Characterization and function analysis of Hsp60 and Hsp10 under different acute stresses in black tiger shrimp, *Penaeus monodon*

Jinxuan Shi^{1,2} · Mingjun Fu^{1,3} · Chao Zhao^{1,3} · Falin Zhou^{1,3} · Qibin Yang^{1,3,4} · Lihua Qiu^{1,3}

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Abstract Heat shock proteins (Hsps) are a class of highly conserved proteins produced in virtually all living organisms from bacteria to humans. Hsp60 and Hsp10, the most important mitochondrial chaperones, participate in environmental stress responses. In this study, the full-length complementary DNAs (cDNAs) of Hsp60 (*PmHsp60*) and Hsp10 (*PmHsp10*) were cloned from *Penaeus monodon*. Sequence analysis showed that *PmHsp60* and *PmHsp10* encoded polypeptides of 578 and 102 amino acids, respectively. The expression profiles of *PmHsp60* and *PmHsp10* were detected in the gills and hepatopancreas of the shrimps under pH challenge, osmotic stress, and heavy metal exposure, and results suggested that *PmHsp60* and *PmHsp10* were involved in the responses to these stimuli. ATPase and chaperone activity assay indicated that *PmHsp60* could slow down protein denaturation and that Hsp60/Hsp10 may be combined to produce a chaperone complex with effective chaperone and ATPase activities. Overall, this study provides useful information to help further understand the functional mechanisms of the environmental stress responses of Hsp60 and Hsp10 in shrimp.

Keywords Hsp60 · Hsp10 · pH challenge · Osmotic stress · Heavy metal exposure · *Penaeus monodon*

Introduction

Heat shock proteins (Hsps), also known as stress proteins and extrinsic chaperones, are a suite of highly conserved proteins produced in all living organisms, especially when exposed to environmental stresses, such as heat shock, UV irradiation, and heavy metals or other toxic chemicals (Roberts et al. 2010). Hsps are involved in a variety of biological functions, such as protein folding, damaged protein repair, nascent protein transport, and immune presentation (Welch 1991; Gething and Sambrook 1992; Freeman et al. 2000; Quintana and Cohen 2011; Pons et al. 2013). Based on their molecular mass, Hsps are generally classified into six major families, i.e., Hsp110, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (Tang et al. 2011; Music et al. 2014).

Hsp60 is a homolog of phage growth E large (GroEL) in *Escherichia coli* (Xu et al. 2014). Over the last several decades, multiple Hsp60 functions have been discovered. For example, Hsp60 is capable of activating the innate immune response to atherosclerosis and other inflammatory disorders (Kol et al. 2000; Grundtman et al. 2011). Ghosh et al. (2008) demonstrated that Hsp60 is involved in apoptosis and cell cycle regulation. Hsp60 is able to substantially accelerate procaspase-3 maturation by different upstream activator caspases (Xanthoudakis et al. 1999). The possible roles of Hsp60 in certain cellular processes, such as sperm cell differentiation, reproduction development, and environment stress response, have also been elucidated by other researches (Meinhardt et al. 1995; Paranko et al. 1996; Kozlova et al. 1997; Neuer et al. 2000; Qian et al. 2012). Much of the research on organism protection under environmental stress in

✉ Lihua Qiu
qiu902@yahoo.com

¹ South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China

² College of Aqua-life Science and Technology, Shanghai Ocean University, Shanghai 201306, China

³ Key Laboratory of South China Sea Fishery Resources Exploitation & Utilization, Ministry of Agriculture, Guangzhou 510300, China

⁴ Tropical Aquaculture Research and Development Center of South China Sea Fisheries Research Institute, Sanya 572000, China

the last decades has examined the involvement of Hsp60 in multiple pathophysiological processes. For instance, Hsp60 was observed to be activated to prevent protein denaturation under heat stress (Martin et al. 1992). Xu et al. (2014) also found that Hsp60 is involved in the heat shock response in the sea cucumber *Apostichopus japonicus*. In addition, Hsp60 plays important roles in salinity stress mediation in *Portunus trituberculatus* (Xu and Qin 2012). Hsp60 participates in immune and stress responses under different environmental stresses in *Litopenaeus vannamei* (Huang et al. 2011). Hsp10 (GroES in *E. coli*), as the co-chaperonin of Hsp60, exerts its biological functions through combining with Hsp60 in many situations (Cappello et al. 2006; Jha et al. 2011; Cappello et al. 2014; Boettinger et al. 2015). Hsp60 and Hsp10 form a folding cage through their rings and produce large and efficient protein-editing machinery that facilitates proper folding and assembly of mitochondrial-imported proteins and corrects misfolded polypeptides (Hansen et al. 2003; Magen et al. 2008). Although the Hsp60/Hsp10 complex system has been well studied in many species; however, the regulatory mechanism of this system, especially in crustaceans, remains unclear (Costa et al. 2011; Johnson et al. 2014; Nisemblat et al. 2014). The chaperon folding cage is composed of two parts: a dome-shaped heptameric ring of Hsp10 (GroES) subunits and Hsp60 (GroEL) cylinder with ATPase (Saibil and Ranson 2002). The cylinder contains three domains: an equatorial ATP-binding domain, an intermediate hinge domain, and an apical domain that binds nascent polypeptides and Hsp10 (GroES) (Saibil et al. 2013). Hsp10 and nascent polypeptides undergo cycles of binding and release, regulated allosterically by the Hsp60 ATPase (Hayer Hartl et al. 2015). The ATP hydrolysis drives conformational changes of cage, creating a protected environment favoring protein folding (Clare and Saibil 2013). The chaperone activity is generally determined by ability to prevent thermal aggregation of citrate synthase (CS), luciferase (Luc), alcohol dehydrogenase (ADH) or malate dehydrogenase (MDH) (Tsai et al. 2012). In the present study, mitochondrial citrate synthase was applied to identify the mitochondrial Hsp60 chaperon activity with a view to better understand the defense mechanism of Hsps when black tiger shrimps (*Penaeus monodon*) are under stress.

The black tiger shrimp (*P. monodon*) is one of the most important aquatic commercial animals in Asia, especially in South China. Considering the enormous economic losses induced by environmental stressors in the shrimp industry, studies focusing on heat shock proteins have become increasingly popular because of the important roles these proteins play in resistance to stress (Cesar and Yang 2007; Cui et al. 2010; Li et al. 2012; Qian et al. 2012). Unfortunately, only three Hsps,

namely, Hsp90, Hsp70, and Hsp21, have been reported in *P. monodon* (Lo et al. 2004; Huang et al. 2008; Jiang et al. 2009). In order to understand the mechanisms of stress tolerance and disease resistance of *P. monodon* better, we characterized the complete complementary DNA (cDNA) sequences of Hsp60 and its co-chaperonin, Hsp10, in this species and investigated the messenger RNA (mRNA) expression of the two genes when exposed to pH challenge, osmotic stress, and heavy metal.

Materials and methods

Experimental animals

Healthy black tiger shrimps (*P. monodon*) weighing an average of 21 ± 1 g were obtained from the experimental base of South China Sea Fisheries Research Institute in Shenzhen (Guangdong, China). The shrimps were reared in tanks containing aerated 3.3 % seawater for 3 days at 25 ± 1 °C before commencement of the experiments. About two thirds of the water in each tank was renewed daily. The shrimps were fed with commercial diets during acclimation until 24 h before treatment.

