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Molecular cloning and sequence analysis of heat shock proteins 70 (HSP70) and 90 (HSP90) and their expression analysis when exposed to benzo(a)pyrene in the clam *Ruditapes philippinarum*

Tong Liu, Luqing Pan *, Yuefeng Cai, Jingjing Miao

The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China

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ABSTRACT

HSP70 and HSP90 are the most important heat shock proteins (HSPs), which play the key roles in the cell as molecular chaperones and may involve in metabolic detoxification. The present research has obtained full-length cDNAs of genes HSP70 and HSP90 from the clam *Ruditapes philippinarum* and studied the transcriptional responses of the two genes when exposed to benzo(a)pyrene (BaP). The full-length *Rp*HSP70 cDNA was 2336 bp containing a 5′ untranslated region (UTR) of 51 bp, a 3′ UTR of 335 bp and an open reading frame (ORF) of 1950 bp encoding 650 amino acid residues. The full-length *Rp*HSP90 cDNA was 2839 bp containing a 107-bp 5′ UTR, a 554-bp 3′ UTR and a 2178-bp ORF encoding 726 amino acid residues. The deduced amino acid sequences of *Rp*HSP70 and *Rp*HSP90 shared the highest identity with the sequences of *Paphia undulata*, and the phylogenetic trees showed that the evolutions of *Rp*HSP70 and *Rp*HSP90 were almost in accord with the evolution of species. The *Rp*HSP70 and *Rp*HSP90 mRNA expressions were detected in all tested tissues in the adult clams (digestive gland, gill, adductor muscle and mantle) and the highest mRNA expression level was observed in the digestive gland compared to other tissues. Quantitative real-time RT-PCR analysis revealed that mRNA expression levels of the clam *Rp*HSP70, *Rp*HSP90 and other xenobiotic metabolizing enzymes (XMEs) (AhR, DD, GST, GPx) in the digestive gland of *R. philippinarum* were induced by benzo(a)pyrene (BaP) and the absolute expression levels of these genes showed a temporal and dose-dependent response. The results suggested that *Rp*HSP70 and *Rp*HSP90 were involved in the metabolic detoxification of BaP in the clam *R. philippinarum*.

1. Introduction

Heat shock proteins (HSPs), ubiquitously distributed and evolutionarily conserved proteins, are found in a variety of living organisms (Lindquist and Craig, 1988). They exert a protective effect against a variety of stressful stimuli and protect cells from damage. HSPs can be broadly categorized into five major families basing on their molecular weight, amino acid sequence homologies and functions: HSP100, HSP90, HSP70, HSP60 and the small HSP family (Joly et al., 2010). HSP70 and HSP90 are the most conserved and extensively studied proteins in the families and they play the key roles in the cell as molecular chaperones, such as in membrane translocation, degradation of misfold proteins and other regulatory processes (Boorstein et al., 1994; Morimoto, 1998; Hayward et al., 2004; Mayer and Bukau, 2005; Li and Du, 2013). Furthermore, many studies indicated that HSP70 and HSP90 could also function as potent activators of the metabolic detoxification, and they can be induced by various environmental stressors, such as toxic and carcinogenic compounds, pesticides, heavy metals, and infection (Parsell and Lindquist, 1993; Basu et al., 2002; Fangue et al., 2006; Colinet et al., 2010; Xu and Qin, 2012).

Several cDNAs encoding HSP70 and HSP90 have been described in almost all vertebrates such as mammals, birds, reptiles, amphibians, and bony fishes. It is well known that they play a vital role in normal cellular function and contribute to correct protein folding (Gething and Sambrook, 1992; Morimoto, 1998). A fair amount of evidence from the studies on mammalian models has shown that HSPs were involved in metabolic detoxification (Burcham, 2013). In fish, the expression of HSP70 and HSP90 can be modulated by a range of stressors (Deane and Woo, 2011). In terms of chemical insults, several studies have reported that fish HSP70 and HSP90 can be induced by the exposure to heavy metals or organic chemicals (Bierkens, 1998; Kiang and

Abbreviations: cDNA, DNA complementary to RNA; HSP70, heat shock protein 70 gene; HSP90, heat shock protein 90 gene; XMEs, xenobiotic metabolizing enzymes; BaP, benzo(a) pyrene; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; qRT-PCR, quantitative RT-PCR; RACE, rapid amplification of cDNA ends; UTR, untranslated region; ORF, open reading frame; kDa, kilodalton; HSP, heat shock proteins; mRNA, message RNA; ATPase, adenosine triphosphatase; RNase, ribonuclease; DNase, deoxyribonuclease; LB, Luria–Bertani; TPR, tetratricopeptide repeat; HOP, HSP70/HSP90 Organizing Protein; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DD, dihydrodiol dehydrogenase; GST, glutathione S-transferase; GPx, glutathione peroxidase.

