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The cloning of a heat shock protein 90β gene and expression analysis in *Botia reevesae* after ammonia-N exposure and *Aeromonas hydrophila* challenge

Chuanjie Qin[a,*], Ting Shao[b], Huiguo Duan[a]

[a] College of Life Science, Neijiang Normal University, Key Laboratory of Sichuan Province for Fishes Conservation and Utilization in the Upper Reaches of the Yangtze River, Neijiang 641100, PR China
[b] College of Life Science, Sichuan Normal University, Chengdu 610101, PR China

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ABSTRACT

The objective of this study was to clone the full-length cDNA of *Heat shock protein 90* (HSP90) in *Botia reevesae* and to determine the effects of pathogenic bacterial challenge after acute sublethal ammonia exposure on HSP90β expression. The HSP90β cDNA of *B. reevesae* contained 2358 bp, including a 1947 bp open reading frame, a 36 bp 5′-untranslated region (UTR), a 375 bp 3′-UTR. Sequence comparisons indicated that the predicted amino acid sequence of *B. reevesae* HSP90 showed a high degree of similarity with other known HSP90β genes, and contained five HSP superfamily signatures, thus suggesting that *B. reevesae* HSP90β is a cytosolic member of the HSP90 family. Quantitative real-time PCR analysis revealed that HSP90 transcripts could be detected in all of the tissues tested, and was strongly expressed in liver, gill and kidney tissues ($p < 0.05$) of *B. reevesae* after sublethal ammonia-N exposure and *Aeromonas hydrophila* challenge. Additionally, following sublethal ammonia-N exposure and *A. hydrophila* challenge, expression of HSP90β mRNA transcripts was increased in gill and kidney tissues by 6–24 h ($p < 0.05$), and was more reduced in the liver than the levels observed in response to ammonia-N exposure or *A. hydrophila* challenge alone. This result indicated that after ammonia-N stress, *B. reevesae* could trigger elevated HSP90β expression in specific tissues to respond to pathogenic bacteria.

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1. Introduction

Heat shock proteins (HSP) are a subset of molecular chaperones, and play key role in the process of protein metabolism under normal and stress conditions, including the refolding of denatured protein, maintenance of structure integrity and other regulatory processes (Hartl, 1996; Feder and Hofmann, 1999). HSP90 is a ubiquitously expressed and highly conserved protein that is widely distributed in all organisms, from bacteria to humans and including aquatic vertebrates (Craig, 1985; Jolly and Morimoto, 1999). The most well-known function of HSP90 concerns the maintenance of key proteins such as steroid receptors and protein kinases by forming specific complexes (Csermely et al., 1998). In addition, HSP90 play an important role in protecting organisms from damage, they are needed even more after stress such as heat shock, heavy metals, or almost any sudden change in the cellular environment which induces protein damage. To date, the full-length *HSP90* cDNA has been isolated and characterized from many species, including the bay scallop *Argopecten irradians* (Gao et al., 2007), the orange-spotted grouper *Epinephelus coioides* (Chen et al., 2010), and the Pacific abalone *Haliotis discus hannai* (Zhang et al., 2011). In these aquaculture animals, HSP90 was stress-inducible by pathogen challenge, hyperthermia treatment, pH, or ammonia-N or heavy metal exposure, and played an important role in the response to deleterious stress conditions (Gao et al., 2007; Chen et al., 2010).

Most ammonia-N in aquatic ponds is derived from the nitrogenous excretory products of teleost bony fish and the decomposition of biological waste, which can easily accumulate in aquatic systems (Randall and Tsui, 2002; Hegazi et al., 2010). In the confines of aquaria and aquaculture systems, ammonium levels can rapidly rise to unsafe levels, and may generate stress conditions. Ammonia reduced the growth rate, induced oxidative stress inside and outside of the brain of the mudskipper *Boleophthalmus boddarti*,

Abbreviations: HSP, heat shock protein; NO, nitric oxide; ORF, open reading frame; PCR, polymerase chain reaction; RACE, Rapid amplification cDNA ends; ROS, reactive oxygen species; RT, reverse transcription; and UTR, untranslated region.