Tissue distribution analysis

Ten tissue types, including the gills, heart, ovary, stomach, lymph, intestines, hepatopancreas, thoracic ganglion (thorax), muscle, and brain, were collected from three shrimps to determine the tissue distribution of *PmHsp60* and *PmHsp10* transcripts. All of the samples were kept in RNAlater (Ambion, CA, USA) and stored at -80 °C until RNA extraction.

pH challenge

Two solutions of pH 7.0 ± 0.1 and pH 9.0 ± 0.1 were prepared using $1.0 \text{ mol} \cdot \text{L}^{-1}$ HCl and $1.0 \text{ mol} \cdot \text{L}^{-1}$ NaOH. Fifty shrimps were divided amongst the solutions after acclimation to normal seawater (pH 8.0 ± 0.1). pH was measured using a pH electrode (Shanghai Shuangxu Electronics Co., Ltd., Shanghai, China). The gills and hepatopancreas of six shrimps in normal seawater were collected as control samples. Three individuals in the experiment groups were collected 6, 12, 24, 48, and 96 h after the pH challenge. All of the samples were kept in RNAlater and stored at -80 °C until RNA extraction.

Osmotic stress challenge

Fifty shrimps were divided into two groups with different salinity conditions. Low salinity (2.3 %) was achieved through addition of freshwater from the normal level (3.3 %). Salinity was measured using a salinity meter

(WZ211, Shanghai JL Optics Instrument Co., Ltd., Shanghai, China). The second group of shrimps was maintained in 4.3 % seawater, which was obtained through addition of sea salt to normal seawater. The gills and hepatopancreas of three individuals were collected 4, 8, 16, and 32 h post-osmotic stress exposure. Three shrimps reared in normal seawater were collected and served as controls. All of the samples were kept in RNAlater and stored at -80°C until needed.

Heavy metal exposure

Three heavy metals, namely, copper (Cu), zinc (Zn), and cadmium (Cd), were selected as stressors. The concentrations of Cu, Zn, and Cd were set to 0.18, 0.506, and $7.73\text{ mg}\cdot\text{L}^{-1}$, respectively, in accordance with previous studies (Chen and Lin 2001; Wang et al. 2001; Qian et al. 2012). Stock solutions of Cu, Zn, and Cd at concentrations equivalent to 100 times the respective experimental concentrations were prepared by dissolving the water-soluble salts $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, and $\text{CdCl}_2\cdot 2.5\text{H}_2\text{O}$ in deionized Milli-Q water. The gills and hepatopancreas of six shrimps in normal seawater were collected as control samples. Three individuals in the experiment groups were collected 6, 12, 24, 48, and 96 h after heavy metal exposure. All of the samples were kept in RNAlater and stored at -80°C until RNA extraction.

RNA isolation and reverse transcription

Total RNA was isolated from dissected tissues (about 50 mg) using TRIzol reagent (Invitrogen, Shanghai, China), following the manufacturer's protocols. The extracted RNA was re-suspended in DEPC-treated water and stored at -80°C until needed. RNA quantity, purity, and integrity were verified by both native RNA electrophoresis on 1.2 % agarose gel and the ratio of UV absorbance at 260 and 280 nm (NanoDrop Technologies, DE, USA). cDNA was synthesized from total RNA by using a PrimeScript™ Reverse Transcriptase kit (TaKaRa, Dalian, China), following the manufacturer's protocol.

Hsp60 and Hsp10 cloning

Partial sequences of *PmHsp60* and *PmHsp10* were obtained from the splicing transcriptome of *P. monodon* established in our laboratory (unpublished). Two pairs of primers (Table 1) were designed based on the partial cDNA sequences to identify the *PmHsp60* and *PmHsp10* partial sequences. The PCR reaction was performed using a Gradient Mastercycler (Eppendorf, Hamburg, Germany) system with a total volume of 25 μL of PCR mixture

containing 2.5 μL 10 $\times$ reaction buffer with 15 $\text{mmol}\cdot\text{L}^{-1}$ MgCl_2 , 2 μL of 10 $\text{mmol}\cdot\text{L}^{-1}$ dNTP mix, 1.5 μL of 25 $\mu\text{mol}\cdot\text{L}^{-1}$ of each primer, 1 μL template cDNA, 16.5 μL MilliQ water, and 0.5 μL BioReady ExTaq DNA Polymerase (5 $\text{U}\cdot\mu\text{L}^{-1}$) (TaKaRa, Dalian, China). The PCR cycles were conducted as follows: an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were analyzed through electrophoresis in 1.2 % agarose gel and subsequently purified using an agarose gel DNA purification kit (Sangon, Shanghai, China). Afterward, the products were ligated into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced (Invitrogen, Guangzhou, China). The full-length cDNA of *PmHsp60* and *PmHsp10* were amplified through the rapid amplification of cDNA ends (RACE) approach. RACE-PCR was performed using a SMART™ RACE cDNA amplification kit (Clontech, Takara, Dalian, China) with specific primers (Table 1).

Sequence analysis, multiple sequence alignment, and phylogenetic analysis

The full cDNA sequences of *PmHsp60* and *PmHsp10* were analyzed for homology with the BLAST programs (Altschul et al. 1997) at the National Center for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The deduced amino acid sequences were obtained using BioEdit software (<https://www.bioedit.com/>). Motifs were predicted using SMART (<http://smart.embl-heidelberg.de/>). Molecular mass and theoretical isoelectric point were predicted using the compute pI/Mw tool (http://web.expasy.org/compute_pi/), and subcellular localization predictions were performed on the PSORT II server (<http://psort.hgc.jp/form2.html>). Multiple sequence alignments were created using ClustalW software (Thompson et al. 1997) (<http://www.clustal.org/>). Bootstrapped neighbor-joining trees were constructed to illustrate *PmHsp60* and *PmHsp10* phylogenetic relationships using Mega software (version 5.03) (<http://www.megasoftware.net/>). The functional domain was predicted by InterPro (<http://www.ebi.ac.uk/interpro/>).

Spatial and temporal gene expression analysis

The *PmHsp60* and *PmHsp10* gene-specific primers F and R (Table 1), which produce 132 and 164 bp products, respectively, were used during QRT-PCR to detect the temporal expression of the genes in black tiger shrimp. The products were sequenced and confirmed to be the correct form of *PmHsp60* and *PmHsp10* genes. The

Table 1 PCR primers used in the experiment

Primer name	Sequence (5'→3')	Sequence information
Hsp60-F	GGCGAGTCGTCTGCTGCTCATT	Cloning of PmHsp60 partial cDNA sequence
Hsp60-R	CCTCGCTGAGACTGTCCAGGGTTA	Cloning of PmHsp60 partial cDNA sequence
Hsp10-F	TCTCACGGTTCTGCTGCGTTAATC	Cloning of PmHsp10 partial cDNA sequence
Hsp10-R	TTAGATGGACAAAACATTCATGCCC	Cloning of PmHsp10 partial cDNA sequence
Hsp60-5'GSP1	TTGGGACTGCCCCAGCTCTGCTC	5'RACE
Hsp60-5'GSP2	CGAGCGACGGGAGTTCGCAATAAA	5'RACE
Hsp60-3'GSP1	AGCGTATGGCTCGTCTGGCCTCAG	3'RACE
Hsp60-3'GSP2	GAGTGGCTTCCCTCCTCACCACAGC	3'RACE
Hsp10-5'GSP1	CTTCTCCTCCAGGGTAACCTTTGTGCC	5'RACE
Hsp10-5'GSP2	CACGGTCGAACAGGGGAACAACTTCT	5'RACE
Hsp10-3'GSP1	TGGTGGCACAAGGTTACCCTGGAG	3'RACE
Hsp10-3'GSP2	AGTCTGCGGCCTGTTTCAGTTCACAA	3'RACE
Hsp60-exF	GGAATTCCATATGCATCGCGCAGCCTCTTT	Recombinant expression
Hsp60-exR	CGCGGATCCCATCATGCCGCCCATGCCTC	Recombinant expression
Hsp10-exF	GGAATTCCATATGGCTGGTGTCTTGAAGAAGT	Recombinant expression
Hsp10-exR	CCCAAGCTTCTCGTTCTTCATCTTGGCCAG	Recombinant expression
qHsp60F	GCCAACAACACCAATGAA	Quantitative real-time PCR
qHsp60R	CATAACTCCACGCCTGAT	Quantitative real-time PCR
qHsp10F	CTGTTGGTGATGAAGTGA	Quantitative real-time PCR
qHsp10R	ATCTGCCTACGAATTGAC	Quantitative real-time PCR
qEF-1 α F	AAGCCAGGTATGGTTGTCAACTTT	Quantitative real-time PCR
qEF-1 α R	GCTTCGTGGTGCATCTCCACAGAC	Quantitative real-time PCR

