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Cloning, characterization and cadmium inducibility of metallothionein in the testes of the mudskipper *Boleophthalmus pectinirostris*

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ABSTRACT

Metallothioneins (MTs) are cysteine-rich, low molecular weight, and heavy metal-binding protein molecules. MT participates in metallic homeostasis and detoxification in living animals due to its abundant cysteine. In order to investigate the functions of MT during spermiogenesis in the mudskipper (*Boleophthalmus pectinirostris*), we identified the MT complete which contains: an 83 bp 5′ untranslated region, a 110 bp 3′ untranslated region, and a 183 bp open reading frame. The protein alignment between MT sequences of other species shows a high similarity and a strong identity in cysteine residues vital for the metal-binding affinity of MT. The localizations of MT were mainly in the cytoplasm of germinal cells, indicating a role in spermatogenesis and testis protection. After the cadmium (Cd) exposure, the testis presents abnormal morphology and MT mRNA expression, both of which indicate a sensitive response of testis MT to Cd. Therefore, we suggest that MTs play an important role in spermatogenesis and testes protection against Cd toxicity in *B. pectinirostris*.

1. Introduction

The increase of industrial and agricultural activities has resulted in aggravating heavy metal discharge in many water bodies. Metal contamination has received much attention in the last decades, since these pollutants offer serious risks due to their toxicity, persistence and bioaccumulation in the aquatic food chain (Yılmaz et al., 2007). Cadmium (Cd), a widespread non-essential metal, enters aquatic ecosystem mainly through industrial processes and a wide application of phosphate fertilizers (Mishra et al., 2006). The integrity of the testis is necessary for normal male reproductive ability. In addition, the testis is the most sensitive organ to cadmium (Cd) toxicity. Cd could induce non-reversible tissue necrosis at relatively low concentrations (Alsberg and Schwartze, 1919) and reduces luteinizing hormone receptor mRNA and cAMP levels in rat testis (Gunnarsson et al., 2003). Furthermore, Cd has been widely acknowledged as one of the most toxic metals in nature and exerts a persistent threat to the aquatic environment. After Cd exposure, the testis showed a damaged and loose appearance due to the separation of germinal cells from non-germinal cells in *Charybdis japonica* (Mao et al., 2012). The freshwater crab *Sinopotamon henanense* studies indicated that after treatment with 116 mg/L Cd for 7 days, the sperm membrane was almost dissolved, the chromatin in the nucleus was more heavily condensed, chromatin irregularities were observed. What's more, serious damage appeared in the acrosome (Ma et al., 2013).

The metallothioneins (MTs) are a group of low molecular weight stress proteins with potent metal-binding abilities due to their rich cysteine content, which are widely distributed in plants, animals, fungi and some prokaryotes (Zhou et al., 2012). Generally, MTs have three key functions: metal ion homeostasis, detoxification and cytoprotection (Kaegi and Schaeffer, 1988; Schroeder and Cousins, 1990; Suzuki, 1993). During Cd exposure in aquatic crustaceans, MTs bind Cd so that Cd could be stored as a non-toxic CdMT complex (Pedersen et al., 1994; Webb, 1986). MTs are not only cytoplasmic protein, it could also accumulate in lysosomes, yet could be transported to the nucleus and to the intermembrane space of mitochondria (Ye et al., 2001). In addition, MTs are released from cells into the extracellular space and taken-up by other cells through a receptor-mediated mechanism. Through this process, MTs transfer heavy metals from the extracellular space to the cytoplasm (Hao et al., 2007; Moltedo et al., 2000; Wolff et al., 2006). MTs could be induced by metal pollution in many taxa, including mammals, fish, and aquatic invertebrates (Amiard et al., 2006; Bebianno and Serafim, 1998). Besides, MTs could be also induced by other agents such as hormones, pharmaceuticals,

Abbreviations: Cd, cadmium; MTs, metallothioneins; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcription polymerase chain reaction; SD, standard deviation; UTRs, untranslated regions

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alcohols, cytokines, alkylating agents, irradiation, infection, reactive oxygen species (ROS) and other diverse chemical and physical treatments (Moltó et al., 2005; Pedersen et al., 1996). In the past study, metallothionein could be considered as a biomarker to detect cadmium pollution (Huang et al., 2014; Lin et al., 2004; Mao et al., 2012; Ren et al., 2011; Wang et al., 2014; Xiang et al., 2013). In this study we found that the expression of MT quantity is positively correlated with Cd concentration and time. Therefore, we suggest that MT is an excellent biomarker candidate for evaluating Cd pollution.

