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A novel biomarker for marine environmental pollution of HSP90 from *Mytilus coruscus*

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ABSTRACT

Heat shock protein 90 (HSP90) is a conserved molecular chaperone contributing to cell cycle control, organism development and the proper regulation of cytosolic proteins. The full-length HSP90 cDNA of *Mytilus coruscus* (McHSP90, KT946644) was 2420 bp, including an ORF of 2169 bp encoding a polypeptide of 722 amino acids with predicted *pI*/MW 4.89/83.22 kDa. BLASTp analysis and phylogenetic relationship strongly suggested McHSP90 was a member of HSP90 family, and it was highly conserved with other known HSP90, especially in the HSP90 family signatures, ATP/GTP-Binding sites and 'EEVD' motif. The mRNA of McHSP90 in haemolymph was upregulated in all treatments including *Vibrio alginolyticus* and *Vibrio harveyi* challenge, metals stresses (copper and cadmium) and 180 CST fuel exposure. All the results implied the expression of McHSP90 could be affected by *Vibrio* challenge and environmental stress, which might help us gain more insight into the molecular mechanism of HSP against adverse stresses in mollusca.

1. Introduction

In almost all eukaryotes and prokaryotes, heat shock proteins (HSPs) are ubiquitous and highly conserved proteins, which have strong cytoprotective effects and behave as molecular chaperones for other cellular proteins (Joly et al., 2010; Mjahed et al., 2012). Under external environmental stress, such as heat shock, heavy metals, pathogenic infections or almost any sudden changes inducing protein damage in the cellular environment, HSPs play crucial roles in protecting organisms from damage and restoring the damaged proteins to their functional three-dimensional structure (Welch, 1992; Song et al., 2006; Gao et al., 2007). According to their molecular weight, HSPs have been classified into several different families: HSP90 (85–90 kDa), HSP70 (68–73 kDa), HSP60, HSP47 and low molecular mass HSPs (16–24 kDa) (Joly et al., 2010). Heat shock proteins 90 (Hsp90s), the approximately 90-kDa family of stress proteins, are the most abundant molecular chaperones in eukaryotic cytoplasm, which basically consist of three isoforms: hsp84, hsp86 and glucose regulated protein (grp94) (Csermeley et al., 1998). They may refold the misfolded proteins into proper conformation, interact with cellular signaling proteins, and associate with steroid hormone receptors and maintain them in a non-functional state until hormone binding. Therefore, Hsp90s are employed to mediate important roles in cellular protection, tumor repression, cell cycle control, cellular signaling and antigen recognition in most eukaryotes (Scholz et al., 2001). Meanwhile, as HSP90s can be regulated by a range of stressors, they play important roles in protecting organisms from damage, being involved in immune response, and promoting the roles in environmental monitoring (Yong et al., 2008). These stressors include heat or cold shock, hyperosmotic stress, food-deprivation, reduced oxygen level, heavy metal concentrations and organic or inorganic chemical substances (Gao et al., 2007).

In recent years, marine pollution has become more and more serious than before, aquatic organisms are now suffering from increasing environmental stresses (Silvia and Elena, 2005). For the sensitive reaction to marine or aquatic environment, marine bivalves in mollusks have been regarded as the ideal indicators in the assessment of environmental pollution because they are sedentary, ubiquitous, filter-feeders inhabiting in the coastal and estuarine areas (Goldberg et al., 1983; Pascal et al., 2004). The effects of pollutants on the immune and stress responses have been reported in many bivalve species, such as *Mytilus edulis*, *M. galloprovincialis*, *Crassostrea virginica*, *Crassostrea gigas*, *Ostrea edulis*, *Crassostrea angulata* and *Haliotis rufescens* (Galloway and Depledge, 2001; Boutet et al., 2003). However for HSP family most studies mainly focused on HSP70 gene but not HSP90, and little is known in mollusk about the relationship between HSP90 and environmental pollution.