housekeeping gene *elongation factor-1 alpha* (*EF-1 α*) (GenBank: DQ021452.1) was selected as the internal control. Quantitative real-time PCR amplifications were performed in triplicate on a 384-well rotor with a total volume of 10 μ L containing 5 μ L of 2 $\times$ SYBR Premix Ex Taq (TaKaRa, Dalian, China), 2 μ L of the cDNA template, 0.8 μ L of 10 mmol·L⁻¹ of each forward and reverse primer, and 1.4 μ L of MilliQ water. The cycling protocol for real-time PCR was 94 °C for 30 s and 40 cycles of 94 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. Melt curve analysis of the PCR products was performed from 65 to 95 °C at the end of each run to ensure that each single product was amplified. The relative expression levels of the target genes were calculated via the $2^{-\Delta\Delta C_t}$ method and normalized to *EF-1 α* .

Recombinant expression and purification of PmHsp60 and PmHsp10

The open reading frames (ORFs) of *PmHsp60* and *PmHsp10* were amplified with Hsp60-exF/R and Hsp10-exF/R primers,

respectively (Table 1). After amplification, the PCR products were ligated into pMD18-T vector (TaKaRa, Dalian, China) and the sequences were confirmed. pMD18T-PmHsp60 was cleaved with *NdeI* and *HindIII* and then cloned into pET28a+ (Invitrogen, Shanghai, China) at *NdeI* and *HindIII* sites, whereas pMD18T-PmHsp10 was cleaved with *NdeI* and *BamHI*. The resultant recombinant plasmids were transformed into *E. coli* BL21 (DE3) strain. *E. coli* BL21 (DE3) cells were cultured in LB medium with 50 mg·mL⁻¹ kanamycin, and the protein was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when OD₆₀₀ of bacteria concentration reached to 0.6. The recombinant PmHsp60 and PmHsp10 proteins were purified and refolded through Ni-NTA affinity chromatography on the resin with the chelated nickel ions according to the instruction for the His-bind Purification Kit (Novagen, Shanghai, China) as described by Cui et al. (2011). The refolding step was performed using the ion-exchange chromatography refolding method (Suttnar et al. 1994). The resultant protein was separated by reducing 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue R250. The

concentrations of the purified PmHsps were quantified using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China), and the purified PmHsps were stored at -80°C for use in subsequent experiments.

Western blot analysis

PmHsps were separated by 12 % SDS-PAGE, and the products were transferred onto nitrocellulose membranes. Membranes were blocked with 5 % skim milk and 0.3 % Tween 20 in PBS (PBST) at 4°C for 12 h and incubated with His-Tag horseradish peroxidase (HRP) conjugated rabbit polyclonal antibody (Huaan Biotechnology, Hangzhou, China) diluted to 1:2000 at 37°C for 1 h. The membrane was washed thrice with PBST, and immune reactive bands were identified through development of blots with HRP-diaminobenzidine (DAB) (Tiangen, Beijing, China).

PmHsp60 ATPase activity assay

ATPase activity was assayed by measuring phosphate release, and measurement was performed according to the reagent kit instructions (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China). PmHsp protein concentrations were adjusted to $100\text{ }\mu\text{g}\cdot\text{mL}^{-1}$, and the reactions were performed for 10 min at 0, 25, 30, 37, 42, and 60°C . ATPase activity was calculated as follows: $\text{PmHsp60 ATPase activity} = (A_1 - A_2) \div (A_3 - A_4) \div X \times Y \times 6 \div C\text{ }\mu\text{mol Pi/mg protein/h}$, where A_1 , A_2 , A_3 , and A_4 are the absorbances of the sample, control, standard, and blank, respectively, at 636 nm; X is the inorganic phosphate content of the standard, Y is the dilution number of the sample, and C is the protein concentration of the sample.

Chaperone activity assay

Citrate synthase (CS, from porcine heart, Sigma, Shanghai, China) aggregation was applied to identify chaperone activity; this procedure has been described in detail in a previous study (Huang et al. 2008). Briefly, the aggregation level of CS was monitored in HEPES buffer (50 mM HEPES, pH 8.0, 25 mM NaCl, 0.5 mM DTT) at 45°C . Buffer containing different concentrations of recombinant PmHsp60 (or PmHsp60 and PmHsp10) was preincubated for 10 min at 45°C before addition of CS to a final concentration of $20\text{ mg}\cdot\text{mL}^{-1}$. Reactions were continuously monitored at 320 nm by an ELISA reader (Molecular Devices, MDS Analytical Technologies, CA, USA). The absorbance of CS alone at 90 min of heating was defined as 100 % aggregation.

Results

Identification and characterization of the PmHsp60 and PmHsp10 genes

The nucleotide sequence of *PmHsp60* (Fig. 1) was deposited in the GenBank database under accession number KT001211. The cDNA sequence contained a 5'-untranslated region (UTR) of 129 bp, a 3'-UTR of 486 bp with a poly (A) tail, and an ORF of 1737 bp encoding 578 amino acids. The molecular weight of *PmHsp60* was 60.99 kDa, and its theoretical isoelectric point (pI) was 5.48. Multiple sequence alignments indicated that *PmHsp60* has a highly conserved Chaperonin 60 (cpn60)-domain (shown in gray) and a Gly-Gly-Met repeat at the C-terminal (shown as wavy lines). Ring oligomerization interfaces (circles), polypeptide binding sites (e.g., Hsp10), and ATP/Mg binding sites (underline) were found in the *PmHsp60* sequence. No signal peptide was found in the deduced amino acid sequence of *PmHsp60*.

The nucleotide sequence of *PmHsp10* (Fig. 2) was deposited in the GenBank database under accession number KT001212. The *PmHsp10* full-length cDNA was 744 bp, including a 309-bp ORF, a 102-bp 5'-UTR, and a 333-bp 3'-UTR. The ORF encoded a protein composed of 102 amino acids with a predicted molecular mass of 11.05 kDa and a theoretical pI of 7.8. A putative Chaperonin 10 (cpn10) conserved domain (shown in gray) was also detected. A mobile loop (underline) functioning as an interaction surface with the large subunit of the chaperonin complex (e.g., cpn60) was found in the *PmHsp10* sequence.

Phylogenetic analysis of PmHsp60 and PmHsp10

Eleven different Hsp60 sequences and nine Hsp10 sequences were downloaded from NCBI to determine their similarity with the deduced amino acid sequences of *PmHsp60* and *PmHsp10*, respectively. The phylogenetic tree of Hsp60 showed that the proteins could be divided into two clusters (Fig. 3a). All invertebrates and arthropods were clustered together, and *PmHsp60* was closely related to *L. vannamei*, which is in the branch of crustaceans. Similarly, the phylogenetic tree of Hsp10 could also be divided into two clusters of vertebrates and invertebrates (Fig. 3b). As expected, *PmHsp10* was divided into the crustacean cluster and shared greater identity with the *Scylla paramamosain* than with other species under this cluster.

