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ISSN 0792 - 156X

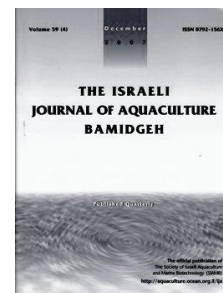
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Identification and mRNA Expression of Heat Shock Proteins in the Mud Crab (*Scylla paramamosain*) in Response to Acute Nitrite Exposure

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Keywords: *Scylla paramamosain*; heat shock protein; HSP40; nitrite stress

Abstract

Heat shock proteins (HSPs) play an important role in protecting organisms against various stressors. Heat shock protein 40 (HSP40) is a class of the heat shock protein family and performs a function as co-chaperone of HSP70. In this study, an HSP40 gene from the mud crab *Scylla paramamosain* (SpHSP40) was identified and characterized. The full-length cDNA of SpHSP40 was 1904 bp, containing an open reading frame (ORF) of 1191 bp, a 5' UTR of 118 bp, and a 3' UTR of 595 bp. The deduced amino acid sequence of SpHSP40 contained all four classical HSP40 family signatures. Quantitative real-time PCR analysis revealed that SpHSP40 transcript was expressed in a wide range of tissues, while strong expression was observed in the hepatopancreas. In order to understand the response of heat shock proteins induced by nitrite exposure, expression levels of HSPs (SpHSP90, SpHSP70, SpHSP60 and SpHSP40) mRNA in the hepatopancreas and gills were investigated. Results show that HSPs (SpHSP90, SpHSP70, SpHSP60 and SpHSP40) were up-regulated displaying a time-dependent pattern in response to nitrite stress. All these results indicate that HSPs play an important role in mediating environmental stress in mud crabs.

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Introduction

Nitrite is a major pollutant in the aquatic environment. When excess feeding supply and nitrogen excretion outstrips the metabolic capability of indigenous flora in aquaculture water, it can increase water nitrite concentrations (Hilmy et al., 1987). Elevated nitrite in aquaculture systems can cause accumulation of nitrite in the gill epithelium and reaches very high concentrations in body fluids (Jensen, 2003). Accumulation of nitrite in aquaculture water is highly toxic to aquatic animals and influences growth, survival, physiological functions, and immune response (Chen & Chen, 1992; Chen and Cheng, 1995a; Deane and Woo, 2007; Guo et al., 2013).

Previous studies have shown that increased nitrite levels cause oxidative stress (Tseng and Chen, 2004; Guo et al., 2013). The organism is incapable of producing ROS to deal with oxidative stress (Lesser, 2006), which can trigger DNA damage, protein oxidation, lipid peroxidation, and a decline of other physiological functions (Stadtman and Levine, 2003). Oxidative stress induced by nitrite stress also can lead to a fundamental biological reaction-heat shock response (Guo et al., 2013; Jia et al., 2015). Heat shock proteins (HSPs) are a group of highly conserved proteins in all living organisms, that are induced by heat shock, toxins, bacterial infection, and a variety of other stresses (Srivastava, 2002). These inducers are responsible for the maintenance of cellular viability by preventing the irreversible loss of vital proteins and facilitating their subsequent regeneration (Parsell and Lindquist, 1993). According to their molecular mass, HSPs have been categorized into different families, including HSP100, HSP90, HSP70, HSP60, HSP40 and several other low molecular mass HSPs. HSP90, is often found in a constitutive dimer, which plays a pivotal role in controlling multiple regulatory pathways such as stress defense, intracellular transport, protein degradation, and cell signaling (Miyata and Yahara, 1992). HSP70 mainly acts as an intracellular molecular chaperone assisting in protein folding and transport (Xu et al., 2014). HSP60 plays an important role in assisting with the protein folding and stress protection in mitochondria (Cechetto et al., 2000).

HSP40 is a class of heat shock proteins that functions as a co-chaperone of HSP70. The HSP40 family members are subdivided into three distinct domains (Kostenko et al., 2014). All types of HSP40 proteins contain the conserved J domain. The conserved J domain of HSP40 can regulate the intrinsically low basal ATPase activity of HSP70 (Minami et al., 1996; Fan et al., 2003). HSP40 members play important roles such as protein translation, folding, unfolding, translocation, and degradation (Li et al., 2009). Previous studies have shown that HSP40 is also involved in mediating the immune responses and environmental stresses (Ohtsuka et al., 1990).