M. coruscus is an important economic shellfish of aquatic invertebrates, widely distributing in eastern coast of East China Sea, especially Zhoushan (China) coast (Liu et al., 2014; Liu et al., 2015). However, the research information of stress response to marine pollution in this species is still limited. *M. coruscus* was regarded as our experimental animal and HSP90 was the candidate gene for its biomarker for pollutants in aquatic invertebrates. The full-length HSP90 cDNA sequence was cloned from *M. coruscus*, and the mRNA expression profiles were analyzed emphatically in haemolymph after *Vibrio* pathogen infection and marine

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contaminants (copper, cadmium and 180 CST) stress based on RT-PCR analysis. All the results will contribute to better understanding of HSP90 diversity in mollusca, selecting appropriate biomarkers to monitor the physiological status of mussels under different environmental conditions, and developing effective management for disease.

2. Material and methods

2.1. Experimental animals

Adult mussels (*M. coruscus*, 5–8 cm in length) were sampled from Zhoushan (Zhejiang province, P.R. China), and immediately transferred to tanks in laboratory. The mussels were maintained at 25 °C with salinity of 27–28‰ for a week before experiment. Seawater was changed daily. Animals were fed with microalgae during the acclimation and experimental period. No mortality was observed in either the experimental or the control groups. Hemolymph was collected from the adductor muscles with syringe. Total RNA was isolated from the haemolymph using Trizol reagent (TaKaRa, China) and treated with DNAse-1 (Sigma, USA) to remove genomic DNA. The concentration was quantified for the ratio of A_{260}/A_{280} by measuring the absorbance at 260 and 280 nm with a UV-spectrophotometer (Bio-Rad, USA). The cDNA synthesis was carried out with M-MLV RTase cDNA Synthesis Kit (TaKaRa, China).

2.2. cDNA of M. coruscus HSP90 identification and full-length amplification

The primers (HSP90-F and HSP90-R, as shown in Table 1) were designed by Primer 5.0 according to the homologous HSP90 sequences of *M. galloprovincialis* (AM236589), *Mytilus trossulus* (KJ871058), *Crassostrea hongkongensis* (HM171376), *Ruditapes philippinarum* (KJ569080), *Haliotis discus hannai* (GU014545) and *Crassostrea ariakensis* (HQ450642). The reaction system was performed in 20 μL volume, including 10 × PCR Buffer 2 μL, dNTPs 0.4 μL, HSP-F 0.8 μL, HSP-R 0.8 μL, template cDNA 0.6 μL, and 0.4 μL of Taq DNA polymerase (TaKaRa, China). The PCR amplification was conducted on the Thermal Cycler (Bio-Rad, USA), and amplification conditions were: 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 120 s at 72 °C, with the final extension of 10 min at 72 °C. The PCR products were subjected to 1.5% agarose gel electrophoresis, purified by NucleoTrap Gel Extraction Kit (TIANGEN, China), and sequenced at Shanghai Invitrogen Biological Technology Company (China).

Full-length cDNA sequence of HSP90 was obtained with other primers (5P, 3P as shown in Table 1) and the primers in the Smart RACE cDNA amplification kit (Clontech, USA). Both 5′-RACE and 3′-RACE were carried out according to the manufacturer's instructions. The PCR products were cloned into the PMD18-T simple vector (TaKaRa, China) and sequenced from both directions. The full-length cDNA was acquired by overlapping the forward and the reverse strand sequences, and further was verified by amplification of the whole length and cluster analysis.

Table 1
PCR primer sequences for HSP90 cloning of *Mytilus coruscus*. Degenerate primers as followed, S: C/G; M: A/C; Y: C/T; W: A/T; R: A/G; K: G/T.