The deduced amino acid sequence of *PmHsp60* showed the highest identity (98 %) with *L. vannamei* (ACN30235) as well as high similarity with Hsp60 from *S. paramamosain* (88 %, AG174967.1), *Drosophila melanogaster* (78 %, NP_511115.2), and *Lissorhoptrus oryzophilus* (76 %, NP_511115.2).

Fig. 1 Nucleotide and deduced amino acid sequence of PmHsp60. The deduced amino acid sequence is shown below the nucleotide sequence. The initiation code (ATG) and the termination code (TAA) are boxed. The conserved cpn60-domain is shown in gray and the classical mitochondrial Hsp60 (mtHsp60) signature motifs were bold. The ATP-binding motif was underlined and the ring oligomerisation interfaces were shown in circles. The typical GGM repeat motif at the C terminus were shown waved lines

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1  CGAGAAGCTTCAGCGCAGAGGACGTCGTGGGGCTCGGGCGAGTCGTCTGCTGCTCATT 60
61  CTAGACGCCGTGAGGCCGCTGAGAGATCGTGTAGAGTGTCTCTCCACTGAAACCTT 120
121 CCTACAAAATGTCATCGCGAGCCTCTTTATTGCGAACTCCCGTCGTCGCCAGGCCACA 180
1  M H R A A S L L R T P V A R Q A T 17
181 AGGCACTACCTGGCGAGACATTATGCAAAGGACGTTAAATTTGGCAGGAGGTGAGGCA 240
18 R H Y L A R H Y A ④ D V K ⑤ G T E V R A 37
241 CTGATGCTGCAGGGCGTCGACGTCTCACCGACGCGGTGGCTGTACCCATGGCCCCAAG 300
38 L M L Q G V D V L T D A V A V T M G P K 57
301 GGTCGAAATGTAATCATTGAGCAGAGCTGGGGCAGTCCCAAGATCACAAGGATGGTGT 360
58 G ⑥ ⑦ ⑧ ⑨ ⑩ ⑪ ⑫ Q S W G ⑬ ⑭ K ⑮ T K D G V 77
361 ACAGTTGCAAGGCTGTTGAACGAAAGACAAGTCCAGAACATTGGAGCTAAGTTGGTC 420
78 T V A K ⑯ V ⑰ L K D K F Q ⑱ G A K ⑲ V 97
421 CAAGATGTTGCCAACACCAATGAAGAGGCTGGTATGGAACACCACAGCCACAGTC 480
98 Q D V A N N T N E E A G D G T T T A T V 117
481 CTGGCTCGTACTATTGCAAAGGAAGGTTTTCAGAGGATTAGCAAAGGTGCCAACCTGTG 540
118 L A R T I A K E G F D R I S K G A N P V 137
541 GAGATCAGGCGTGGAGTTATGTTGGCGTGGATGCCATTGTTGCTCACCTGAAGACCTG 600
138 E I R R G V M L A V D A I V A H L K T L 157
601 TCAAAGCCTGTGCCACTCCTGCTGAGATTGCTCAGGTTCACCCATCTCTGCTAATGGA 660
158 S K P V T T P A E I A Q V A P I S A N G 177
661 GACATTGAAGTAGGCAGTCTTATCTCGGACGCCATGAAAAGTGGTCTGAGGGTGTG 720
178 D I E V G S L I S A A M E K V G R E G V 197
721 ATCACTGTGAAAGATGGCAAGACCTTGAAGGATGAGTTGAGGTCATTGAAGCATGAAG 780
198 I T V K D G K T L K D E L E V I E G M K 217
781 TTCGATCGTGGTTACATTTCTCCTTACTTCATAAATCCAGCAAGGAGCTAAGGTGAA 840
218 F D ⑳ G Y I S P Y F I N S S K G A N P V 237
841 TACCAAGACTGCCTTGTGTTGCTCTCGGAGAAGAAATTTCTCTATCCAGTCCATTATC 900
238 Y Q D C L V L L S E K K I S ㉑ I Q S I I 257
901 CCAGTGCTAGAACTGGCCAATGCCCAAAGGAAACCTCTATTGATCATTGCTGAGGACATT 960
258 P V L E L A N A Q R K P L L I I A E D I 277
961 GATGGAGAAGCCTTGAGCACACTTGTGTAACCGCTTGAAGATTGGCTCCAGGTAGCT 1020
278 D G ㉒ A L S T L V V N R L K I G L Q V A 297
1021 GCTGTAAAAGCTCCAGGCTTGGTGATAACCGCAAGATACTCTCATGACATTGCCATT 1080
298 A V K A P G F G D N R K N T L H D I A I 317
1081 GCAACAGGTGCTATTGTCTTCAATGACGAAGCAAGCATGGTCAAGATTGAAGATGTTGAG 1140
318 A T G A I V F N D E A S M V K I E D V Q 337
1141 GTCCATGATCTTGCCAAAGTTGGAGAAGTGCAGATCACAAGGATGACACACTCCTGCTG 1200
338 V H D L G Q V G E V Q I T K D D T L L L 357
1201 AAGGGCAAGGAAATACTAGTGATATTCAGCGTCGTGTAGAACAAATTAAGGACCAGATT 1260
358 K G K G N T S D I Q R R V E Q I K I 377
1261 GCTGATAGTTCCTCCGAGTATGAGAAGGAGAAAATGCAGGAGCGTATGGCTCGTCTGGCC 1320
378 A D S S S E Y E K E K M Q E R M A R L A 397
1321 TCAGGTGTGGCAGTTGTGAAGTTGGAGTTCCTCGGAGGTTGAAGTGAAGGAGAAGAAG 1380
398 S G V A V V K V G G ㉓ S ㉔ V E V N E K K 417
1381 GATCGTGTAATGCTCTGTGTGCAACAAGGGCTGCAGTTGAAGAGGGCATCGTTCCA 1440
418 D R V N D A L C A T R A A V E E G I V P 437
1441 GGTGAGGAGTTGCCCTTAATTCGTGCTTCTGCTTTAGATACTCTCACTCCAAGCAAC 1500
438 G G G V A L I R C L P A L D T L T P S N 457
1501 GAAGACCAGAAGGTGGCATTGAAATGTCGCAAGGCTATCCAGACTCTTCCACACC 1560
458 E D Q K V G I E I V R K A I Q T P C H T 477
1561 ATTGCTAGCAATGCAGGTGTTGATGCATCAGTTATTGTCAACAAGGTGATGGAAGCTTCT 1620
478 I A S N A ㉕ V D A S V I V N K V M E A S 497
1621 GGAGATGTTGGATATGATGCTGCTACAGGAACCTTCGTTAACCTTGTGGAAGCAGGAATC 1680
498 G D V G Y D A A T G T F V N L V E A G I 517
1681 ATTGATCCCAAGGTTGTCCGTACAGCCCTAAGTATGCTGCAGAGGTGGCTTCCTC 1740
518 I D P T K V V R T A L T D A A G V A S ㉖ 537
1741 CTCACCACAGCTGAGAGTGTCTTACAGAGATCCCCAGGAAGAACAGCTGGTATGGGA 1800
538 L ㉗ ㉘ ㉙ ㉚ ㉛ ㉜ E I P Q E E P A G M G 557
1801 GGCATGGGTGGTATGGCGGAATGGGTGGCATGGGCGGAATGGGAGCATGGGCGGCATG 1860
558 G M G M G G M G G M G G M G G M G G M 577
1861 ATGTAAGCTTCCCATGGATTGGCTAGGAAGAACTCTTAATTTGTAAGTAACATTTTT 1920
578 M * 578
1921 TTGTTATGTACAAAGTTACTTTGGTTCTACAAGAGGTACGGAGAGTACATAGATGCCACA 1980
1981 GAACATGTCTAGTTTACAAGAAAATCAATAAGCGGAGGAAATCTTCAATGATTTAGA 2040
2041 AGTAACCTGGACAGTCTCAGCGAGGAAAGGGACATGCAAAACATGAAGTTGACACAAG 2100
2101 TCAAAGGAATGTTAACCATTCCAAGAAGTACCTCGGCATACCGAATGTGGTCCATTAA 2160
2161 TTTTCATCTAATCATCTTCATCTGAATGTTTGAAGGGTATATGTGCTTCATCTTAGTG 2220
2221 TAAATGTTGAATGTTGTACAAAGTGTAGATAAGAGAATGTGTAGATAATTTGTTATAA 2280
2281 GGAAGATTTGTTGTTCCCTAAATGAAATAAGATTTTGAGAAG.AAAAAAAAAAAAAA 2340
2341 AAAAAAAAAAAAAA 2352

