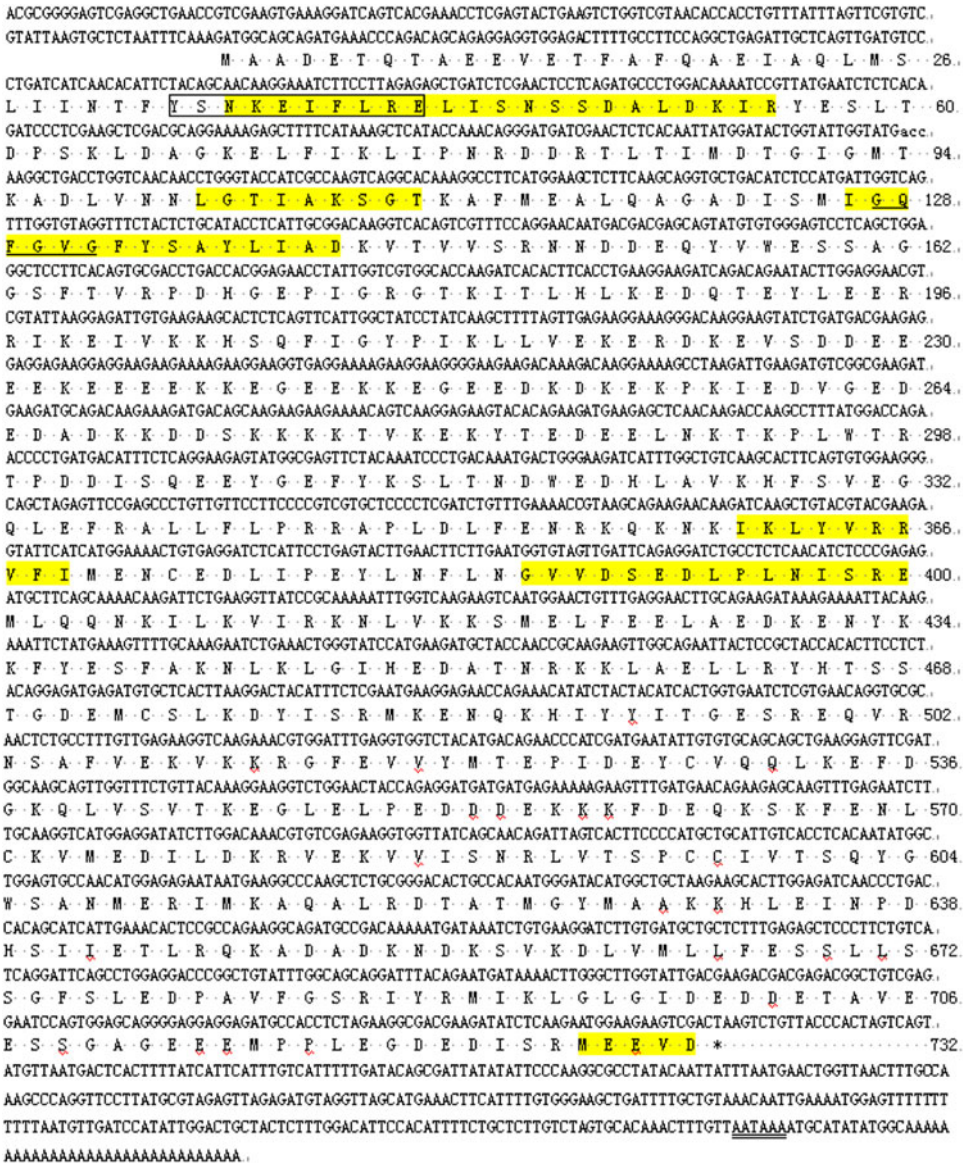

Fig. 1 Nucleotide and deduced amino acid sequences of MnHSP90 from the oriental river prawn. Five amino acid blocks defining the HSP90 protein family and consensus sequence MEED are highlighted as *shaded regions*. The putative polyadenylation signal site is shown in the *open box*. The GXXGXG motif is *underlined*. The RNA instability motif AATAAA is *double underlined*. The stop codon is indicated by an *asterisk*

encoding a 732 amino acid protein polypeptide with predicted molecular mass of 84.3 KDa. In the 3′ UTR, there was one 31 bp poly (A) tail, two polyadenylation signals located 229 bp (ATTTA) and 19 bp (AATAAA), and one upstream poly (A)+ tail.

BLAST analysis indicated that MnHSP90 shared significant homology (72–79%) at the nucleotide with the HSP90 sequences reported in *Eriocheir sinensis* (79%), *M. ensis* (78%), *H. sapiens* (74%), *Mus musculus* (73%) and *Opistophthalmus carinatus* (72%). The deduced amino acid sequence of MnHSP90 in the oriental river prawn showed a high homology with other crustaceans (*E. sinensis* 88%, *Chiromantes haematocheir* 87% and *M. ensis* 87%), fishes (*Salmo salar* 77% and *Oncorhynchus mykiss* 77%), and mammalians (*M. musculus* 80% and *H. sapiens* 80%).

