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Short communication

Identification and analysis of HSP70 from *Sepiella maindroni* under stress of *Vibrio harveyi* and Cd^{2+}

Hui-hui Liu *, Jian-yu He, Chang-feng Chi, Zhen-ming Lv

National Engineering Research Center of Marine Facilities Aquaculture, Zhejiang Ocean University, Zhoushan 316022, PR China

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ABSTRACT

The 70-kDa heat shock proteins (HSP70) play crucial roles in protecting cells against environmental stresses, such as heat shock, heavy metals and pathogenic bacteria. The full-length HSP70 cDNA of *Sepiella maindroni* (designated as SmHSP70, GenBank accession no. KJ739788) was 2109 bp, including an ORF of 1950 bp encoding a polypeptide of 649 amino acids with predicted *pI*/MW 5.24/71.30 kDa, a 62 bp-5′-UTR and a 97 bp-3′-UTR. BLASTp analysis and phylogenetic relationship strongly suggested that the amino acid sequence was a member of HSP70 family. Multiple sequence alignment revealed that SmHSP70 and other known HSP70 were highly conserved, especially in the regions of HSP70 family signatures, the bipartite nuclear targeting sequence, ATP/GTP-binding site motif and 'EEVD' motif. Time-dependent mRNA expression of SmHSP70 in the liver was recorded by quantitative real-time RT-PCR after *Vibrio harveyi* injection and Cd^{2+} exposure. The results indicated that SmHSP70 played a significant role in mediating the environmental stress and immune response against pathogens.

1. Introduction

The organisms are continually challenged by environmental conditions, which cause acute and chronic stresses. To adapt to the changes and survive from different types of injuries, eukaryotic cells have evolved networks of different responses to detect and control diverse forms of stresses (Santoro, 2000). Heat shock proteins (HSPs) are ubiquitous and highly conserved stress proteins occurring in all organisms from bacteria to humans, they have strong cytoprotective effects, and behave as molecular chaperones for other cellular proteins (Joly et al., 2010; Mjahed et al., 2012). According to their apparent molecular mass, HSPs have been classified into several families: HSP90 (85–90 kDa), HSP70 (68–73 kDa), HSP60, HSP47 and low molecular mass HSPs (16–24 kDa). Heat shock protein 70 (HSP70) is an important member of the heat shock protein superfamily and appears in almost all species except for some archaea (Tavaria et al., 1996; Krenek et al., 2013). As a kind of highly conserved protein HSP70 is associated with intracellular chaperone and extracellular immunoregulatory functions to protect cells against environmental stress, and shows tissue-/time-/dose-dependent changes (Liu et al., 2014). Various stresses stimulate the synthesis of HSP70, which presumably acts to restore protein structure and function (Yenari et al., 1999).

In recent years, marine pollution has become more and more serious than before, marine organisms have been suffering from increasing environmental stress via exposure to a plenty variety of pollutants, and even result in mutations or the death of intact organisms and their progenies. Moreover, a large portion of these contaminants can be transferred through the food chain, making them a potential threat to entire ecosystems and even human beings (Wan et al., 2008). Therefore, studies on the resistant molecules that exist in marine organisms and related applications in the assessment of environmental health are necessary (Silvia and Elena, 2005), such as HSP70s, HSP90s, superoxide dismutases (SODs), catalases (CATs), Glutathione S-Transferases (GSTs), and glutathione peroxidase (GSH-PX). As the important molecular for protein folding, multimer dissociation and association, translocation of proteins across membranes and regulation of the heat shock response, several HSP70s have been described in molluscs, including *Crassostrea gigas* (Isabelle et al., 2003), *Ostrea edulis* (Piano et al., 2005), *Mytilus edulis* (Luedeking and Koehler, 2004), *Chlamys farreri* (Wu et al., 2003), and *Argopecten irradians* (Song et al., 2006). Also, the studies examining the effects of environmental stressors on HSP70 gene expression have been reported in different species of molluscs and recognized the relevant physiological and ecological importance of heat shock gene expression in response of changing environments (Hamdoun et al., 2003; Piano et al., 2005; Buckley et al., 2001). However, no information is available on HSP70 expression of

Abbreviations: HSP70, heat shock protein 70; HSPs, heat shock proteins; SmHSP70, heat shock protein 70 of *Sepiella maindroni*; CST, critical solution temperature; RACE, rapid amplification of cDNA ends; ORF, open reading frame; UTRs, untranslated regions.