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1  CCAGCCATTTTACTCAGGAAGGACAGACGCGCGGACTAGACGCTTCTCTCA 60
61  CGGTTCTGCTGCGTTAATCTCCGGCTGCCTTCTGAGTAAATGCTGGTGTGTTGAAG 120
1  M A G A L K 6
121 AAGTTTGTTCCTCGTTCGACCGGTGTGCTGGTCCAAAGGCTGAGGCTTTGACCGTACT 180
7  K F V P L F D R V L V Q K A E A L T R T 26
181 GCAAAGGGAATCTTGATCCAGAAAGTCTGTACCAAGGTCTCACAGGGAAGGTGGTT 240
27  A K G I L I P E K S V P K V L T G K V V 46
241 GCTGTAGGAGAGGAGCCAGCAATGATGCTGGCACCACAATCCCCCATGTGTACTGTT 300
47  A V G E G A R T D A G T T I P P C V T V 66
301 GGTGATGAAGTATGCTTCCTGAGTTTGGTGGCACAAGGTACCTGGAGGAGAAGGAC 360
67  G D E V M L P E F G G T K V T L E E K D 86
361 TATTACCTCTTCAGAGAATCTGAATCTCTGGCCAAGATGAAGAAGAGTAAAGAGCAACC 420
87  Y Y L F R E S E L L A K M K N E * 102
421 CTGTAGGAGTGAATCATTGGGTCAATTCGTAGGCAGATGCTTAAGGAGGCCAAGGAAA 480
481 AAGAAATCCAGCAGATCAGAGATTGCTTGTGTGTAGTCTGCGGCTGTTTCAGTTC 540
541 ACAATGTGAAGATGTATGTTTGTAGTCTAACATGGACCAAGTTTCTATGTATGTATGA 600
601 ATCATCACATATAATTCAATTGTCATTCATCATTTAGGGCATGAATGTTTGTCCATCT 660
661 AACATGTGTATAGAAAGTGAAGAATTGTTTACAAAATATAGATTGTTTCAAAAAA 720
721 AAAAAAAAAAAAAAAAAAAAAA 744

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Fig. 2 Nucleotide and deduced amino acid sequence of PmHsp10. The deduced amino acid sequence is shown below the nucleotide sequence. The initiation codon (ATG) and termination codon (TAA) are marked by boxes. The conserved Cpn10-domain is shown in gray and the predicted mobile loop is underlined

AHE77384.1). *PmHsp60* also shared high homology with other vertebrates (Fig. 4). *PmHsp10* revealed the highest identity with *S. paramamosain* (79 %, AGI74966.1) as well as high similarity with *Lygus hesperus* (61 %, AFX84557.1), *Zootermopsis nevadensis* (59 %, KDR14059.1), and *Harpegnathos saltator* (58 %, XP_011146739.1) (Fig. 5).

Recombinant expression and Western blot analysis of PmHsp60 and PmHsp10

The recombinant plasmids of PET28a-PmHsp60 and PET28a-PmHsp10 were expressed in *E. coli* BL21 (DE3). A PmHsp60-6×His tagged fusion protein about 64 kDa, consistent with the predicted molecular mass of PmHsp60 with the His tags added by the vector, was detected during SDS-PAGE (Fig. 6a). In the full-length *PmHsp10* overexpression experiment, a protein with a molecular mass of 11 kDa was found during SDS-PAGE after isopropyl β-D-1-thiogalactopyranoside (IPTG) induction (Fig. 7a). No protein band was found at the same position in the non-induced controls of the two *PmHsp* genes. The fusion protein was detected through Western blot analysis. Figure 6b clearly shows a specific band of about 64 kDa (arrow) after Western blot analysis. The results of PmHsp10 Western blot analysis showed a specific protein band of about 11 kDa (arrow) (Fig. 7b). Purified and refolded PmHsps were obtained through affinity chromatography, and only one target band was detected by SDS-PAGE (Figs. 6c and 7c).

Tissue distribution of PmHsp60 and PmHsp10 analysis

Different tissues were collected to determine the distributions of *PmHsp60* and *PmHsp10* transcripts, and the results are shown in the Fig. 8a. The highest expression of the *PmHsp60* transcript was detected in the hepatopancreas and the lowest expression was noted in the gills, heart, lymph, stomach, intestines, thorax, and muscle. Similarly, the highest expression level of the *PmHsp10* transcript was found in the hepatopancreas, whereas the lowest expression was found in the lymph, gills and thorax (Fig. 8b).

Transcription analysis of PmHsp60 and PmHsp10 post-environmental stress

In the pH challenge experiments, the expression level of *PmHsp60* mRNA was upregulated and reached peak values (3.8-fold higher compared with that of the control) in gills 96 h after pH-7.0 exposure. *PmHsp10* was induced at 6 h, and maximum mRNA expression levels were found at 6, 48 and 96 h after pH 7.0 challenge (Fig. 9a). In the hepatopancreas, the expression level of *PmHsp60* mRNA was upregulated at 12 and 48 h. No obvious variation in the expressions of *PmHsp10* was observed after pH 7.0 challenge (Fig. 9b). Compared with the results of the pH 7.0 challenge, the *PmHsp* genes were induced immediately by pH 9.0 stress. The relative expressions of *PmHsp60* and *PmHsp10* transcripts were rapidly upregulated within 6 h after pH 9.0 exposure and declined at 12 h. Moreover, maximum expression levels were detected at 48 h in gills. At their peak, *PmHsp60* and *PmHsp10* expression levels were, respectively, 24- and 20-fold higher relative to the corresponding levels of the controls (Fig. 9c). In the hepatopancreas, *PmHsp60* and *PmHsp10* transcription levels were upregulated compared with that of the control within 12 h under pH 9.0 stress and subsequently declined to the same levels at 24 (*PmHsp60*) and 48 h (*PmHsp10*); peak values were observed at 96 h (Fig. 9d).

In the salinity stress experiment, *PmHsp10* mRNA expression reached maximum levels after 4 h of low salinity exposure and declined after 8 h in the gills (Fig. 10a). In the hepatopancreas, *PmHsp10* was induced at 4 h and reached maximum expression levels at 4, 8 and 32 h of low salinity stress (Fig. 10b). The *PmHsp60* transcript was only slightly upregulated under low salinity stress in both tissues. The *PmHsp60* transcript level increased to maximum levels (twofold higher compared with that of the control) at 32 h in the gills under high salinity; moreover, maximum *PmHsp10* expression level was 3.2-fold higher than that of the control at 16 h (Fig. 10c). Both *PmHsp60* and *PmHsp10* transcripts were elevated at 4 h, and this level was maintained for 16 h in the hepatopancreas when the shrimps were stressed in pH 9.0 solution (Fig. 10d).