The mud crab (*Scylla paramamosain*) is widely distributed along the coast of South China. In recent years, mud crab cultivation has become increasingly popular in South China, with production exceeding 140 thousand tons. However, with the expansion of culture, the mud crab aquaculture industry has encountered great challenges including devastating diseases which cause economic losses (Weng et al., 2007; Guo et al., 2013). In mud crab, characterization of HSP90, HSP70, and HSP60 was reported in National Center for Biotechnology Information (NCBI). However, little information is available regarding molecular features and functions of HSP40. The main purpose of this study was: (1) to clone the full-length cDNA of HSP40 from the mud crab; (2) to investigate the tissues expression patterns of HSP40; (3) to clarify the time-course expression profiles of HSP90, HSP70, HSP60, HSP40 in response to acute nitrite exposure. Results obtained may give us more insight into the molecular mechanism of HSPs against adverse stress in the mud crab.

Materials and methods

Mud crabs (50±3 g) were obtained from the mud crab cultivating area of Taishan in Guangdong province. They were acclimated in tanks maintained at 10‰ salinity and 25°C for two week prior to the experimental treatments. Oyster meat was fed to them twice a day until 24 h before onset of the experiments.

Mud crabs were exposed to 0 and 15 mg/L nitrite (N) for 72 hours. Nitrite test solutions were prepared by adding NaNO₂ to 10‰ saltwater until the desired concentration was attained. After exposure for 0, 12, 24, 48, and 72 h, six mud crab from each group were randomly sampled and sacrificed. Hepatopancreas and gill samples were collected and immediately frozen in liquid nitrogen before storage at -80°C.

Total RNA was extracted from selected tissues of the mud crabs, using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The A₂₆₀/A₂₈₀ ratios of all RNAs prepared were measured by Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA integrity was checked by electrophoresis on 1% agarose gels. Subsequently, Rnase-free water was used to dissolve total RNA which was then stored at -80°C. Then, single-stranded cDNA was synthesized from 1µg total RNA using PrimeScript RT reagent Kit With gDNA Eraser (Takara, Dalian, China) following the manufacturer's instructions. The cDNA templates were then stored at -80°C for later analysis.

To determine the full-length cDNA of SpHSP40, two specific primers, R1 and R2 (Table 1) were selected based on EST sequence. Full-length cDNA of SpHSP40 was obtained through rapid amplification of cDNA ends (RACE). RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer's instructions. Forward and reverse primers were designed based on the partial cDNA sequence obtained above (Table 1). The nested PCR program for 3' and 5' RACE were as follows : 5 cycles at 94°C for 30 s, 72°C for 2min; 5 cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 2 min; 25 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 2 min, and a 10 min final extension at 72°C. The nested PCR products were then cloned and sequenced as described above.

Table 1 The sequences of primers in this experiment.

| Primers names | Nucleotide sequence (5'-3') |
|---------------|-----------------------------|
| HSP40-F | ACTCAAAAAGCATATA |
| HSP40-R | GTGGTTGTGACGCCTTCCTCG |
| HSP40-3gsp | GGAAGAGGCACGCAAGCAGAAGG |
| HSP40-5gsp | CATAGTAACCTGTTTCTTTACCACAT |
| RT-HSP40-F | GCCGAAATCAAGTCTGTATTAGGTG |
| RT-HSP40-R | AAGTCTACAAGGAACTGGAGGAGC |
| RT-18S-F | CCTCGTTCATGGGAGACAAT |
| RT-18S-R | CTAGTCGACGGATCTCCAGC |
| RT-HSP60-F | CTCCCTGTCCCGCCAAGT |
| RT-HSP60-R | TGTTGATGAAGTATGGGGAGATGTA |
| RT-HSP70-F | AGGACAAGGTGAGCGAAGAGG |
| RT-HSP70-R | TTGGTGATGATGGGGTTACAGA |
| RT-HSP90-F | TCTTGCCTCGCCGTGCTC |
| RT-HSP90-R | TTCGGGAATCAACTCCTCACA |

The nucleotide sequence and deduced amino acid sequence of SpHSP40 cDNA were performed using the BLAST program at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The open reading frame (ORF) was predicted using Open Reading Finder (ORF) (<http://www.ncbi.nlm.nih.gov/gorf>). Translation and protein analysis were performed by ExPaSy tools (<http://www.expasy.org/tools/>). The conserved domains of SpHSP40 were predicted by the SMART program available from the EMBL website (<http://smart.embl-heidelberg.de/>). The phylogenetic tree was determined based on Molecular Evolution Genetics Analysis (MEGA) software version 4.1 by the neighbor-joining method and 1000 replications of bootstrap.