* Corresponding author at: School of Life Science, Neijiang Normal University, Neijiang, Sichuan 641000, PR China.
E-mail address: qinchuanjie@126.com (C. Qin).

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changed the histopathology of the gill epithelia of fish, stimulated Na^+/K^+-ATPase activity and obviously disturbed the ion balance in the goldfish *Carassius auratus* L. and the rainbow trout *Oncorhynchus mykiss* (Sinha et al., 2012a,b; Ching et al., 2009; Wilkie, 1997; Richards et al., 2003). Additionally, ammonia could depress the immune response in *Portunus trituberculatus*, *Litopenaeus stylirosyris*, *Macrobrachium rosenbergii*, *Penaeus japonicas* and *Chlamys farreri*, and increase *HSP90* mRNA expression levels in the ridgetail white prawn *Exopalaemon carinicauda* (Yue et al., 2010; Liu and Chen, 2004; Cheng and Chen, 2002; Jiang et al., 2004; Wang et al., 2012; Li et al., 2012). So the accumulate ammonia may be partly responsible for frequent incidences of disease outbreak by adversely affecting immunity, thus enhancing susceptibility to infection (Pipe and Coles, 1995). However, the effect of pathogen infection after ammonia-N exposure on HSP90 expression in teleosts has received relatively little attention. Therefore, it was essential to investigate how ammonia stress affected HSP90-mediated resistance to pathogens in aquaculture systems.

In the upper Yangtze River and its tributaries, *Botia reevesae* is an economically important fish. Because of the abundant surface mucus, which is far more abundant than that found in rice eel, mud fish or other scaleless fish, *B. reevesae* is known as "*Xuanyu*" in the regions near the upper Yangtze River (Qin et al., 2013a). Even in deteriorating water conditions, pathogen infections in *B. reevesae* breeding areas are rare. Therefore, the aim of this study was to analyze the nucleotide sequence of a HSP90 transcript from the liver of *B. reevesae* and to compare its sequence to HSP90 transcripts from other organisms. Furthermore, the *Aeromonas hydrophila* was common pathogenic bacteria, and led to diseases of fish in deleterious aquaculture systems. So HSP90 mRNA expression induced in response to *A. hydrophila* was evaluated after ammonia exposure. This study could provide insights into the role of HSP90 in resistance to bacterial infection under conditions of ammonia stress.

2. Methods and materials

2.1. RNA isolation from liver, reverse transcription (RT) and cloning of partial HSP90 cDNA

Total RNA was isolated from *B. reevesae* livers and further enriched for RNA with a Unizol kit (Biostar, Shanghai, China) according to the manufacturer's protocol. We synthesized first-strand cDNA using Superscript^{TM} III RNAse H^- reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to transcribe poly$(A)^+$ RNA using oligo-d(T) 18 as primers according to reaction conditions based on the manufacturer's instructions.

To clone *HSP90* cDNA from *B. reevesae*, two degenerate primers were designed based on multiple alignments of known fish HSP90 sequences, including *O. mykiss* (NM_001124231.1), *D. rerio* (NP_571385.2), *Megalobrama amblycephala* (AGI97008.1) and *Bufo gargarizans* (ABD75383.1). Polymerase chain reactions (PCR) were performed using the primers HSP90-F (5′-CGTGGTGTGGTDGACTCTG-3′) and HSP90-R (5′-CCTTCATG AGCTTGCAGAGGTTCTC-3′) and liver cDNA was used as a template for amplification. The PCR reactions were performed as follows: 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 30 s, and elongation at 72 °C for 30 min, and then a 5 min extension at 72 °C and cooling to 4 °C. PCR products were purified and ligated into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA). Ligated vectors were transformed into competent *Escherichia coli* TOP10 cells and grown on LB-agar plates. Transfected clones containing inserts of the expected size were screened by colony PCR and sequenced (Shenggong Corp., Shanghai, China).