Distribution of MnHSP90 mRNA in tissues

The mRNA transcripts of MnHSP90 were widely detected in all examined tissues (Fig. 2a). The highest expression was observed in the thoracic ganglia (TG), which was 2–10 times higher than that in other tissues. MnHSP90 was moderately expressed in muscle (MU), intestine (IN) and heart (HE), while it was least expressed in haemocytes (HA) and gills (GI). There were significant differences of MnHSP90 expression between thoracic ganglia (TG) and other tissues ($P < 0.05$).

MnHSP90 cDNA expressions in the ovary and hepatopancreas

The MnHSP90 expressions in the hepatopancreas (Fig. 2b) and ovary (Fig. 2c) were changed as the ovary developed.

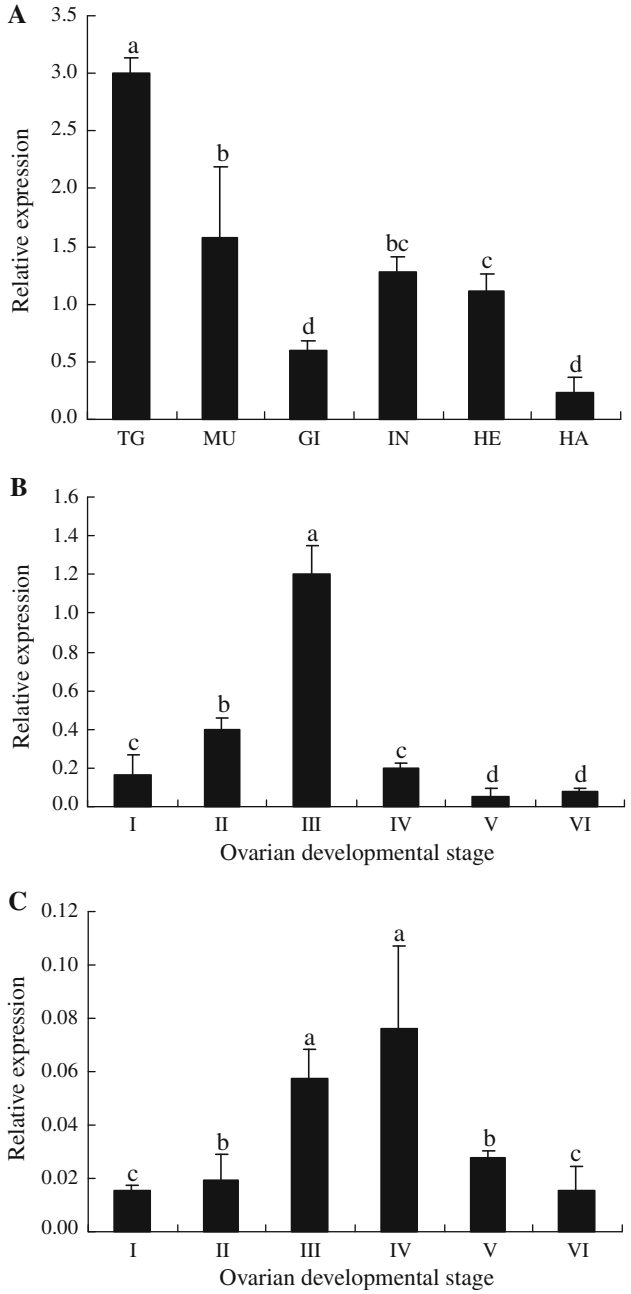

Fig. 2 MnHSP90 mRNA expressions in various tissues of the oriental river prawn. **a** MnHSP90 transcripts in haemocytes (HA), gill (GI), thoracic ganglia (TG), heart (HE), muscle (MU), and intestine (IN) at the ovarian stage II. **b** MnHSP90 mRNA expression in the hepatopancreas during ovarian development. **c** MnHSP90 mRNA expression in ovaries during ovarian development. The relative expression of MnHSP90 was measured by the SYBR Green qPCR. Values are shown as mean ± SE ($n = 3$). Values with the same superscript are not significantly different ($P > 0.05$)

The relative expressions of MnHSP90 mRNA in the hepatopancreas and ovary reached the maximum at stage III and stage IV, respectively, then dropped with the advance of ovarian development. However, there was no significant difference in MnHSP90 expressions in the ovaries between stage III and IV. And the relative transcript of MnHSP90 mRNA in ovaries was significantly lower than that in the hepatopancreas.

Discussion

The HSP90 acts as a homodimer to facilitate maturation and signal transduction proteins in the regulatory pathway in flatfish Senegalese sole *Solea senegalensis* Kaup [28]. HSP90 genes have also been isolated from crab [29, 30] and marine shrimp [24, 31], but the expression of HSP90 genes in freshwater crustacean has not been investigated. In the present study, we cloned the full length of the MnHSP90 gene cDNA (GenBank accession N0 GU319963) from a freshwater crustacean species for the first time in an attempt to provide a fundamental basis to understand the molecular mechanism of this protein in regulating the ovary maturation of *M. nipponense*.