The mRNA levels of SpHSP40 in different tissues were detected using quantitative real-time RT-PCR. Tissues (hemocytes, gill, muscle, heart, stomach, hepatopancreas, and intestine) were carefully collected separately from six healthy fish. The relative mRNA level was compared with muscle expression. Total RNA extraction, DNase I treatment and cDNA synthesis were conducted according to the method described above.

To further investigate the expression profiles of HSPs in response to nitrite exposure, SpHSP90, SpHSP70, SpHSP60, and SpHSP40 were used by RT-PCR. Specific primer pairs of SpHSP90, SpHSP70, and SpHSP60 were designed based on available published mud crab mRNA sequences in NCBI using Primer Premier 5 (Table 1). The 18s rRNA used as an internal control. The specificity of the primer was examined by conventional PCR and melting curve sequence analysis. Real-time PCR was amplified in an ABI 7500 real-time PCR machine (Applied Biosystems, USA) using SYBR Premix Ex Taq™ (Takara, Dalian, China) following manufacturer's recommendations. Before the qRT-PCR experiments, the specificity and efficiency of the primers above were determined. The reaction mixtures were 20 µL, containing 2 µL diluted cDNA sample (50 ng/µL), 0.4 µL ROX, 10 µL 2× SYBR Premix Ex Taq, 0.4 µL each of primer (10µM), and 6.8 µL dH₂O. The real-time PCR

conditions were as follows: 94°C for 10 min, then 45 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, followed by 10 min at 72°C. The standard equation and correlation coefficient were determined by constructing a standard curve using a serial dilution of cDNA. cDNA of each sample isolated from six fish in each treatment was detected by qRT-PCR analysis. Each sample was amplified in triplicate. After the program finished, the threshold cycle (Ct) values were obtained from each sample. Relative gene expression levels were evaluated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

All data were expressed as means \pm standard deviation. Significant differences were evaluated by a one-way ANOVA followed by Duncan's multiple range tests. Statistical analysis was performed using SPSS 18.0 software (SPSS, Chicago, IL, USA). P value < 0.05 was considered to be statistically significant.

