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Identification and mRNA expression of antioxidant enzyme genes associated with the oxidative stress response in the Wuchang bream (*Megalobrama amblycephala* Yih) in response to acute nitrite exposure

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ABSTRACT

Aquatic organisms possess cellular detoxification systems to deal with pollutants. To explore the influence of reactive oxygen species (ROS) generated in response to nitrite on oxidative stress defenses and the antioxidant system in *Megalobrama amblycephala*, the full length cDNA sequences were determined for three antioxidant-related genes, namely catalase (*MaCAT*), selenium-dependent glutathione peroxidase (*MaGPx1*) and Cu/Zn superoxide dismutase (*MaCu/Zn-SOD*). Encoded polypeptides that exhibited high identity and similarity with corresponding proteins in other fish species. Expression levels of these antioxidant genes were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) technique. *MaCAT*, *MaGPx1* and *MaCu/Zn-SOD* expression was greatest in the liver and qRT-PCR was used to assess expression of these genes in juvenile fish during 72 h of exposure to 15 mg/L nitrite. Prolonged nitrite exposure resulted in the formation of excess ROS that caused oxidative damage to lipids and proteins and reduced the activities of antioxidant enzymes. Fish exposed to nitrite also showed liver damage. This study provides transcriptional data for *MaCAT*, *MaGPx1* and *MaCu/Zn-SOD* that suggest expression is related positively with oxidative stress induced by nitrite exposure, indicating that imbalance between ROS and antioxidant defenses is one mechanism underlying nitrite toxicity in *M. amblycephala*.

1. Introduction

In aquaculture, fish often encounter high temperatures, crowded conditions, water quality deterioration and threats posed by pathogenic bacteria and viruses. These adverse environmental factors can disturb the balanced harmony between the fish and its environment. Thus, fish can be more or less susceptible to pollutants that directly or indirectly promote the intracellular generation of reactive oxygen species (ROS) and/or the alteration of antioxidant defenses, which causes oxidative stress and leads to potential oxidative damage to cellular macromolecules (Livingstone, 2001; Monserrat et al., 2007). Considering that all primary interactions of chemicals with organisms take place at the molecular level, it has been reasoned that understanding ecotoxicology at the molecular level is critical to gaining mechanistic knowledge and predictive power required to face contemporary challenges.

Fish possess systems for generating as well as protecting against the adverse effects of free radicals (Kelly et al., 1998). These defenses consist of antioxidant molecules like glutathione or antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and these are required to eliminate excessive ROS and protect the host (Almar et al., 1998; Mates, 2000; Muradian et al., 2002; Chelikani et al., 2004). *Megalobrama amblycephala* is considered to be a principal species in Chinese freshwater polyculture systems and total annual production is enormous (Shen et al., 2010). Indeed, *M. amblycephala* is currently the most widely cultivated freshwater fish in China. As a major species frequently cultured in Chinese freshwater fish polyculture system, *M. amblycephala* protospecies are likely to encounter environmental stresses caused by overstocking and water pollution (Li et al., 1993). Studies have revealed that fish in polyculture system were often at high risk of excessive nitrite concentrations, which can cause a variety of physiological disturbances (Jensen, 1995). Elevated nitrite levels during unbalanced nitrification can seriously damage fish health and may lead to mass mortalities (Svobodova et al., 2005). Previous studies suggest that intracellular ROS production is a possible mechanism underlying nitrite toxicity (Jensen, 2003; Wang et al., 2004, 2006; Xian et al., 2012). When the production of ROS exceeds the ability of the antioxidant system to counter these molecules, lipid peroxidation (LPO) and protein oxidation can be initiated, and most cellular components are prone to oxidative damage (Sies, 1991). Taken together, all

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these changes can ultimately lead to cellular and tissue injury and dysfunction (Marks et al., 1996). So far, there are no reports of the response mechanisms of *M. amblycephala* to nitrite stress in terms of antioxidant enzyme gene expression changes that occur during the resultant oxidative stress.

