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Molecular cloning and differential expression patterns of copper/zinc superoxide dismutase and manganese superoxide dismutase in *Hypophthalmichthys molitrix*

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ABSTRACT

Copper/zinc superoxide dismutase (Cu,Zn-SOD) cDNA and manganese superoxide dismutase (Mn-SOD) cDNA were first cloned from silver carp *Hypophthalmichthys molitrix* using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) method. The open reading frame (ORF) of Cu,Zn-SOD is 465 bp and encodes a 154 amino acids (aa) protein, whereas the ORF of Mn-SOD is 675 bp and encodes a 224 aa protein. Multiple polypeptide sequence alignment showed high identity both of Cu,Zn-SOD (70–87%) and Mn-SOD (80–96%) with the species compared. Both Cu,Zn-SOD and Mn-SOD were detected in heart, brain, liver, kidney, spleen, muscle, gill and blood. Cu,Zn-SOD and Mn-SOD were expressed throughout the embryogenesis, indicating their important roles during embryonic development specially at the cleavage stage. Acute hypoxia suppressed expression of Cu,Zn-SOD and Mn-SOD in liver significantly, up-regulated them in gill relatively, indicating that tissue-specific expression of Cu,Zn-SOD and Mn-SOD is an important stress response adapted to hypoxia.

1. Introduction

Reactive oxygen species (ROS) are important in the initiation and promotion of cells to neoplastic growth which are produced in both unstressed and stressed cells. Generation of ROS is unavoidable consequence of oxygen metabolism in aerobic organisms and an excess of ROS can lead to oxidative, loss of cell function, and ultimately apoptosis or necrosis [1,2]. Organisms have well-developed defense systems against ROS, involving both limiting the formation of ROS as well as instituting its removal [3]. Under unstressed conditions, the formation and removal of ROS are in balance. However, the defense system, when presented with increased ROS formation under stress conditions, can be overwhelmed.

Superoxide dismutase (SOD), catalase and glutathione peroxidase are three main antioxidant enzymes in organisms. Superoxide dismutases (SODs) are considered as the first defense line against oxidative stresses by catalyzing ROS to hydrogen peroxide which is consequently converted to water by catalase [4,5]. Combining with different metals, they are classified into three distinct groups: copper/zinc SOD (Cu,Zn-SOD), manganese SOD (Mn-SOD), and iron SOD (Fe-SOD). These SODs are located in different compartments of the cell. Fe-SOD is located in the chloroplast, Mn-SOD in the mitochondrion and the peroxisome, and Cu,Zn-SOD in the chloroplast, the cytosol, and possibly the extracellular space. The Fe and Mn SODs are similar in their primary, secondary and tertiary structures, and the two enzymes most probably have arisen from the same ancestral enzyme, but are distinct from the Cu,Zn-SOD [6]. As reported, the Cu,Zn-SOD has experienced significant changes in its evolutionary rate. In contrast, the clock for the Mn/Fe-SOD enzyme is ticking quite regularly [7].

Due to chronic changes in the environment, fish are subject to an enhanced oxidative stress and as biochemical indicators of aquatic pollution [8,9]. Environmental pollution may enhance oxidative stress and thus disturb the natural antioxidant enzyme system of fish [10,11]. Previous studies on fish SODs have proved its sensitivity as early warning bioindicator particularly in relation to environment pollution by the biochemical assay, as well as molecular clock and genetic marker for evolutionary studies and population genetics analysis [12–14]. Molecular cloning and functional expression of SODs are the currently hot topic in fishes. Up to now, both Cu,Zn-SOD and Mn-SOD were cloned in *Danio rerio* [15,16], *Hemibarbus mylodon* [17,18], *Takifugu obscurus* [19], *Pagrus*

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major [20]and so forth. Nevertheless, there was no SODs report on silver carp (*Hypophthalmichthys molitrix*) which accounts for an important proportion in Chinese aquaculture. Silver carp is famous also for its role in controlling eutrophication according to the nontraditional biomanipulation theory [21].

In this study, Cu,Zn-SOD and Mn-SOD full-length cDNAs of silver carp were cloned and molecular characterized, the tissue-specific and embryogenetic expression patterns were analyzed by real-time RT-PCR. In addition, acute hypoxia induced expression patterns of the SODs were analyzed. It's also the first time to compare the two major kinds of superoxide dismutase expression during the same progress in fish.

2. Materials and methods

2.1. Experimental fish and embryos

All silver carps used in this study were cultured in Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science, Jingzhou, China. Fish (mean wt. 186.0 ± 24.7 g) were stocked in tanks and allowed to acclimate for 30 days before starting the trail. The tanks were connected to a cycling water system with an aerator to keep dissolved oxygen stable. To study the embryogenetic expression profiles, fertilized embryos and early larvae from different development stages (including zygote, cleavage, morula, blastula, gastrula, neurula, tail bud, somite emergence, muscle contract, hatching, and early larva, each 50 specimen) were collected and stored in liquid nitrogen.

2.2. Exposure and tissue collection

Three acclimated silver carps were randomly loaded into each fiberglass tank (0.5 m × 0.6 m × 0.4 m) with full-filled aerated water (6.10 ± 0.45 mg/L dissolved O_2/L). Eight tanks were used in the trail with one of them as control group. The tanks were hermetic by using the plastic mulch which can totally forbid the oxygen in the air except for the control one. The control temperature was maintained at 27 ± 1.6 °C. Samples were collected every 3 h after-hypoxia (hah) until the emergence of the suffocation. The dissolved oxygen (DO, mg/L) of the tanks were recorded. During sampling, fish were anaesthetized with a 0.05% solution of MS-222 (Sigma). The tissue samples (including heart, brain, liver, kidney, spleen, muscle, gill, blood) were dissected from the fish, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. The experimental procedures were performed on the standards of the China Council on Animal Care.