* Corresponding author.
E-mail address: liuhuihui2004@126.com (H. Liu).

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cephalopods at transcriptional level under aquatic pathogenic bacterial infection and environmental stress.

Sepiella maindroni is an important economic cephalopod in the East Sea of China. In view of few related reports of this squid, *S. maindroni* was regarded as our experimental animals, and HSP70 was the candidate gene for its intracellular chaperone and extracellular immunoregulatory functions in aquatic invertebrates. The full-length HSP70 cDNA sequence was cloned from *S. maindroni* (designated as SmHSP70), and the mRNA expression profiles were analyzed emphatically in the liver after *Vibrio harveyi* infection and cadmium stress based on SYBR Green quantitative RT-PCR analysis. All of the results will contribute to better understanding of HSP70 diversity in mollusca and the biochemical resistance mechanisms used by marine consumers to cope with their allelochemically defended prey.

2. Material and methods

2.1. Experimental animals

The juvenile cuttlefishes (*S. maindroni*, 2.0–4.0 cm in length) were collected from the Dongji aquaculture farm in Zhoushan, Zhejiang province, P.R. China, immediately transferred to the laboratory, and acclimated with salinity of 28–30‰ for a week at 23–25 °C before experiment in May 2014. Seawater was changed daily. Animals were fed with microalgae during the acclimation and experimental period. No mortality was observed in either the experimental or control groups. Total RNA was isolated from the liver with Trizol reagent (TaKaRa, China) and the ratio of A_{260}/A_{280} was determined. The cDNA synthesis was carried out with M-MLV RTase cDNA Synthesis Kit (TaKaRa, China).

2.2. cDNA of S. maindroni HSP70 identification and full-length amplification

The primers HSP70-F/HSP70-R (shown in Table 1) were designed according to the sequence of *Pteria penguin* (ABJ97377), *Cyclina sinensis* (AET13646), *Perna viridis* (ABJ98722), *A. irradians* (AAS17723), *Azumapecten farreri* (AAO38780), *Mytilus coruscus* (AGY56119), and *Meretrix meretrix* (ADT78478). The total RNA from the liver of *S. maindroni* was used as template after reverse transcription and the ratio of A_{260}/A_{280} was 1.90. The reaction system was performed in 20 μL volume, including 2 μL 10× PCR Buffer, 2 μL of $MgCl_2$ (25 mmol/L), dNTPs 0.4 μL (2.5 mmol/L), HSP70-F 0.8 μL (10 mol/L), HSP70-R 0.8 μL (10 μmol/L), template cDNA 0.6 μL, Taq DNA polymerase (TaKaRa, China) 0.4 μL (1 U) and 13.0 μL of PCR-Grade water. The PCR amplification was conducted on a Thermal Cycler (Bio-Rad, USA), and amplification conditions were: 4 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 30 s at 56.5 °C, and 60 s at 72 °C, with a final extension of 10 min at 72 °C. The PCR products were gel-purified and sequenced at Shanghai Invitrogen Biological Technology Company (P.R. China). Gene specific primers for rapid-amplification of cDNA ends including 5′-RACE (5P1 and 5P2) and 3′-RACE (3P1 and 3P2, Table 1), were designed based on the known partial sequence. Full-length cDNA sequence of HSP70 was performed with the specific 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 obtained by overlapping the forward and reverse strand sequences. The resulting sequence was verified by the amplification of the whole full length and further subjected to cluster analysis.

Table 1
PCR primer sequences for SOD cloning of *S. maindroni*. Degenerate primers were as follows, S: C/G; M: A/C; Y: C/T; W: A/T; R: A/G; and K: G/T.

