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Acute temperature and cadmium stress response characterization of small heat shock protein 27 in large yellow croaker, *Larimichthys crocea*

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ABSTRACT

Small heat shock proteins (sHSPs) are a group of molecular chaperones and play a crucial role in cell response to various stresses. In this study, we cloned and sequenced a small heat shock protein 27 (*Lc*HSP27) cDNA from large yellow croaker, *Larimichthys crocea*. The full-length cDNA of *Lc*HSP27 was 1227 bp, including a 5′-terminal untranslated region (UTR) of 54 bp, a 3′-terminal UTR of 561 bp and an open reading frame (ORF) of 612 bp encoding a polypeptide of 203 amino acids. Three conserved phosphorylation sites of serine were identified in the deduced *Lc*HSP27 amino acid sequence at positions 15, 91and 95 respectively, and a typical α-crystallin domain was at positions 96–193. Phylogenetic analysis revealed that *Lc*HSP27 was categorized together with the HSP27 obtained from other fish. And a closer phylogenetic relationship of HSP27 was found with HSP22, then HSP30. Quantitative real-time reverse transcription PCR (qRT-PCR) analysis indicated the strongest expression of *Lc*HSP27 in heart. However, expression of *Lc*HSP27 in other examined tissues including muscle, brain, liver, spleen, kidney, gill, and blood was very weak. The impact of temperature and cadmium (Cd^{2+}) stress on *Lc*HSP27 expression was tested in liver and brain. The results showed that the levels of *Lc*HSP27 expression increased significantly after low temperature (19 °C) and high temperature (27 °C and 31 °C) stress both in liver and brain. And low temperature stress induced a higher *Lc*HSP27 expression in liver. More important, *Lc*HSP27 expression showed a dramatic up-regulation after a combined stress of temperature and cadmium ($p<0.05$). These results reveal that HSP27 may play an important role in the large yellow croaker response to temperature and cadmium stress.

1. Introduction

Small heat shock proteins (sHSPs) are a group of molecular chaperones that contain highly conserved amino acid sequence and the α-crystallin domain at the C-terminus (Montfort et al., 2002). The common roles of sHSPs are chaperone activity, thermo-damage protection, apoptosis inhibition, cell development regulation, cell signal transduction, and various cellular stress responsion (Leroux et al., 1997), such as oxidative (Broome et al., 2006), toxic (Airaksinen et al., 2003a, 2003b), and pathogenic stress (Mayr et al., 2000).

As an important member of sHSPs, HSP27 plays crucial roles in cytoprotection, including inhibition of protein aggregation and procaspase9 activation (Bruey et al., 2000; Paul et al., 2002). In fish, the HSP27 sequence has been obtained from Pacific bluefin tuna (*Thunnus orientalis*) (Ojima and Oohara, 2008), zebrafish (*Danio rerio*) (Mao et al., 2005) and goldfish (*Carassius auratus*) (Wang et al., 2007), and two mRNA splice variants of HSP 27 were identified from rainbow trout (*Oncorhynchus mykiss*) (Ojima, 2007). Mao et al. (2005) demonstrated that zebrafish HSP27 contained three conserved phosphorylation sites of serine and a cysteine important for regulation of apoptosis but lacked a typical C-terminal tail domain essential for complete chaperone activity and stability in mammalian sHSPs (Lindner et al., 2000). It was demonstrated that HSP27 expression showed a close relationship with heat stress (Mao et al., 2005), embryo development (Mao and Shelden, 2006), or water pollution (Wang et al., 2007) in fish. However, most researches focused on the structure and stress response characterization of conservative HSP70 in fish and other species and the detailed structure and stress response characterizations of HSP27 in most fish need to be better understood.