* Corresponding author.
E-mail address: panlq@ouc.edu.cn (L. Pan).

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Tsokos, 1998; Boone and Vijayan, 2002; Mukhopadhyay et al., 2003; Deane and Woo, 2006).

In invertebrates, HSP70 and HSP90 have been cloned from different species of bivalves including *Crassostrea gigas* (Boutet et al., 2003a; Gourdon et al., 2000; Choi et al., 2008), *Crassostrea virginica* (Rathinam et al., 2000), *Crassostrea hongkongensis* (Zhang and Zhang, 2012), *Ostrea edulis* (Boutet et al., 2003b; Piano et al., 2004), *Mytilus galloprovincialis* (Franzellitti and Fabbri, 2005), *Chlamys farreri* (Wu et al., 2004; Gao et al., 2007), *Argopecten irradians* (Song et al., 2006; Gao et al., 2008), *Meretrix meretrix* (Xin et al., 2011), *Laternula elliptica* (Kim et al., 2009), *Haliotis tuberculata* (Farcy et al., 2007) and *Haliotis asinina* (Gunter and Degnan, 2007). A clear time-dependent expression pattern of HSP70 mRNA in the digestive gland and gill of the oyster was observed after exposure to Cu^{2+} and malachite green (Zhang and Zhang, 2012). The HSP90 gene from *C. gigas* and *C. farreri* responded to various heavy metal stresses with a time and dose-dependent expression pattern (Choi et al., 2008; Gao et al., 2007). To our knowledge, most studies of HSP70 and HSP90 are focused on mammals and typical model organisms, while molecular features and function of HSP in mollusks have seldom been studied.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants derived from incomplete combustion of carbon. In China, concentrations of environmental PAHs are increasing particularly in aquatic environment (Feng et al., 2009). Benzo(a)pyrene (BaP) is the most studied high molecular mass PAH and it induces AhR-mediated CYP1 enzymes, which, in turn, metabolize BaP to mutagenic and carcinogenic metabolites (ATSDR, 1995; Piskorska-Pliszczynska et al., 1986). The clam *Ruditapes philippinarum* (*R. philippinarum*), a commercial species native from southern Siberia to China distributes at shore areas and total or half burrow in the sediment. The clam filters large amounts of water by the siphon extended out to the water for their nutritional and respiratory needs, so it accumulates environmental pollutants and is often considered as an indicator organism in pollution assessments of coastal sediment. The objective of this study was to characterize the complete HSP70 and HSP90 cDNA sequence of *R. philippinarum* and investigate the response of mRNA expression of the two genes in the clams when exposed to BaP.

2. Materials and methods

2.1. Organisms and BaP exposure experiments

Healthy *R. philippinarum* (shell length: 4.11 ± 0.22 cm) were collected from Red Island (Yellow Sea, Qingdao, China) and acclimated in tanks containing aerated sand-filtered seawater (salinity: 31‰, pH 8.1) at 12 ± 0.5 °C for one week before the exposure test. The water in each tank was fully renewed once a day and the clams were fed with dried powder of *Spirulina platensis* (30 mg for each individual) per day during the acclimation.

The clams were randomly divided into three experimental groups with three replicates. BaP concentrations of 0, 1 and 4 μg/L were used for exposure based on contents of BaP in coastal seawater of Qingdao (Pan et al., 2008; Liu et al., 2010). All the conditions were maintained the same as those used for acclimation. Six clams for each replicate were sampled at days 0, 1, 5, 10 and 15. Digestive glands from the six clams were excised and mix grinded, and total RNAs were extracted immediately and stored at −80 °C until expression analysis. The digestive glands of untreated clams were collected from six clams to clone the HSP70 and HSP90 genes and the gill, digestive gland, adductor muscle and mantle of untreated clams were sampled from another six clams to determine the tissue distribution of HSP70 and HSP90.