The relative mRNA expression levels of *PmHsp* genes in response to copper exposure are shown in Fig. 11. In the gills,

Fig. 3 Phylogenetic analyses of PmHsp60 (a) and PmHsp10 (b). Alignment of amino acid sequences were produced by Clustal W, and the bootstrap neighbor-joining phylogeny tree was constructed by MEGA 5.03. GenBank accession numbers encoding Hsp60 are as follows: *Litopenaeus vannamei* (ACN30235), *Scylla paramamosain* (AGI74967.1), *Drosophila melanogaster* (NP_511115.2), *Lissorhoptus oryzophilus* (AHE77384.1), *Homo sapiens* (ABB01006.1), *Mus musculus* (NP_034607.3), *Misgurnus anguillicaudatus* (AHB82279.1), *Bos Taurus* (NP_001160080.1), *Paralichthys olivaceus* (ABB76384.1), *Danio rerio* (NP_851847.1), *Gallus gallus* (NP_001012934.1); GenBank accession numbers encoding Hsp60 are as follows: *Scylla paramamosain* (AGI74966.1), *Lygus hesperus* (AFX84557.1), *Zootermopsis nevadensis* (KDR14059.1), *Harpegnathos saltator* (XP_011146739.1), *Danio rerio* (NP_571601.1), *Homo sapiens* (NP_002148.1), *Mus musculus* (NP_032329.1), *Gallus gallus* (NP_990398.1), *Oryzias latipes* (NP_001098232.1), *Paralichthys olivaceus* (ABB76382.1)

the *PmHsp60* transcript level significantly increased at 12 h and decreased 24 and 48 h after the shrimps were exposed to copper stress. This parameter reached maximum levels at 96 h (10.7-fold higher compared with that of the control). *PmHsp10* expression level was slightly induced before 48 h and reached maximum levels (7.8-fold higher compared with that of the control) at 96 h in the gills of shrimps under copper stress (Fig. 11a). In the hepatopancreas, *PmHsps* expression were maintained at levels similar to that of the control (Fig. 11b).

Similar tendencies of the *PmHsp* transcripts in the gills and hepatopancreas were observed during zinc stress exposure. In the gills, the maximal enhancement of *PmHsp60* and *PmHsp10* expression levels was reached at 12 h, which are about 7.2- and 4.8-fold higher compared with that of the control (Fig. 11c). In the hepatopancreas, upregulation of *PmHsp60* and *PmHsp10* transcripts was detected at the beginning of zinc exposure. Maximum *PmHsp60* and *PmHsp10* mRNA expression levels were observed at 24 h, which are 8.8- and 2.6-fold

higher compared with that of the control, under zinc treatment (Fig. 11d). In the cadmium exposure experiment, the *PmHsp60* transcript significantly increased at 24 h in the gills and reached maximum expression (6.3-fold higher compared with that of the control) thereafter. Furthermore, the maximum mRNA expression level of *PmHsp10* was 2.7-fold higher compared with that of the control at 12 h in the gills (Fig. 11e). In the hepatopancreas, the two *PmHsp* genes were induced slightly, and the highest expression levels of *PmHsp60* (2.0-fold higher compared with that of the control) and *PmHsp10* (2.3-fold higher compared with that of the control) were detected at 6 and 48 h, respectively (Fig. 11f).

ATPase activity of PmHsp60

The ATPase of *PmHsp60* is shown in Fig. 12. *PmHsp60* revealed ATPase activity from 0 to 60 °C, and maximum activity was found at 37 °C. *PmHsp60* ATPase activity increased at all temperature conditions when PmHsp10 was added to the

Fig. 4 Multiple alignment of the deduced amino acid sequences of Hsp60 from *Penaeus monodon* and other species. Identical and similar amino acid residues in all the proteins are indicated by black and gray

highlights respectively. The amino acid positions are shown on the right. The species names and GenBank accession numbers of Hsp60s are shown in Fig. 3

reaction system. The maximum increase in activity was found over the temperature range of 37–42 °C, and the lowest increase in activity was detected at 60 °C.

Chaperone activity of PmHsp60

Heat-induced citrate synthase aggregation assay was performed to determine the chaperone activity of PmHsp60

(Fig. 13). At a 1:1 Hsp60/CS ratio (w/w), Hsp60 offered about 21 % protection. The extent of protection increased as the Hsp60 concentration increased. Approximately 57 % protection was observed at a 2:1 Hsp60/CS ratio. At a 4:1 ratio, over 60 % protection was observed. Only approximately 38 % protection was observed when the Hsp10/CS ratio was 4:1. However, over 76 % protection was observed at a 4:4:1 Hsp60/Hsp10/CS ratio.

Fig. 5 Multiple alignment of the deduced amino acid sequences of Hsp10 from *Penaeus monodon* and other species. Identical and similar amino acid residues in all the proteins are indicated by black and gray highlights respectively. The amino acid positions are shown on the right. The species names and GenBank accession numbers of Hsp60s are shown in Fig. 3

Discussion

In the present study, the full-length cDNAs of *PmHsp60* and *PmHsp10* were cloned from *P. monodon*. A Cpn60 conserved domain and seven Gly-Gly-Met repeats at the C terminus were found in the *PmHsp60* sequence, in accordance with previous studies (Xu et al. 1997; Sanchez et al. 1999). The functional domains of the motif, such as ring oligomerization interfaces and ATP/Mg binding sites, were detected and similar characteristics were found in other species (Xu and Qin 2012; Xu et al. 2014). This finding suggested that *PmHsp60* belongs to the mitochondrial Hsp60 (mtHsp60) chaperone

family. As the *PmHsp60* co-chaperone, *PmHsp10* was cloned in the current study. A mobile loop was also found in the *PmHsp10* sequence. The mobile loop mediates the interaction with the Hsp60 apical domains form a protein folding mechanism (Hayer Hartl et al. 2015). These motifs and structures could be used to research the functions of the Hsp60/Hsp10 complex in future studies.

Phylogenetic analyses of *PmHsp60* and *PmHsp10* were conducted by the neighbor joining method. The phylogenetic trees obtained revealed that *PmHsp60* shows the highest identity with *L. vannamei* and the *PmHsp10* is similar to *S. paramamosain*. Sequence alignment and phylogenetic

Fig. 6 Expression of recombinant *PmHsp60* in *E. coli* and western blot analysis. **a.** SDS-PAGE of expressed protein. Lanes M, pre-stained molecular weight marker; 1, non-induced cells transformed empty plasmid; 2, 3-h-induced cells transformed empty plasmid; 3, 6-h-induced cells transformed empty plasmid; 4, non-induced cells

transfected recombinant; 5, 3-h-induced cells transformed recombinant; 6, 6-h-induced cells transformed recombinant. **b.** Western blotting of *PmHsp60*. Lanes 1, non-induced; 2, 3-h-induced *PmHsp60*-6xHis fusion protein; 3, 6-h-induced *PmHsp60*-6xHis fusion protein. **c.** purified *PmHsp60*-6xHis fusion protein

Fig. 7 Expression of recombinant PmHsp10 in *E. coli* and western blot analysis. **a.** SDS-PAGE of expressed protein. Lanes M, pre-stained molecular weight marker; 1, non-induced cells transformed empty plasmid; 2, 3-h-induced cells transformed empty plasmid; 3, 6-h-induced cells transformed empty plasmid; 4, non-induced cells

transfected recombinant; 5, 3-h-induced cells transformed recombinant; 6, 6-h-induced cells transformed recombinant. **b.** Western blotting of PmHsp10. Lanes 1, non-induced; 2, 3-h-induced PmHsp10-6×His fusion protein; 3, 6-h-induced PmHsp10-6×His fusion protein. **c.** purified PmHsp10-6×His fusion protein

analysis further suggested that PmHsp60 and PmHsp10 respectively belong to the Hsp60 and Hsp10 families. This finding provides some molecular evidence of the evolutionary and phylogenetic development of the black tiger shrimp.