Heavy metals can enter seawater by industrial and consumer waste and are dangerous because they tend to bioaccumulate. Among which, cadmium is considered to be one of the main and most dangerous heavy metal contaminant to mari-cultured animals (Waisberg et al., 2003). Even in low concentrations, cadmium could be absorbed from the gill, and accumulated in organisms especially in the kidney, liver, brain and gonad tissues (Pedersen and Bjerregaard, 2000; Fort et al., 2001; Scott et al., 2003; Barbier et al., 2004; Matz and Krone, 2007). Some researches demonstrated that the expression level of the HSPs could be induced by cadmium stress. For example, a concentration-dependent increase in HSP27 was detected in MDCK and LLC-PK1 renal epithelial cells treated with cadmium (Bonham et al., 2003). King et al. (2009) demonstrated that HSP27 can provide a protective influence in primary cortical neurons in the face of toxic concentrations of amyloid. Up-regulation of HSP27 and metallothionein has been detected in MDA-MB231 cells after

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cadmium exposure (Sirchia et al., 2008). HSP27 is highly phosphorylated in response to the cadmium exposure in bovine adrenal chromaffin cells (BACCs) (Leal et al., 2007). Acute exposure to cadmium was found to trigger the up-regulation of genes encoding the chaperone proteins HSP90A, HSP27, HSP40, GRP78, HSP72, and HO-1 in cultured human pneumocytes (A549 cell line) (Croute et al., 2005). Cadmium is a widely-disseminated metal which can be imported and accumulated in cells. More important, liver and brain tissue have been reported the most sensitive tissues to cadmium stress (Yeh et al., 2011). However, the more details of HSP27 response to cadmium stress are poorly studied in fish.

The large yellow croaker, *Larimichthys crocea*, is an economically important mari-cultured species in the southeast of China. However, the cultured environment of this fish is a very complex system in which the health status of fish showed a close relationship with temperature and heavy metal stress. Therefore, a better understanding of the stress response of large yellow croaker may contribute to developing strategies for long-term sustainability.

In the present study, the full-length cDNA of HSP27 was obtained from *L. crocea*, and the temporal expression levels of the gene after temperature and cadmium stress were examined and compared in order to better understand the potential role of HSP27 in fish response to environmental stress.

2. Materials and methods

2.1. Fish collection

Healthy cultured large yellow croaker (*L. crocea*, Sciaenidae, body mass 100 ± 20 g), were collected at the Experimental Fish Farm of Ningde Centre of Popularization of Fisheries Technology, Fujian province, China. Before the experiments, the fish were acclimated for at least two weeks in 1 m^3 re-circulating seawater tanks in salinity (28 psu), temperature (23 °C) and density conditions similar to those of culture net cages from which the specimens were obtained, and the fish were fed with a commercial feed twice per day. Blood was collected from eugenol anaesthetized fish, by cutting the tail and the blood cells were separated in two times the volume of anticoagulant solution (0.48% citric acid, 1.32% sodium citrate and 1.47% glucose) at 800 *g* for 5 min at 4 °C and were stored in liquid nitrogen immediately (Yao et al., 2010). Kidney, spleen, liver, gill, brain, heart, and dorsal muscle were dissected out and preserved in liquid nitrogen for RNA extraction.

2.2. Temperature stress

Temperature stress was performed by removing the fish to other containers with sea water at a temperature of 19 ± 0.5 °C, 23 ± 0.5 °C, 27 ± 0.5 °C and 31 ± 0.5 °C respectively. Five fish were randomly collected at 6, 12, 24 and 48 h separately during the temperature stress. The liver and brain of fish at each sampling time were separated, frozen immediately in liquid nitrogen, and stored at −80 °C until RNA extraction for later gene expression. No mortality was observed during the experiment.

2.3. Cadmium stress

Cadmium stress was performed by injecting a 200 μL $CdCl_2$ (0.25 μg/μL) per fish. Then the fish were exposed to seawater at 19 °C, 23 °C, 27 °C and 31 °C respectively. Fish were injected with 200 μL of sterile NaCl as Cd^{2+} stress controls. Five fish were used for each group, and every treatment was composed of three replicates. Fish were cultured at 23 °C as blank control. Livers and brains of each group were collected at 0, 6, 12, 24 and 48 h after stress and were frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. No mortality was observed during the collection.