Results

The full-length of SpHSP40 was 1904 bp including a 118 bp 5'-untranslated region, a 1191-bp open reading frame, and a 595-bp 3'-untranslated region (Fig.1).

```

TGCTCTCCCTCCCGCTGTCTGCTGGAAGTTTCCCGAGATCAGAGAATATTCTCGCTC-
TCTGCGAAGAACGGCGCCCTTTTCCACATCCAGCCCTAAATACATCAAGAATAAAG-
20- atggTgaagaacaaggttactatgatatgcttggggTgaacccaactgcatcacaagat
M V K E T G Y Y D M L G V K P T A S Q D-
gaactcaaaaaagcatataggaatTggccctcaagTatccacctgaacaaacccaat 40-
E L K K A Y R K L A L K Y HPD K N P N-
gaaggagaaaagtTcaagTtgatctcaccagcatatgaagTcctTtagcaatgaagaaaaa 60-
E G E K F K L I S Q A Y E V L S N E E K-
cgcaaaatTtagcaccaggaggagaaacagccctcaaggaaggagTtagTggagTgga 80-
R K I Y D Q G G E Q A L K E G G S G G G-
agctTctctcccccaggacatctTtgacatTtctTcggagTggaatgagggcagga 100-
S F S S P M D I F D M F F G G G M R R G-
cgagaaaaagagagTgaaggaTgtTatTcaccaaatgagTgtTtcatTtagaggaactTtac 120-
R E K R V K D V I H Q M S V S L E E L Y-
aatggagctactcgaagTtggccctgcagaagcatTtattTgcaacaaatgcgaagTt 140-
N G A T R R L A L Q K H V I C N K C E G-
caggcggaagaagaaccccagagaagTgcccTtcatTtagagcactggaatgcagTc 160-
Q G G K K P P E K C P S C R G T G M Q V-
cgaatccagcagctaggccaggcatTggtatcccagTgcaagcagTgtTggaagTgt 180-
R I Q Q L G P G M V S Q V Q S M C G E C-
cagggcgaggTgaacgcatcagTctTaaaggcagTgtaagacatTgagggcagaag 200-
R G Q G E R I S L K D R C K T C E G R K-
gtgTcaaaagagagaagatctTtagagTtcatTggaacaaggcagTggaagTggacag 220-
V V K E R K I L E V H V D K G M E D G Q-
aagatTgtTtctcggTgaaggggaccaagagccagccctTgagccagagatattatc 240-
K I V F S G E G D Q E P G L E P G D I I-
atcTctTtagatgagaagatcaccacaatTcagacgcTcgaatgatTcacaatg 260-
I V L D E K D H P T F R R V M N D L T M-
cagatTcacataaacctTgTggaagcatTgTgTgTtccagaaacctatTaaactTta 280-
Q I H I T L V E A L C G F Q K P I K T L-
gatgataagactatcTgtTtagcaccatTcctTggcgaagTgtaagaatTgcgaaatc 300-
D D R T I V V S T I P G E V I K N A E I-
aagTctgTattagTgagggcagTcctcagTacaagaatccatTtgaagggagcTgtTg 320-
K S V L G E G M P Q Y K N P F E K G R L-
ctcctccagTtctTgtagactTcccatcaaaatTcctTtTgatcgaatccgaaagctT 340-
L L Q F L V D F P S Q I P L D R I R K L-
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E K I L P P R P E I I I P D E G E E V N-

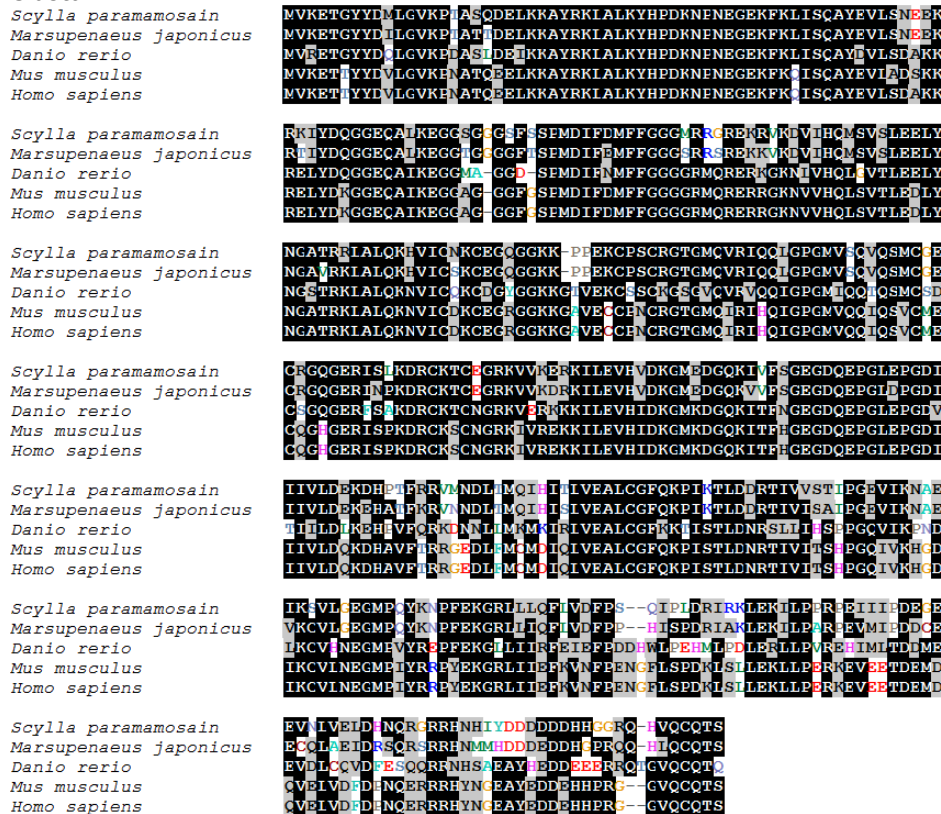
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L V E L D H N Q R G R R H N H I Y D D D-
GatgataccatcagggagcagcaacatTccagTgccagacaagctag 396-
D D D H H G G R Q H V Q C Q T S - -
GCTCCACAGCTTTATATGATGATGTCGGTAACTTAAATACTTGACAGACCTTCTTGATCC
CTGTCAGCAGGTATATGATGACTTGGGCTGAAAGACTGGAGTGTGATCAGCACAGCAT
GAAAGGTAACCAGGATAITGATAITTTATGTTTGGAGTTTGTGTTCTGTGCTGCTTCTA
ATTATGTTCAATCATTTCACATTTCACTTAGTACATGTCAGGAAGGGTGTCTGTGTAGAAGG
GAGGATATGATGATGAGGCTTCAGTATTTAAAAATATAACCCCTTTTCTCTCATAGGAGAT
GCAATCTCTGTAGAGAGCACATCCCAAAGCTGGTGGTTTTATGCTTCTCTTAGATGTTGTA
GAGGACACTACATATTTTATTTTATTTTGAATTTTGACGTAATAAGCCCTCCATTTAGTTT
CTTTCTTTCCATGGTATTTGTTCCACTATGATGATGGAACAGTCCACAGATAGATCTGAAGG
ACACCAATTCCTTTAAATCAATGATATAATGACAGATCATTCAAAGTGAATTTGCATCA-
TGAAAAAAAAAAAAA-

```

Fig.1. Nucleotide sequence of SpHSP40 cDNA and the deduced amino acid sequence. Conserved J-domain is underlined. There is a square border around HPD tripeptide. G/F region is has a dotted underline. CR domain is double underlined. The poly-(A) tail is underlined at the end of the nucleotide sequence.