2.2. RNA isolation and reverse transcription

Total RNA was extracted from about 100 mg tissues of *R. philippinarum* using the RNAiso Plus reagent (TaKaRa, China) according to the manufacturer's protocols and the contaminating genomic DNA was eliminated by using RNase-free DNase (TaKaRa, China) following the manufacturer's instructions. RNA quantity, purity and integrity were verified by both native RNA electrophoresis on 1.0% agarose gel in 50× TAE buffer (Tris 2 mol L^{-1}, acetic acid 1 mol L^{-1}, EDTA 0.1 mol L^{-1}, pH 8.0), and the UV absorbance ratio at 260 nm and 280 nm (Ultrospec 2100 pro, Amersham, USA). cDNA was synthesized from 2 μg of total RNA by M-MLV reverse transcriptase (Promega, USA) at 42 °C for 60 min with an oligo dT-adaptor primer following the protocol of the manufacturer.

2.3. Cloning the full-length cDNA of HSP70 and HSP90 using RACE-PCR

The partial sequences of *Rp*HSP70 and *Rp*HSP90 were obtained from the splicing transcriptome of the clam *R. philippinarum* established by our laboratory (unpublished). The sequences of primers were listed in Table 1 and all the primers used in the study were obtained from BGI (Qingdao, China). The PCR reaction was performed using a Gradient Mastercycler (Eppendorf, Germany) in a total volume of 25 μL PCR mixture containing 2.5 μL 10× reaction buffer with 15 mmol L^{-1} $MgCl_2$, 2 μL of 10 mmol L^{-1} dNTP mix, 1 μL of 25 μmol L^{-1} of each primer, 2 μL template cDNA, 16 μL MilliQ water, and 0.5 μL of BioReady rTaq DNA Polymerase (5 U μL^{-1}) (TaKaRa, China). To identify the partial *Rp*HSP70 and *Rp*HSP90 genes of the splicing transcriptome of the clam *R. philippinarum*, PCR cycles were conducted at 95 °C for 3 min followed by 31 cycles of 94 °C for 30 s, 56.5 °C for 1 min, 72 °C for 3 min, and a final cycle of 72 °C for 7 min. The PCR products were analyzed by electrophoresis in 1.0% agarose gel, followed by purification using an agarose gel DNA purification kit (TaKaRa, China), and then they were ligated into the pMD18-T vector (TaKaRa, China) and sequenced (BGI, China).

The full-length of *Rp*HSP70 and *Rp*HSP90 was obtained by the procedures of rapid amplification of cDNA ends (RACE) method using the SMART™ RACE cDNA amplification kit (Clontech, USA). 5′ and 3′ RACE-PCRs were performed using specific primers (Table 1). Amplification methods were conducted by following the protocol of manufacturers.

2.4. Sequence analysis

The amino acid sequences, protein molecular mass and isoelectric point (pI) were predicted using lasergene 5.1 (DNA STAR inc., Madison, USA). The homology searches of nucleotide and protein sequences were

Table 1
Primer names and sequences used in this study.

Primer name	Sequences (5′ → 3′)
5′RACE-HSP70-1	ATTTCGTTCACCTCCAACCTTCT
5′RACE-HSP70-2	TACGCAAGATATTGAGACCAGAA
3′RACE-HSP70-1	CACTATTACAAATGACAAAGGAC
5′RACE-HSP90-1	CTTGATGGGATAGCCAATGAACT
5′RACE-HSP90-2	CTGGGAATGTTTCTTCACTACCTCT
3′RACE-HSP90-1	CTTGGTCTTGGCATTGATGAGGA
3′RACE-HSP90-2	GGATTCTCCCTTGAAGAGCCAACA
RT-HSP70F	TGAAAGACAATAAACGTGCTG
RT-HSP70R	AATTTCAATGCTGGCTTGTGT
RT-HSP90F	AATTCGCCATGTTGTATCGTC
RT-HSP90R	CTTTGCTGCCATGTAACCC
RT-AhRF	GGCAACCGATATAATTTGGAC
RT-AhRR	TTACCACAAACAGAAAACCGTA
RT-DDF	AAAAGCGGAAGTGCCTGAGATGC
RT-DDR	TACGGACGCTGGAAAGGGAGATA
RT-GSTF	AACAAGGAGACCCACCAGGATTG
RT-GSTR	CGATATGCTTCAGAATGGCGTTA
RT-GPxF	GACATCCGATGGAACTACGAA
RT-GPxR	ACAACTACAGAACCGGAAGC
RT-18SF	ACTCAACACGGGGAACCTC
RT-18SR	TTAACCAGACAAATCGCTCCAC