In this study, the deduced amino acid sequence of MnHSP90 included five conserved amino acid motifs which are characterized for the HSP90 protein family signature (i.e., NKEIFLRELISN[S/A]SDALDKIR, LGTIA [K/R]SGT, IGQFGVGFYSA[Y/F]LVA[E/D], IKLYVRR VFI, and GVVDS[E/D]DLPL N[I/V]SRE) [31–33]. In the motif of IGQFGVGFYSA[Y/F]LVA[E/D], the MnHSP90 has Ile^{14} while the HSP90 in other organisms has Val at the homologous position. This structural similarity is consistent with the HSP90 amino acid of *Tigriopus japonicus* (ACA03524) and *Apis mellifera* (XP_395168). We identified a GxxGxG motif in the deduced amino acids, which is essential to the ATP binding in the HSP90 molecular chaperone [34, 35]. The BLAST analysis indicates that the MnHSP90 shares significant homology at the levels of nucleotide (72–79%) and amino acids (78–88%) with HSP90 in other organisms. The conserved characteristics and high similarity with known HSP90s indicate that the MnHSP90 belongs to the HSP90 family. Based on the presence of sequence MEEVD on its C-terminus, the MnHSP90 is concluded to be a cytosolic HSP90 homolog, similar to the HSP90 proteins in other species [36, 37].

MnHSP90 were ubiquitously detected in all examined tissues, with the highest expression in the thoracic ganglia which is a part of the central nervous system in shrimp. The finding can be compared with what found in rabbit [38] and bovine [39] where the highest expressions of HSP90s occur in brain. The current study was the first to report the HSP90 expression in the thoracic ganglia in a crustacean.

Interestingly, among the tested tissues, the thoracic ganglia are the only organ reported to secrete hormones to induce ovary maturation in crustacean [40, 41]. In another study, the HSP90 mRNA expression in the ovary of *M. ensis* starts to increase in stage II, and the level of its expression varies with the stage of ovary development [24]. This result is similar to what we observed on *M. nipponense*. The results of these two compatible studies suggest that the high expression of HSP90 in the thoracic ganglia supports our hypothesis that HSP90 plays a role in regulating ovary development since the development of thoracic ganglia can stimulate the ovary maturation [40].

Subepidermal adipose tissues, hepatopancreas and ovaries are thought to be the sites for VTG synthesis in crustaceans [42]. The hepatopancreas and ovary are the primary organs for VTG synthesis in oriental river prawn [25]. In this study, we found that the MnHSP90 was detected not only in the ovaries but also in the hepatopancreas. Interestingly, the expression of MnHSP90 mRNA in the hepatopancreas was much higher than that in the ovaries of animals at the same ovarian stage. The hepatopancreas is a gland in crustacean, which combines the digestive functions of the liver and pancreas. However, in the oriental river prawn, the hepatopancreas is proved to be a main organ for VTG synthesis [25], which is consistent with the result in *Macrobrachium rosenbergii* [43] and *Fenneropenaeus chinensis* [44].

In *M. ensis* and *Penaeus japonicus*, the transcription of VTG in the hepatopancreas and ovaries peaked at a late vitellogenic stage [45–48]. In the present study, the expression of MnHSP90 in these two major organs for VTG synthesis was high in stage III (early vitellogenic stage) and IV (late vitellogenic stage) ovaries but decreased in stage V (mature stage) ovaries. This expression pattern of MnHSP90 in the ovary and hepatopancreas during the ovary development is consistent with that found in *M. ensis* [24] and *P. monodon* [23]. Our results showed that the relative transcripts of MnHSP90 mRNA in the ovary and hepatopancreas were related to the stage of ovarian development. The high expression of MnHSP90 prior to the transcription of VTG indicates an active role of HSP90 in the transcriptional regulation of VTG synthesis [24].

The regulatory mechanism of HSP90 for VTG synthesis is controversial. In oviparous vertebrates, there exists a HSP90-ER-E-VTG regulation channel for VTG synthesis. In crustacean, on the other hand, estrogen (E) does not exist. Therefore, the traditional school does not support that a similar regulatory mechanism in oviparous vertebrates exists in crustacean. However, the detection of estrogen in the ovary of crustaceans has challenged the conventional thought in this regard [49, 50]. Wu and Chu [24] reported a strong correlation between estrogen hormones and HSP90 expression in a shrimp *M. ensis*, suggesting that the expression of VTG may be under the regulation of estrogen hormones through the mechanism similar to that in vertebrates. The detection of MnHSP90 expression in the hepatopancreas and ovary of oriental river prawn supports the hypothesis that HSP90 regulates maturation and reproduction in freshwater crustacean.

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