The ORF encoded a polypeptide of 396 amino acids with a theoretical isoelectric point of 6.87 and predicted molecular weight of 44.7 kDa. The deduced amino acid sequence of SpHSP40 contains a conserved J-domain in the N-terminus by SMART analysis. The SpHSP40 polypeptide sequence does not have a signal peptide. The J-domain was located from Thr^{5rd} to Lys^{60st} in the deduced amino acid of SpHSP40, where a highly conserved histidine-proline-aspartic acid (HPD) motif was also found (Fig.2). Adjacent to the J-domain is a glycine/phenylalanine-rich (G/F) region with a conserved DIF (Asp-Ile-Phe). In addition, a cysteine-rich Zn²⁺ binding domain (CR) contacting CXXC-CXX sequence was found in the deduced amino acid sequence of SpHSP40. These results suggest that SpHSP40 belongs to type I HSP40 family.

Fig.2. Multiple alignments of deduced amino acid sequence of SpHSP40 with other HSP40 using Clustal X.



The deduced amino acid sequence of SpHSP40 shared significant homology to other known HSP40 protein families, such as 86% identity with *Marsupenaeus japonicus*, 68% identity with *Homo sapiens*, 67% identity with *Mus musculus*, 62% identity with *Danio rerio*. Multiple sequences alignment revealed that SpHSP40 shared a high degree of identity of HSP40 (Fig.2).

To understand the evolutionary relationship, the phylogenetic tree of HSP40 was constructed using MEGA4.1. As shown in Fig.3, the phylogenetic tree revealed that HSP40 sequences were divided into two major clusters. According to the phylogenetic tree, SpHSP40 was closely related to those of other invertebrates.

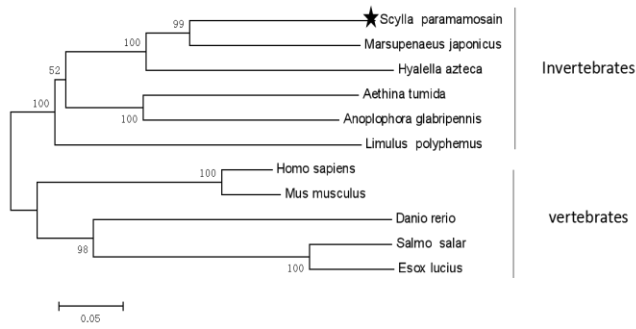


Fig.3. Phylogenetic analysis of SpHSP40 with other members of the HSP40. The phylogenetic tree was constructed using MEGA software 4.1 by the Neighbor-joining method and 1000 replications of bootstrap.

The tissue distribution of SpHSP40 mRNA was investigated with RT-PCR. As shown in Fig.4, the SpHSP40 mRNA was widely expressed in all selected tissues (hemocytes, gill, muscle, heart, stomach hepatopancreas, and intestine). The highest expression of SpHSP40 was observed in the hepatopancreas, and the lowest in muscle.