Primer	Sequences
For HSP70 cDNA clone	
HSP70-F	5′-GTTATGACCTCGTTGATTAAGAG-3′
HSP70-R	5′-TTKSWSARRCGACCTTTGTC-3′
For 5′-/3′-RACE	
Adaptor primer	5′-TCATTGCTCTTTCTCC-3′
5P1	5′-CCAGGTTGGTTATCGGAGTATG-3′
5P2	5′-AGGTCTGTGTTTGTTTTGTTGG-3′
3P1	5′-CTTCACAACATACTCCGATAACCAACCT-3′
3P2	5′-GAGCACTGGTAAAGAGAACAAGATCACC-3′
For qRT-PCR	
qSmHSP70-F	5′-ACTCCGATAACCAACCTG-3′
qSmHSP70-R	5′-GAGGCGACCTTTGTCATTT-3′
β-Smactin-F	5′-GCCAGTTGCTCGTTACAG-3′
β-Smactin-R	5′-GCCAACAATAGATGGGAAT-3′

2.3. Sequence analysis

The cDNA sequence was spliced by the software of DNAstar v7.0. The amino acid sequence of SmHSP70 was deduced by the Expert Protein Analysis System (http://www.expasy.org/) and the homology search was conducted with BLASTp program of NCBI (http://www.ncbi.nlm.gov/BLAST/). The conserved domains were predicted using SMART (http://smart.embl-heidelberg.de/) online tool. The theoretical MW and predicted *pI* were determined by Expasy-ProtParam online tool (http://www.expasy.org/tools/protparam.html). Multiple sequence alignments were performed with ClustalW v1.8 (http://pbil.ibcp.fr/htm/index.php). The phylogenetic tree was constructed by Bootstrapped Neighbor-Joining rule method from a distance matrix with MEGA v4.0 software.

2.4. SmHSP70 mRNA expression in liver after V. harveyi challenge

The liver was selected as a candidate tissue for investigating the temporal expression profile of SmHSP70 challenged by *V. harveyi*. The live bacteria were cultured on LB plates at 28 °C overnight, then a single colony was inoculated in 5 mL of LB broth at 28 °C for 12 h. The bacterial suspension was centrifuged at 6000 ×*g*, 4 °C for 10 min to collect bacteria, and the resuspended live *V. harveyi* in 100 μL of PBS (pH 7.4, OD 600 = 0.4) were injected into muscles of *S. maindroni*. The cuttlefishes, which were injected with 100 μL PBS, acted as the control group. Total 35 infected cuttlefishes were cultured in 10 L filtered fresh well-aerated seawater, the livers were randomly collected at 2, 4, 8, 12, 24, 48 and 72 h post-injection and frozen immediately in liquid nitrogen and stored at −70 °C. The control group was disposed as above.

To analyze the temporal expression patterns of SmHSP70 gene at mRNA level in livers after *V. harveyi* challenge, real-time PCR (qRT-PCR) was performed with SYBR PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa, China) as recommended by the manufacturer's instructions in the 7500 Real Time PCR System (Applied Biosystems, UK). For each time point in the challenge experiment 5 biological replicate was used. The reaction mixture of 20 μL contained qSmHSP70-F 0.8 μL (10 μmol/L), qSmHSP70-R 0.8 μL (10 μmol/L), 2× SYBR® Premix Ex TaqTM II (TaKaRa, China) 10 μL, cDNA sample 0.8 μL, ROX II 0.4 μL, and ddH_2O 7.2 μL. The standard cycling conditions were: 95 °C for 1 min (initial polymerase activation), followed by 40 cycles of 10 s at 95 °C and 45 s at 59.6 °C. The PCR specificity was checked with dissociation curve analysis from 55 to 95 °C, β-actin of *S. maindroni* (β-Sm actin-F and β-Sm actin-R) was used as the internal standard, and all the primers were 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 means ± S.E. (N = 5). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed *t*-test. Differences were considered significant at $P < 0.05$.