The aim of this present study was to clone and sequence the gene products in juvenile *M. amblycephala* that encode CAT, GPx1 and Cu/Zn-SOD, and to investigate the effects of nitrite exposure on the expression levels of these genes and their enzymatic activities in the liver. In addition, oxidative stress markers and histological changes were investigated to identify the extent of the oxidative stress caused by acute nitrite toxicity.

2. Materials and methods

2.1. Sample collection

Megalobrama amblycephala juveniles (average mass 13.56 ± 2.25 g) were obtained from the Yixing fish farm at the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Prior to the experiment, fish were fed with a commercial diet for two weeks before processing. Adult fish were anesthetized in water containing 100 mg/L of MS-222 (Sigma). Six tissues (kidney, spleen, liver, intestine, gill and muscle) from nine adult fish were removed and kept at −80 °C. In the nitrite stress tests, Wuchang breams were randomly divided into two nitrite treatment groups performed in triplicate: one at 0.02 mg nitrite/L (control) and one at 15 mg nitrite/L (55% 96-h LC_{50}); the latter concentration was prepared by adding a stock solution of sodium nitrite according to preliminary studies. Fish in aerated running water served as controls (0.02 mg nitrite/L). Water temperature was kept at 25 ±0.5 °C using aquarium heaters while pH was maintained at 7.8 ± 0.1. Levels of nitrite were determined every day by a spectrophotometric method described by Bendschneider and Robinson (1952). Six 400-L tanks were required to rear the fish for each nitrite concentration (n = 30 per tank). For each tank, the livers from three individuals were sampled at 0, 3, 6, 12, 24, 48 and 72 h after nitrite exposure, respectively, and then kept at −80 °C.

2.2. Molecular cloning

Total RNA was extracted from liver samples using TRIzol reagent (TaKaRa) according to the manufacturer's protocol. A 530-bp fragment of the *MaCAT* gene, an 871-bp fragment of *MaGPx1* and a 478-bp fragment of *MaCu/Zn-SOD* were obtained from a *de novo* transcriptomic library (Gao et al., 2012) of *M. amblycephala*, and these were used to clone full-length cDNA sequences of *MaGPx1*, *MaCAT* and *MaCu/Zn-SOD* by rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) using the RNA PCR Kit (AMV) ver. 3.0 (TaKaRa, Japan) according to the manufacturer's manual. The 5′- and 3′-end sequences were obtained using the 5′-/3′-Full RACE kit (TaKaRa). The gene-specific primers were designed based on the partial sequences obtained above (Table 1). The 5′-RACE and 3′-RACE PCR products were ligated and cloned into the pMD-18 T vector (Takara, Japan). After transforming into competent *Escherichia coli* DH5a cells, recombinant bacteria were identified by blue/white screening and then confirmed by PCR. Six positive clones were picked and sequenced on an ABI PRISM 3730 Automated Sequencer using BigDye terminator ver. 3.1 (Applied Biosystems, USA). Finally, the RT-PCR and 5′- and-3′-RACE products were assembled to form a full-length cDNA sequence containing the entire open reading frame using the SeqMan software.

2.3. Sequence analysis

Homology searches of nucleotide and protein sequences were conducted using the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The protein prediction was performed using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/), while motif identification was performed with the Motifs Scan program (http://hits.isb-sib.ch/cgi-bin/motif scan/). The deduced amino acid sequences of *MaCAT*, *MaGPx1* and *MaCu/Zn-SOD* and other vertebrates in the NCBI GenBank were analyzed using the Clustal W Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). A neighbor-joining phylogenetic tree was constructed using MEGA software version 3.1.

Table 1
Sequences of the primers used in this study.