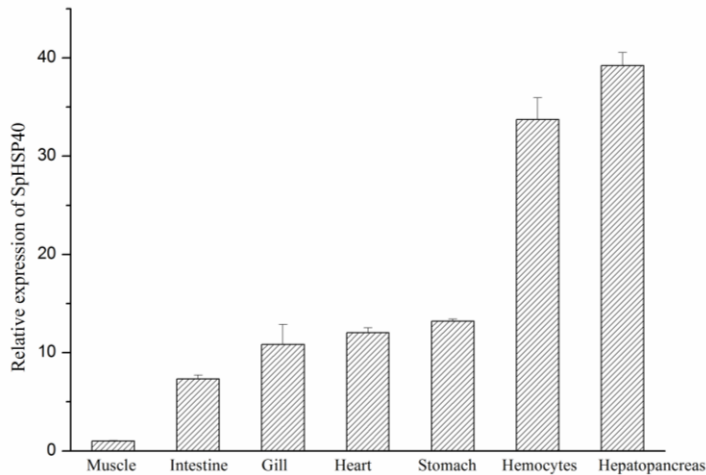
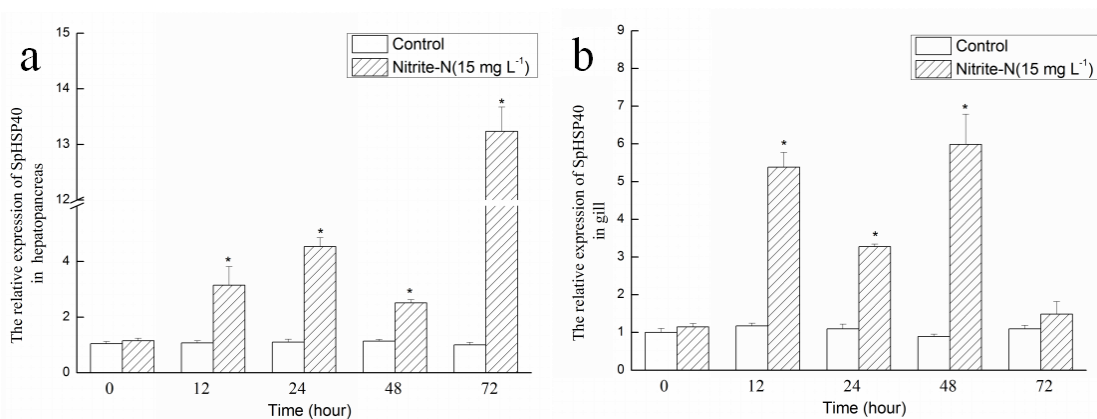


Fig.4. Tissue-specific mRNA expression of the SpHSP40 determined by quantitative real-time PCR. The relative SpHSP40 mRNA expression of each tissue was calculated by the $2^{-\Delta\Delta CT}$ method using 18S rRNA as a reference gene. Data are presented as mean \pm SD (N= 6).

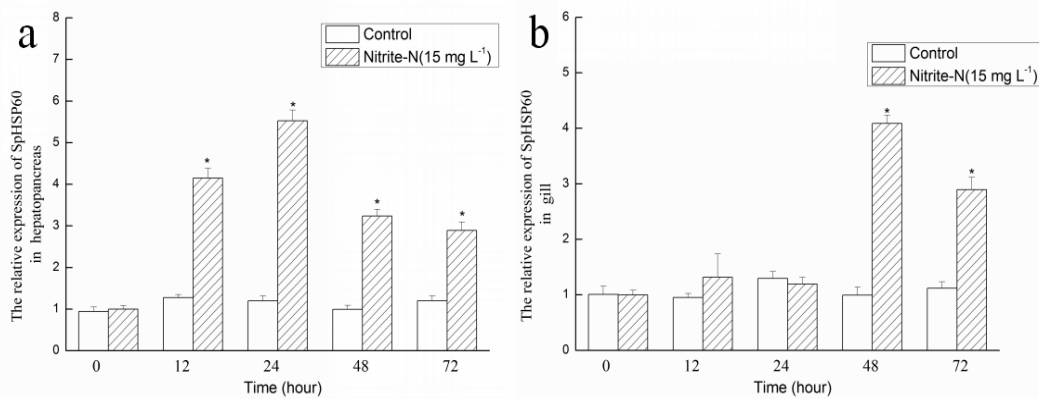
In order to provide a better understanding of HSP responses induced by nitrite exposure, mRNA transcripts of HSP (SpHSP90, SpHSP70, SpHSP60, and SpHSP40) in hepatopancreas and gill were investigated with RT-PCR. As shown in Fig. 5a, the transcript level of SpHSP40 in hepatopancreas increased significantly at 12 h and reached the highest level at 72 h after nitrite exposure. In the gill, the expression level of SpHSP40 mRNA was significantly up-regulated at 12 h, 24 h, and 48 h, and returned to its original level after 72 h (Fig.5b).

Fig.5. Relative expression levels of SpHSP40 in hepatopancreas (a) and gill (b) in response to nitrite stress. Data are presented as the mean \pm SD (N=6). Asterisks indicated results that are significantly different from the control ($P < 0.05$).