2.2. RT-PCR and rapid amplification cDNA ends (RACE)

The full-length HSP90 cDNA of *B. reevesae* was obtained using RT-PCR and RACE methods. The first-strand cDNA synthesized with MMLV reverse transcriptase and the 3′-CDS primer A (5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN-3′) for 3′-RACE and the 5′-CDS primer A (5′-$(T)_{25}$VN-3′) for 5′-RACE (Clontech, USA). Reaction conditions were based on the manufacturer's instructions. For 5′-RACE, the primer set included 5′-RACE HSP90-AS (5′-TTCTCAACAAACAGAGTGATTGGGTAT-3′) and the universal primer A mix (UPM, 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′). For 3′-RACE, the primer set included 3′-RACE HSP90-S (5′-ATGGCAAGACTCTGGTGTCCGTC-3′) and the UPM. The PCR reaction conditions were based on the manufacturer's recommended instructions for the Advantage^{TM} 2 PAC kit (Clontech, USA).

2.3. Cloning, sequencing and sequence analysis

The PCR fragments were subjected to 1.5% agarose gel electrophoresis and cloned amplified cDNA fragments into the pGEM-T easy vector according to the manufacturer's instructions (Promega, USA). Recombinant bacteria were identified by blue/white screening and the screening results were confirmed by PCR. Plasmids containing the insert were purified using minipreps and were used as templates for DNA sequencing. Clones were sequenced using the T7 (forward) and SP6 (reverse) primers.

BLAST (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for nucleotide and protein sequence similarities. The *B. reevesae* HSP90 amino acid sequence was predicted using the Expert Protein Analysis System (http://us.expasy.org). The signal peptide was predicted using the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP), and motifs were identified using the Motif scan program (http://hits.isb-sib.ch/cgi-bin/PFSCAN).

2.4. Analysis of HSP90 mRNA expression in different tissues

Expression of HSP90 mRNA in brain, gill, heart, intestinal, kidney, liver, muscle, skin, spleen and visceral adipose tissue were analyzed by quantitative PCR. All tissues were extensively rinsed with phosphate buffered saline to remove blood. Total RNA was extracted as described above. The amounts of extracted and purified total RNA were quantified, and 5 μg RNA was used for reverse transcription. The first-strand cDNA was synthesized as described above, and the products were diluted 10-fold. Two gene-specific primers (Q-HSP9βs, 5′-CGAACTGTTGCGTTATCA-3′; and Q-HSP9βa, 5′-CAGCACCTCAAATCCTCT-3′) were designed to amplify a 114 bp product of the HSP90 transcript. PCR products were sequenced to verify the specificity of the PCR primers. Two primers (β-actin F, 5′-CCCACACTGTGCCCATCTAT-3′; and β-actin R, 5′-AAGTCCAGACGGAGGATGG-3′) were used to amplify a 61 bp fragment that was used as a reference gene. A SYBR Green RT-PCR assay was conducted to determine *HSP90* mRNA expression levels. Thermocycling and PCR reaction conditions were based on the SYBR *Premix Ex Taq* manufacturer's instructions (TaKaRa, Dalian, China) and reactions were carried out using a LightCycler® Nano Real-Time PCR System (Roche, Basel, Switzerland). The comparative C_T method was used to analyze HSP90 expression levels. The amplification efficiency of β-actin and HSP 90 β was 1.891 (R^2 = 0.9943) and 1.983 (R^2 = 0.989), respectively. The ΔC_T value was calculated for each sample to represent the difference in C_T between the target and internal control, and this value was subtracted from a calibrator C_T value to yield the $\Delta\Delta C_T$. The HSP90 expression level was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

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