2.4. RNA extraction and cDNA synthesis for gene cloning

Total RNA was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Total RNA was incubated with RNase-free DNase I (Roche, USA) to remove the contaminating genomic DNA. First strand cDNA was synthesized from 2 μg of total RNA by M-MLV reverse transcriptase, following the manufacturer's protocol with oligo $d(T)_{15}$ (TIANGEN, Beijing) at 37 °C for 60 min.

2.5. Cloning and sequencing of LcHSP27 cDNA

To obtain HSP27 cDNA from large yellow croaker, four degenerate primers were designed based on the highly conserved sequence of known fish HSP27 genes in the GenBank database. The first round PCR was performed using primers HSP27 F1 and HSP27 R1 with 1 cycle of denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s, followed by a 10 min extension at 72 °C. Heart cDNA was used as template. The nested PCR was then performed using primers HSP27 F2 and HSP27 R2 under the same conditions. All PCR products were cloned into pGEM-T Easy vector (Promega, USA) and sequenced in Invitrogen Corp (Shanghai, China). All primer sequences are listed in Table 1.

Based on the obtained sequence of large yellow croaker HSP27, the 3′- and 5′-ends were obtained by RACE approaches, using gene-specific primers and adapter primers (Table 1). The 3′ end RACE PCR reaction was performed with gene-specific primer HSP27 3-F1 and adapter primer AOLP. The PCRs used heart cDNA as template with annealing temperature of 58 °C. Nested PCR was conducted with a gene-specific primer HSP27 3-F2 and adaptor primer AP (Table 1) with annealing temperature of 56 °C.

For the 5′ end, heart mRNA was transcribed by M-MLV reverse transcriptase with gene-specific primer HSP27 5-R1. Then the cDNA was purified with a DNA purification kit (TaKaRa, Dalian) and tailed with poly (C) at the 5′ end of cDNA by terminal deoxynucleotidyl transferase (TdT) (Fermentas, USA). PCR was performed initially with primer HSP27 5-R2 and adapter primer AAP using the tailed cDNA as template and with an annealing temperature of 55 °C. Nested PCR was conducted with a gene-specific primer HSP27 5-R3 and adapter primer AP with annealing temperature of 58 °C. PCR products were gel purified, cloned, and sequenced as described above.

2.6. Sequences analysis and construction of a phylogenetic tree

The cDNA clones were sequenced and the full-length HSP27 sequence was assembled with DNASTAR (DNASTAR Inc., Madison, WI, USA). The sequence homology and the deduced amino acid sequence

Table 1
Sequence and experimental conditions for primers used in the present study.

Primers	Sequences (5′–3′)	Annealing temperature (°C)
HSP27 F1	AGCCGBMTCTTCGAYCAGRC	55
HSP27 R1	CAGRGABGAGGTSAYCTTCT	55
HSP27 F2	ACMCACTGGCCBGGRTAYDT	57
HSP27 R2	AGDGTGTATTTYCTGGTG	51
HSP27 3-F1	CAGATGAGCAGCGGTATGTC	58
HSP27 3-F2	GGAAATCACTGGCAAGCAC	56
HSP27 5-R1	CGTGCTTGCCAGTGATTT	53
HSP27 5-R2	TTGACATCCAGGGACACC	55
HSP27 5-R3	TGACATACCGCTGCTCATCT	58
HSP27 real F	CGTGCTTGCCAGTGATT	63
HSP27 real R	CTTGACCCGCCAGATGA	63
β-actin real F	GCTGTGCTGTCCCTGTATG	63
β-actin real R	CAGAGCGTAACCCTCGTAG	63
AOLP	$GGCCACGCGTCGACTAGTACT_{16}$	
AAP	$GGCCACGCGTCGACTAGTACG_{10}$	
AP	GGCCACGCGTCGACTAGTAC	