Primer	Sequences
For HSP90 cDNA clone	
HSP90-F	5′- TGATGAGYTTGATCATCAAYAC -3′
HSP90-R	5′-TGYTTYTTRGCCATRYADCCCAT-3′
For 5′ and 3′ RACE	
5P-R	5′–TTTCTTGGCTGCCATGTAACCC–3′
3P-F	5′–AAGACCTGGAAATCCGAATCAT–3′
For qRT-PCR	
McHSP90-F	5′- GGAAATCCGAATCATACCAGAC -3′
McHSP90-R	5′- ACCTCTGCCGATGGACTCAC -3′
β-actin-F	5′-ATGAAACCACCTACAACAGT-3′
β-actin-R	5′-TAGACCCACCAATCCAGACG-3′

2.3. Sequence analysis

The cDNA sequences (sequencing was repeated for three times) were spliced by the software of DNAstar v7.0. The BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) was used to analyze the cDNA sequence. The conserved domains were predicted using SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi) online tool. The theoretical MW and *pI* were determined by Expasy-ProtParam online tool (www.expasy.org/tools/protparam.html). Multiple sequence alignments were performed with ClustalW in the software of Genedoc (http://www.genome.jp/tools/clustalw/). The phylogenetic tree was constructed by Boostrapped Neighbor-Joining rule method from a distance matrix with the software of MEGA 4.0 (Kumar et al., 2004).

2.4. The temporal expression of HSP90 mRNA in haemolymph after Vibrio harveyi and Vibrio alginolyticus challenge

For the aquatic pathogenic bacterial challenge experiment, 100 mussels were obtained and kept in aerated tanks. The live *V. alginolyticus* and *V. harveyi* that were isolated from marine shellfish before, were injected into the adductor respectively of 100 μL diluted with PBS (pH 7.4, final concentration of O.D 600 = 0.4). Forty-five individuals of the challenged mussels by different *Vibrio* were randomly collected at 2, 4, 6, 8, 12, 24, 36, 48 and 72 h post-challenge. The mussels that were injected with the same amount of PBS, were used as the blank group. Hemolymph (about 0.35 mL for each individual) from the blank and the challenged group were collected from the adductor muscles with a syringe. The hemolymph from five mussels of the same time were pooled together as one sample and immediately centrifuged at 800*g*, 4 °C for 10 min to harvest the hemocytes for total RNA extracting. Two micrograms of total RNA from each group (n = 5) were reverse transcribed in the final volume of 40 μL with a PrimeScript™ RT reagent kit (Perfect Real Time) (TaKaRa) following the manufacturer's instructions. Real-time PCR was performed in a reaction mixture of 20 μL, containing McHSP90-F 0.8 μL, McHSP90-R 0.8 μL, 2×SYBR® Premix Ex TaqTM II (TaKaRa) 10 μL, cDNA sample 0.8 μL, ROX II 0.4 μL, ddH_2O 7.2 μL. The standard cycling conditions were: 95 °C for 1 min (initial polymerase activation), followed by 40 cycles of 30 s at 95 °C, 45 s at 65 °C. The PCR specificity was checked with dissociation curve analysis from 55 to 95 °C, and β-actin of *M. coruscus* was taken as the internal standard (primers as shown in Table 1). The $2^{-\Delta\Delta CT}$ method was used to analyze the mRNA expression level. All data were given in terms of relative mRNA expressed as mean ± S.E. (N = 5). Statistical analysis of differences was done with SPSS 13.0 by one-way analysis of variance (ANOVA) followed by an unpaired, two-tailed *t*-test. Differences were considered significantly at $P < 0.05$.

2.5. The temporal expression of HSP90 mRNA in haemolymph after contaminated treatments

The mussels were divided into 3 groups with 50 animals each. Two groups were exposed to 10 L seawater with heavy metal press, Cu^{2+} (final concentration of 20 μg/L) from $CuSO_4 \cdot 5H_2O$ and Cd^{2+} (final concentration of 200 μg/L) from $CdCl_2 \cdot 5H_2O$ respectively. The other group was cultured in 10 L seawater with 180 CST fuel (final concentration of 40 μg/L). Five normal mussels were reared in an individual tank as control. All mussels were kept in static tanks at 25 °C. Fresh seawater was changed daily and resupplied with the corresponding concentration of pollutants. Sampling was performed in the three groups were on 3, 5, 10, 15, 20, 25 and 30 d after the stimulation. The methods of haemolymph collection, total RNA extraction, cDNA synthesis and real-time PCR analysis were performed as described above.