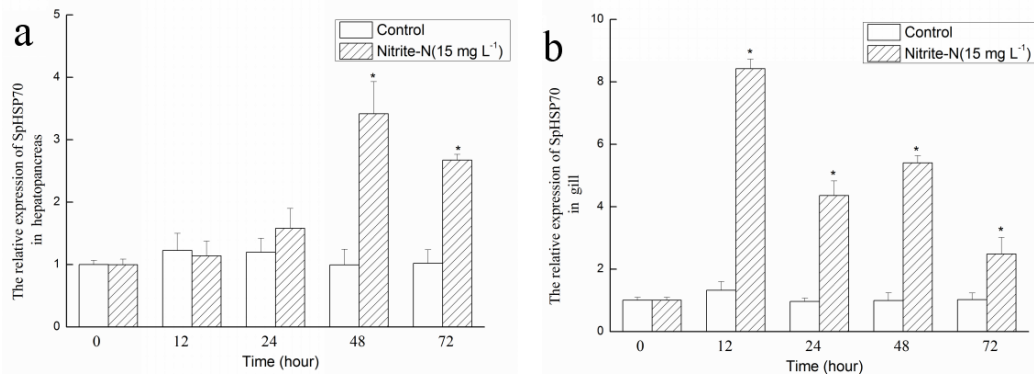
As shown in Fig. 6a, a gradual increase of SpHSP60 transcript expression in hepatopancreas was observed from 12 h to 24 h after nitrite exposure, and reached a peak level at 24 h with the highest value of 5.5-fold greater than that of the control, followed by a slight fluctuation from 48 h to 72 h. In the gills, there were no significant differences at 12 h and 24 h in the expression level of SpHSP40 mRNA, after nitrite exposure. There was a significant increase in the expression level of SpHSP40 mRNA at 48 h and 72 h after nitrite exposure (Fig. 6b).

Fig.6. Relative expression levels of SpHSP60 in hepatopancreas (a) and gill (b) in response to nitrite stress. Data are presented as the mean \pm SD (N=6). Asterisks indicated results that are significantly different from the control ($P < 0.05$).



After exposure to nitrite, no significant change was observed during the first 24 h (Fig. 7a). As time progressed, the expression level of SpHSP70 mRNA in hepatopancreas increased significantly, and the level of SpHSP40 was 3.4-fold at 48 h and 2.6-fold at 72 h compared with that in the control group. In the gill, the expression level of SpHSP70 mRNA was up-regulated and reached the highest level after 12 h, but then decreased until the end of the experiment.

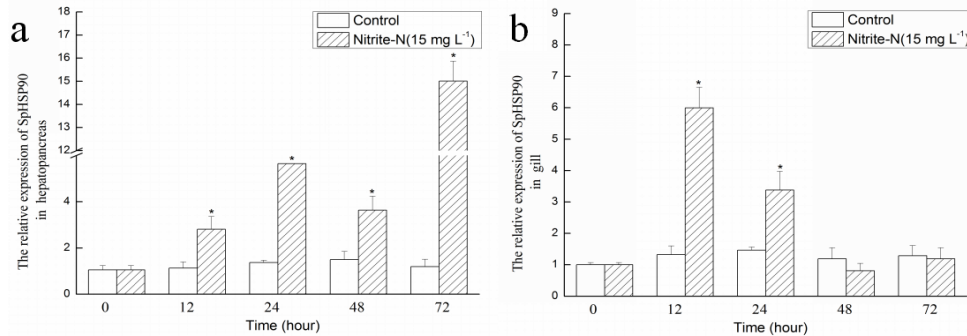
Fig.7. Relative expression levels of SpHSP70 in hepatopancreas (a) and gill (b) in response to nitrite stress. Data are presented as the mean \pm SD (N=6). Asterisks indicated results that are significantly different from the control ($P < 0.05$).



As shown in Fig.8a, the expression level of SpHSP70 mRNA in the hepatopancreas increased significantly from 12 h to 72 h and peaked at 72 h with the highest value being 15 times greater than that of the control group. In the gills, the expression level of SpHSP70 mRNA was up-regulated at 12 h and 24 h after nitrite exposure. However, the SpHSP70 expression dropped gradually and returned to its original level at 48 h and 72 h (Fig.8b).

As shown in Fig.8a, the expression level of SpHSP70 mRNA in the hepatopancreas increased significantly from 12 h to 72 h and peaked at 72 h with the highest value being 15 times greater than that of the control group. In the gills, the expression level of SpHSP70 mRNA was up-regulated at 12 h and 24 h after nitrite exposure. However, the SpHSP70 expression dropped gradually and returned to its original level at 48 h and 72 h (Fig.8b).

Fig.8. Relative expression levels of SpHSP90 in hepatopancreas (a) and gill (b) in response to nitrite stress. Data are presented as the mean \pm SD (N=6). Asterisks indicated results that are significantly different from the control (P<0.05).