2.3. Full-length cDNA cloning of Cu,Zn-SOD and Mn-SOD

Full-length cDNAs of Cu,Zn-SOD and Mn-SOD were obtained by the procedures of reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) method. First, total RNA was isolated from silver carp liver using Trizol agent (Invitrogen, Carlsbad, CA, USA) referring to the manufacturer's protocol. The quality of RNA was measured at A260 nm and the purity from the ratio A260 and A280 nm (Eppendorf Biometer, Hanburg, Germany). Furthermore, the total RNA samples were treated with Dnase I (TaKaRa, Otsu, Japan) to ensure the elimination of genomic DNA contamination. Reverse transcription of the total RNAs was then carried out with RNA PCR Kit (AMV) Ver3.0 (TaKaRa, Otsu, Japan). Degenerate primers (CZS1-F and CZS1-R, MS1-F and MS1-R) based on the conserved regions of the two genes from sorts of fishes were designed and shown in Table 1. The PCR were performed by denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and elongation at 72 °C for 1.5 min and an additional elongation at 72 °C for 8 min after the last cycle. The products of the PCR were visualized by electrophoresis of ethidium bromide stained agarose gel. Target DNA fragments were excised from agarose, purified with the AxyPrep DNA gel extraction kit (Axygen, CA, USA), cloned into pMD18-T vector (TaKaRa, Otsu, Japan), and sequenced.

Table 1
Degenerate and specific primers used in the experiment.

Name	Sequence (5′–3′)
CZS1-f	GACCAACCGATAGTGAAAGACAC
CZS1-R	AGAAAGCAGAAATCAGAGGAAAT
CZS-3	GGAGGATGACTTGGGGAAGGGAAAC
CZS-5	AGAGCAGGACACTACTGAGCGATG
CZS2-F	CCAACCGATAGTGAAAGACACGT
CZS2-R	CTCATTGCCTCCCTTCCCCAAGT
MS1-F	GAGATTATGCAGCTTCATCACA
MS1-R	AGTACAAAAAAGACACTCGGTT
MS-3	GCATTGAAATTTAACGGTGGTGG
MS-5	GCAAAGGGTCTTGGTTAGCGCAA
MS2-F	TGTGACGACCCAAGTCTCCCTT
MS2-R	CTGTGGCTCTCCTCCACCATTC
β-actin-F	TGTGACGACCCAAGTCTCCCTT
β-actin-R	CTGTGGCTCTCCTCCACCATTC

Based on the sequences of conversed region we got in silver carp, 5′-and 3′-RACE PCR were performed to define the putative 5′-and 3′- ends of Cu,Zn-SOD and Mn-SOD, respectively. The gene-specific-primers (CZS-3 and CZS-5, MS-3 and MS-5) were designed according to the conserved region sequences we obtained above (Table 1). RACE and RACE-PCR were conducted with the SMART RACE cDNA Amplification Kit and Advantage 2 PCR Kit (Clontech, CA, USA). Touchdown PCR was adopted to improve the specificity of SMARTer RACE amplification. The conditions for the PCR were 15 cycles of (94 °C 1 min; 70 °C 1 min, −1 °C each cycle; 72 °C 2 min), followed by 20 cycles (94 °C 1 min; 55 °C 1 min; 72 °C 2 min) and 72 °C for 10 min after the last cycle. The products of the PCR were visualized by electrophoresis of ethidium bromide stained agarose gel, Target DNA fragments were excised from agarose, purified with the AxyPrep DNA gel extraction kit, cloned into pMD18-T vector, and sequenced.

2.4. Sequence analysis

The identity and similarity of the two silver carp SODs were compared with other species by using the online BLAST program from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein analyses were performed using ExPASy online tools (http://us.expasy.org/tools). The phylogenetic tree was constructed by aligning protein sequences with the ClustalW methord. Bootstrap analyzed with 1000 replicates using Mega 4.0. The following sequences (acronym, GenBank accession number) were included in the analysis: *Homo sapiens* (*Hs*Cu,Zn-SOD, NP000445; *Hs*Mn-SOD, CAA42066), *Mus musculus* (*Mm*Cu,Zn-SOD, NP035564; *Mm*Mn-SOD, NP038699), *D. rerio* (*Dr*Cu,Zn-SOD, NP571369; *Dr*Mn-SOD, NP956270), *H. mylodon* (*Hm*Cu,Zn-SOD, ACR56338; *Hm*Mn-SOD, ACR23311), *T. obscurus* (*To*Cu,Zn-SOD, ABV24054; *To*Mn-SOD, ABV24053), *Epinephelus coioides* (*Ec*Cu,Zn-SOD, AAW29025; *Ec*Mn-SOD, AAW29024), *Rachycentron canadum* (*Rc*Cu,Zn-SOD, ABI96913; *Rc*Mn-SOD, ABC71306).

2.5. Tissue and embryogenetic expression analysis

Cu,Zn-SOD and Mn-SOD mRNA contents in tissues were detected on the samples from the control group to illustrate the spatial expression patterns of Cu,Zn-SOD and Mn-SOD in silver carp. Heart, brain, liver, kidney, spleen, muscle, gill and blood were dissected, blotted and weighed. Total RNA was extracted as described.