Primer	Sequence (5′-3′)
CAT-F1 (3′RACE out primer)	TGGGTGGAGACAAATGAAGATCAC
CAT-F2 (3′RACE inner primer)	GATCAGATGAAACTGTGGAAGCAG
CAT-R1 (5′RACE out primer)	TCATCAGTAAACACCACGTCCTGG
CAT-R2 (5′RACE inner primer)	TGCTTCCACAGTTTCATCTGATCC
GPx-F1 (3′RACE out primer)	TAAGGTGGTCCTGATCGAGAATG
GPx-F2 (3′RACE inner primer)	GCTTCTCAGATAAAGGGCTTGTG
GPx-R1 (5′RACE out primer)	GGAAGTTTCTCCTTGAGGAAGACG
GPx-R2 (5′RACE inner primer)	TAGGTTCAAAGCCATTGCCTGGAC
Cu/Zn-SOD-F1 (3′RACE out primer)	AGATTCGTAAATCCCAGGACTCC
Cu/Zn-SOD-F2 (3′RACE in primer)	TCTGTTGTCATTCATGAGGGAGAG
Cu/Zn-SOD-R1 (3′RACE out primer)	TTAATTTTGGGTGTTGCCCAGTC
Cu/Zn-SOD-R2 (3′RACE in primer)	ATTCAGCAAGCTTTCCACGTTTC
CAT- RT-F (Real-time primer)	GTTTCCGTCCTTCATCCACTCT
CAT- RT-R (Real-time primer)	GACCAGTTTGAAAGTGTGCGAT
GPx- RT-F (Real-time primer)	CTTTTGTCCTTGAAGTATGTCC
GPx- RT-R (Real-time primer)	CTTGAGGAAGACGAAGAGAGGG
Cu/Zn-SOD-F (Real-time primer)	AGTTGCCATGTGCACTTTTCT
Cu/Zn-SOD-R (Real-time primer)	AGGTGCTAGTCGAGTGTTAGG
β-Actin F (Real-time primer)	TCGTCCACCGCAAATGCTTCTA
β-Actin R (Real-time primer)	CCGTCACCTTCACCGTTCCAGT

2.4. Tissue-specific distribution of MaCAT, MaGPx1and MaCu/Zn-SOD

The distribution of *MaCAT*, *MaGPx1* and *MaCu/Zn-SOD* mRNA was detected in several tissues (kidney, spleen, liver, intestine, gill and muscle) by quantitative real-time PCR (qRT-PCR). The qRT-PCR primers are listed in Table 1. The specificity of each primer pair was verified via the detection of a single amplicon of the expected size after normal PCR amplification and the presence of just one peak in the melt curves of the qRT-PCR reactions. Amplification of *β-actin* was used as the qRT-PCR internal control (Ming et al., 2010). A SYBR Green qRT-PCR assay was conducted to determine gene mRNA expression. The PCR temperature profile and reaction conditions were specified by the manufacturer of the SYBR Premix Ex Taq (TaKaRa, Dalian, China) and reactions were performed on an ABI step of the qRT-PCR system (Applied Biosystems, USA). The ΔC_T was the difference in C_T values between the target tissue and the internal control for each sample and it was subtracted from the calibrator C_T to yield $\Delta\Delta C_T$. The expression levels of the antioxidant genes were calculated using the equation: $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). The tissue distribution analysis was carried out on tissues from nine fish (n = 9).

2.5. Gene expression profiles of MaGPx1, MaCAT and MaCu/Zn-SOD following nitrite exposure

The mRNA expression levels of *MaGPx1*, *MaCAT* and *MaCu/Zn-SOD* from *M. amblycephala* were measured at 0, 3, 6, 12, 24, 48 and 72 h by qRT-PCR in an unexposed control group and a group exposed to 15 mg nitrite/L. The gene-specific primers, *β*-actin primers and PCR reaction conditions were exactly as described above. Each transcript was analyzed in nine individuals in each group.

2.6. Indices of oxidative stress

The expression of specific lesions known to arise specifically during oxidative stress, e.g. lipid peroxidation (LPO), carbonyl protein (CP) and oxidized bases in the DNA, are found in many aquatic animals exposed