In the present study, recombinant PmHsp60 and PmHsp10 were expressed in *E. coli* BL21 (DE3). A PmHsp60-6×His tagged fusion protein of about 64 kDa, was detected via SDS-PAGE. A protein with a molecular mass of 11 kDa was found during SDS-PAGE after induction with 1 mM IPTG at 37 °C. There is no any nonspecific band that was detected in the PmHsps western results. PmHsp60 and PmHsp10 were rapidly obtained in large amounts in vitro. The results of the present study thus far provide a molecular foundation for proteomics and immunohistochemistry analyses to better understand the biological functions of PmHsps.

Aquatic animal health is well-known to be usually influenced by the external environment. Numerous economic losses in the aquaculture industry are caused by environmental stressors, such as heavy metal stress, heat stress, hypoxic stress, osmotic stress, and pH challenge. Thus, Hsps, as a series of molecular chaperones induced by environmental stress, play important roles in the innate immune responses of aquatic species. The highest transcripts of *PmHsps* were found in the hepatopancreas whereas the lowest transcripts were found in the gills of shrimp during distribution analysis (Fig. 8). The two tissues have similarly been reported to be environmentally sensitive tissues in other species (Zhou et al. 2009; Li et al. 2012; Shekhar et al. 2013). Therefore, *PmHsp60* and *PmHsp10* transcript levels in the hepatopancreas and gills were investigated upon exposure of *P. monodon* to

Fig. 8 Relative expressions of PmHsp60 (**a**) and PmHsp10 (**b**) transcripts in different tissues. Relative expressions of PmHsp60 and PmHsp10 in different tissues, gill, heart, ovary, lymph, stomach, intestines, hepatopancreas, thoracic ganglion (thorax), muscle and brain, by quantitative RT-PCR analysis. Relative expressions of PmHsp60 and PmHsp10 were calculated with the $2^{-\Delta\Delta Ct}$ method. Each bar represent the mean±SD ($n=3$). Different lowercase letters indicate statistically significant differences ($P<0.05$)

Fig. 9 Relative expression levels of PmHsp60 and PmHsp10 in gills and hepatopancreas after pH challenge. **a.** In gill after pH7.0 challenge. **b.** In hepatopancreas after pH7.0 challenge. **c.** In gill after pH9.0 challenge. **d.** In hepatopancreas after pH9.0 challenge. The untreated (0 h) group was considered as control. The EF-1 α RNA was used as an internal control and the relative expression levels of PmHsp60 and PmHsp10 were obtained relative to EF-1 α expression. Each bar represent the mean $\pm$ SD ($n=3$). Different lowercase letters indicate statistically significant differences ($P<0.05$)

different environmental stresses, including pH challenge, osmotic stress, and heavy metal exposure.

In the pH challenge experiment, the *PmHsp60* and *PmHsp10* transcripts were induced under pH 7.0 in the gills, and no obvious variation in their expression was observed in the hepatopancreas under the same conditions. However, the relative expressions of *PmHsp60* and *PmHsp10* mRNA in gills increased significantly 6 h after pH 9.0 exposure and reached maximum levels (24-fold higher compared with that of the control) at 48 h. Compared with the stable expressions of *PmHsp60* and *PmHsp10* at pH 7.0 in the hepatopancreas, transcript expressions peaked at 96 h after pH 9.0 exposure. We further found that the change tendencies of the *PmHsp60* and *PmHsp10* transcripts were similar in most cases. These results suggest that PmHsp60 and PmHsp10 may be involved in the defense against pH stress, especially alkalization and that gills are sensitive tissues to pH stress. A distinct change in the expressions of these genes has also

been observed in other organisms post-acid shock. *Helicobacter pylori* Hsp70 is upregulated preferentially by pH rather than heat shock (Huesca et al. 1998). Phosphatidylinositol 3-kinase/Akt and MAPK regulate Hsp70 and Hsp27 induction in primary cultures of human esophageal microvascular endothelial cells in response to acid exposure (pH 4.5) (Rafiee et al. 2006). Four Hsps expression patterns were compared in response to pH challenge in *L. vannamei* (Qian et al. 2012). Acid or alkaline exposure may induce reaction oxygen species (ROS) and cooperatively upregulate manganese superoxide dismutase (MnSOD), catalase, glutathione peroxidase (GPx), and thioredoxin (Wang et al. 2009). Glutathione S-transferase (GST) is induced when ROS is produced (Dorts et al. 2009). Moreover, the Hsp60 folding machinery is crucial for the folding and functions of MnSOD (Magnoni et al. 2014). In the mitochondria, the combination of mitochondrial phospholipid hydroperoxide glutathione peroxidase and Hsp60/10 provides protection against damage resulting

Fig. 10 Relative expression levels of PmHsp60 and PmHsp10 in gills and hepatopancreas of shrimps under osmotic stress. **a** In gill after low salinity (2.3 %) exposure. **b** In hepatopancreas after low salinity (2.3 %) exposure. **c** In gill after high salinity (4.3 %) exposure. **d** In hepatopancreas after high salinity (4.3 %) exposure. The untreated (0 h) group was considered as control. The EF-1 α RNA was used as an internal control and the relative expression levels of PmHsp60 and PmHsp10 were obtained relative to EF-1 α expression. Each bar represent the mean $\pm$ SD ($n=3$). Different lowercase letters indicate statistically significant differences ($P<0.05$)

from simulated ischemia/reoxygenation injury (Hollander et al. 2003). These results suggest that Hsp60 and Hsp10 combine with antioxidant enzymes, such as MnSOD, GPx, and GST, to achieve protection against pH stress. However, the detailed mechanisms of such a combination require more investigation.

Osmotic stress is the most common environmental stress factor for aquatic organisms. The shrimps were exposed to low (2.3 %) and high salinity (4.3 %) to investigate the functions of *PmHsp60* and *PmHsp10*. *PmHsp10* expression in gills was found to reach maximum levels below 4 h under low salinity exposure; however, this expression was induced continuously in the hepatopancreas. The *PmHsp60* transcript was upregulated slightly in these two tissues under low salinity stress. Under high osmotic stress, *PmHsp60* and *PmHsp10* mRNA expression reached maximum levels in the gills at 32 and 16 h, respectively. In the hepatopancreas, the transcripts of these two PmHsps were induced at 4 h and maintained until 16 h. The *PmHsp60* and *PmHsp10* transcript variations observed suggest that Hsp60 and Hsp10 may be involved in the refolding of misfolded proteins and maintenance of ionic homeostasis under osmotic stress. Hsps induced by salinity stress have

been determined in numerous previous studies. For example, DnaK, together with GroEL, GroES, and a number of other Hsps, are induced during salt stress in *Lactococcus lactis* (Kilstrup et al. 1997). DnaK is required for K⁺ transport maintenance at high osmolality (Meury and Kohiyama 1991). T cells in experimentally induced salt-driven hypertension present a CD4 clonal response to *Hsp70*, which is overexpressed in the kidneys (Pons et al. 2013). *Hsp21* was identified as a differentially expressed gene from a forward SSH cDNA library constructed from gut tissues of *P. monodon* showing significant increases in gene expression under osmotic stress (Shekhar et al. 2013). Ion uptake in crustaceans is mostly attributed to Na⁺/K⁺-ATPase and Ca²⁺-ATPase activity (Meyran and Graf 1986; Lima et al. 1997; Furriel et al. 2004; Masui et al. 2005). Several previous studies have found that Hsps could be involved in Na⁺/K⁺-ATPase stabilization (Burdon and Cutmore 1982; Deane and Woo 2005; Chatterjee et al. 2009). Hsp60 is essential to the functions of Ca²⁺-ATPase KIPmr1p (Uccelletti et al. 2005). Results of previous research and the expression levels of Hsp60 and Hsp10 determined in the present study suggest that these proteins play important roles in ion balance under osmotic stress. However, details