Discussion

In this study, the full-length HSP40 cDNA sequence from *Scylla paramamosain* was cloned successfully. Multiple sequence alignment showed that SpHSP40 was similar to other species. HSP40 proteins are divided into three subgroups according to their domain organization. Previous studies showed that the G/F domain may be important for substrate specificity and binding to HSP70 (Fan et al., 2005; Knox et al., 2011). The cysteine-rich Zn²⁺ binding domain is a prerequisite for substrate binding to their HSP70 partners (Fan et al., 2005). In our study, SpHSP40 protein possesses a conserved structural characteristic of the J-domain, G/Fdomain, cysteine-rich Zn²⁺ binding domain, and conserved C-terminal domain, suggesting that SpHSP40 is a member of the type I HSP40 family. Type I HSP40 can perform a function as a co-chaperone of Hsp70 to suppress protein aggregation (Knox et al., 2011). A phylogenetic tree was constructed based on the amino acid sequence of SpHSP40 and other HSP40 sequences from both vertebrates and invertebrates. We found that SpHSP40 was conserved. These findings suggested that SpHSP40 possessed the major structural and similar regulatory functions as found in typical HSP40.

The SpHSP40 mRNA expression in different tissues could be helpful to further understand its function. In our study, the level of SpHSP70 mRNA was constitutively expressed in all examined tissues (hemocytes, gill, muscle, heart, stomach, hepatopancreas, and intestine). This constitutive expression of HSP40 has also been reported in *Pinctada martensii* (Li et al., 2016) and *Paralichthys olivaceus* (Dong et al., 2006). Furthermore, SpHSP40 was highly expressed in hemocytes and the hepatopancreas. Similar results were observed in *Pinctada martensii* (Li et al., 2016). The hepatopancreas is an important tissue for immune defense (Gao et al., 2008). In invertebrates, hemocytes play a key role in the recognition and elimination of pathogens, which could cause high expression of SpHSP40 in hemocytes. All these results indicate that SpHSP40 is dominantly expressed in immunity-associated tissues, which play important roles in the immune system of the mud crab.

Environmental stress induces suppressive or adverse effects on the immune system of aquatic animals. Nitrite stress is one of the most serious threats for aquaculture. Organisms could not remove overproduced ROS products induced by nitrite stress, which then led to DNA damage, protein oxidation, and lipid peroxidation (Lesser, 2006). Nitrite exposure of 20 mg/L was reported to induce overproduction of ROS, resulting in DNA damage and cell apoptosis of *Penaeus monodon* (Xian et al. 2001). Prolonged nitrite exposure resulted in the formation of excess ROS, causing oxidative damage to lipids and proteins, and impairment of antioxidant defenses (Sun et al. 2014). As we know the antioxidant defense system plays an important role in immune defense against ambient stressors. In addition, heat shock proteins are considered to be the first line of defense against environmental stresses in order to maintain protein homeostasis. Previously, it has been demonstrated that nitrite stress changed HSP70 transcription in *L. vannamei* hemocytes (Guo et al., 2013). HSP70 levels were shown to be stimulated by nitrite exposure (Jia et al. 2015). HSP40 and HSP70 mRNA levels in *P. martensii* were induced by thermal, low salinity, and bacterial challenges (Li et al. 2016). In the present study, SpHSP90, SpHSP70, SpHSP60, and SpHSP40 mRNA levels were up-regulated displaying a time-dependent pattern after nitrite exposure. HSPs protect proteins from denaturation, assist in the folding of nascent proteins, and remove irreversibly damaged

proteins (Geething and Sambrook, 1992). HSP was considered to play a critical role in protecting cells against oxidative stress. The rapid induction of HSPs in organisms could enhance cell resistance to environmental stress (Pelham, 1986). Our results suggest that HSP plays an important role in protecting organisms against nitrite stress.

In conclusion, we cloned the SpHSP40 gene from the mud crab for the first time. Sequence characterization and phylogenetic analysis demonstrated that SpHSP40 was highly homologous to the known HSP40 sequences from other crustaceans. SpHSP40 is constitutively expressed in various tissues, with a high-level expression in immune-related tissues (hepatopancreas and hemocytes). The expression of SpHSP90, SpHSP70, SpHSP60, and SpHSP40 were up-regulated, displaying a time-dependent pattern in response to nitrite stress. This study shows us the molecular mechanism of HSPs against adverse stresses in the mud crab. It also contributes to developing strategies for monitoring environmental pollutants.

Acknowledgements

This research was supported by the China Agriculture Research System (CARS-48), Marine Fisheries Technology and Industry Development Special Projects of Guangdong Province (A201501B14), Marine Fisheries Technology and Extension System of Guangdong Province (A201701B01). Central Public-interest Scientific Institution Basal Research Fund, CAFS (2017HY-ZD0304 and 2017HY-ZD1007). the Youth Science and Technology Innovation Talents Funds in Special Support Plan for High Level Talents in Guangdong Province (2016TQ03N275).

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