Fig. 11 Relative expression levels of PmHsp60 and PmHsp10 in gills and hepatopancreas of shrimps under heavy metal exposure. **a** In gill after copper exposure. **b** In hepatopancreas after copper exposure. **c** In gill after zinc exposure. **d** In hepatopancreas after zinc exposure. **e** In gill after cadmium exposure. **f** In hepatopancreas after cadmium exposure. The untreated (0 h) group was considered as control; the untreated (0 h) group was considered as control. The *EF-1 α* RNA was used as an internal control and the relative expression levels of PmHsp60 and PmHsp10 were obtained relative to *EF-1 α* expression. Each Bar represent the mean $\pm$ SD ($n=3$). Different lowercase letters indicate statistically significant differences ($P<0.05$)

of the regulation and stabilization mechanisms of these proteins under salinity stress are limited in crustaceans and require further research.

Increasing evidence has demonstrated that heavy metal pollution impairs the growth and development of aquatic organisms (Calabrese et al. 1977; Ahsanullah et al. 1981; Wong et al. 1995). Previous studies have also shown that *P. monodon* is influenced by heavy metal exposure (Chen HC Water quality criteria for farming the grass shrimp and *Penaeus monodon*. In 1985; Guhathakurta and Kaviraj

2000; Chen and Lin 2001; Mokhtar et al. 2009). Hsps, as a series of proteins that respond to environmental stress, play important roles in the defense against heavy metals. In the present study, the *PmHsp60* transcript increased significantly at 12 h, and both *PmHsp60* and *PmHsp10* expression levels in gills reached peak values (10.7- and 7.8-fold compared with the control, respectively) at 96 h after copper exposure. Compared to the gills, the variation of *PmHsps* transcript was imperceptible in hepatopancreas. These results suggest that PmHsp60 may be involved in

Fig. 12 ATPase activity of PmHSP60. PmHSP60 (black circle) and PmHSP60+PmHSP10 (white circle) activity determination were performed at 0, 25, 30, 37, 42 and 60 °C. The light scattering of the solution was monitored at 636 nm

the protection against copper exposure and that the gills are tissues that sensitively responded to copper stress.

Zinc is physiologically essential for the normal growth and development of aquatic organisms. However, zinc may harm marine organisms when concentrations exceed actual physiological requirements (Wong et al. 1995). In the present study, the expression profiles of *PmHsp60* and *PmHsp10* were detected under zinc stress. The expression patterns observed showed that both *PmHsp60* and *PmHsp10* genes were induced significantly in the gills and reached maximum levels (7.2- and 4.8-fold higher compared with that of the control, respectively) at 12 h. In the hepatopancreas, the *PmHsp60* and *PmHsp10* transcripts were clearly upregulated at the beginning of zinc exposure. Maximum *PmHsp60* (8.8-fold higher compared with that of the control) and *PmHsp10* (2.6-fold

Fig. 13 Chaperone activity of PmHSP60 and PmHSP10. Thermally induced aggregation of citrate synthase (20 mg/ml) alone (black circle); PmHSP60/citrate synthase (w/w) at 1:1 (white circle); 2:1 (inverted black triangle) and 4:1 (upright triangle). PmHSP10/citrate synthase (w/w) at 4:1 (black box); PmHSP60/PmHSP10/citrate synthase (w/w/w) at 4:4:1 (white box). The light scattering of the solution was monitored at 320 nm

higher compared with that of the control) mRNA expression levels were found 24 h after zinc treatment. These results indicate that more Hsps are required to defend zinc-induced stress and maintain normal physiological processes.

Cadmium is a widespread environmental pollutant which has received an increased worldwide concern. Cadmium toxicology has been studied in various aquaculture organisms (Wu et al. 2006; Seebaugh et al. 2012; Ma et al. 2013). and previous studies have demonstrated that Hsps are involved in the defense against cadmium toxicity (Jing et al. 2013; Jung and Lee 2013; Mazzei et al. 2015). However, very few studies have been conducted in *P. monodon*. In the current study, we investigated the expression patterns of *PmHsp60* and *PmHsp10* in shrimps exposed to cadmium stress. The *PmHsp60* transcript increased significantly in gills at 24 h and reached peak levels (6.3-fold higher compared with that of the control) at 24 h. The maximum mRNA expression level of *PmHsp10* in gills was 2.7-fold higher compared with that of the control at 12 h. In the hepatopancreas, both *PmHsp* genes were induced slightly under cadmium stress. The highest *PmHsp60* expression was significantly induced by cadmium exposure, in accordance with previous studies (Qian et al. 2012). For example, the relative mRNA expression of *LvHsp60* shows sensitive responses to cadmium exposure and reaches 21.9-fold that of the control group within 24 h. Similar phenomena have also been observed in mouse and chicken (Knoflach et al. 2011; Chen et al. 2012). These results suggest that Hsp60 may play an important role in cadmium tolerance and can be used as biomarker for cadmium pollution. *PmHsp60* and *PmHsp10* expressions were up- and downregulated with similar tendency. These results suggest that PmHsp60 and PmHsp10 probably perform their biological functions as a complex in response to environmental stress.

Previous studies have demonstrated that Hsp60 is one of the main Hsps to environment stress responses (Tsan and Gao 2004; Li et al. 2011). The ATPase and chaperone activities of Hsp60 play important roles in the folding of denatured proteins and responses to stress (Gupta et al. 2008). Figure 12 shows that the ATPase activity of PmHsp60 increased from 0 to 37 °C and decreased from 42 to 60 °C. Moreover, ATPase activity increased at every tested temperature after addition of PmHsp10 to the reaction system. Hsp10 interacts with a single ring of Hsp60 and forms a folding cage, and ATP binds to an open GroEL (Hsp60) ring before GroES (Hsp10) encapsulation (Magen et al. 2008; Tyagi et al. 2009). These results suggest that the Hsp60/Hsp10 complex produces an efficient ATPase machinery. The chaperone activity assay showed that Hsp60 facilitated proper protein folding activity (Fig. 13), and the extent of protection increased with variations of the mixing ratio (i.e., weight of chaperone/weight of target). Hsp60 was a more effective chaperone than Hsp10, and the Hsp60/Hsp10 complex demonstrated greater activity. Huang et al. (2011)

also found that Hsp60 presents chaperone activity in *L.vannamei*, similar to our experimental results. The data suggest that Hsp60/Hsp10 functions as a major chaperone complex in response to environmental stresses.

In summary, the full-length cDNAs of *PmHsp60* and *PmHsp10* were cloned from *P. monodon*, and their expression profiles were detected when the shrimps were exposed to pH challenge, osmotic stress, and heavy metal stress. *PmHsp60* and *PmHsp10* were found to be involved in responses to pH, osmotic, and heavy metal stress. The results obtained from this study provide useful evidence of the ability of Hsp60/Hsp10 to defend shrimp against cellular stress and demonstrate how the complex participates in environmental stress responses.

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