Boleophthalmus pectinirostris is regarded as a good model organism which is a commercially important aquaculture species living along coastal area in China, and is unavoidably exposed to the inshore sediment toxicity. Therefore, this specie may serve as a bio-indicator of aquatic heavy metals pollution. The mudskipper *B. pectinirostris*, a burrow-dwelling fish inhabiting intertidal mudflats, is an exceptional model among fish because of their amphibious behavior as well as numerous physiological and morphological specializations adapted to amphibious life (Chen et al., 2007; Hong et al., 2007).

In this study, we used *B. pectinirostris* as a model animal to explore the functions of detoxification through specific detoxification and regulatory mechanisms. We cloned the gene metallothionein with degenerate primers and RACE, analyzed its sequence, compared MT with its homologs from other species, and constructed a phylogenetic tree. In addition, we employed RT-PCR, in immunohistochemistry, histochemistry analysis, and real-time quantitative PCR to analyze MT expression and its response to the effects of Cd.

2. Material and methods

2.1. Experimental animals

Research work was carried out in strict accordance with the requirement by 'Governing Regulation for the Use of Experimental Animals in Zhejiang Province' (Government Order no. 263, released in August 17, 2009, effective from October 1, 2010). The Institutional Animal Care and Use Committee at the Zhejiang Laboratory Animal Research Center and Ningbo University approved this study.

We purchased mature *B. pectinirostris* 20 ± 5 (g) from Ningbo Aquatic Products Market (Ningbo, China). The liver, testes, muscle, kidney, spleen and gill were dissected from the mature mudskipper, quickly dropped into liquid nitrogen, and subsequently stored at -80 °C.

The aquarium size was 35 cm × 45 cm × 15 cm. We changed the natural seawater daily and kept the fish at a constant aeration. After a recovery period of 2 weeks, fish were divided into four groups, each had 20. Eighty fish were exposed to a series of Cd concentration gradients (the final Cd concentrations were 0, 0.005, 0.1, 2 mg/L, prepared with $CdCl_2 \cdot 2.5H_2O$) for 24 h, 48 h, 72 h and 96 h, respectively. Stock solution of the Cd^{2+} with a concentration of 2 mg/L was prepared by dissolving $CdCl_2 \cdot 2.5H_2O$ in distilled water. Appropriate volumes of stock solution were added to 10 L city water to get a series of expected Cd^{2+} solution. Twenty fish which were not exposed to Cd served as the control group.

2.2. Cloning of metallothionein from Boleophthalmus pectinirostris

Total RNA was extracted with Trizol reagent from the testis. Reverse transcriptions were executed using an oligo(dT) primer according to the manual book of BioRT cDNA First Strand Synthesis Kit (Bioer). A pair of degenerate primers (forward: 5′-AGTGCKCYAAGACTGGAACCTGC-3′, reverse: 5′-GCTWGTGTCR CAVGTCTT CC-3′) was used for cloning MT cDNA (Leignel et al., 2008). PCR was conducted as follows: 5 min at 94 °C, 35 cycles running (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) with 10 min at 72 °C for the final extension. Ultimately, the products were detached by agarose electrophoresis, DNA gel green was used for showing the bands, from which the expected products were subsequently obtained and purified using AxyPrep DNA Gel Extraction Kit (Axygen) and AxyPrep PCR Cleanup Kit (Axygen). The purified fragments were then cloned into pMD18-T vectors (Takara), propagated in *Escherichia coli* DH5α (Takara) and sequenced by Biosune Company, Shanghai, China.

To get the full sequence of MT cDNA, RACE were conducted in order to get the 5′ and 3′ cDNA ends, using Smart RACE cDNA Amplification Kit (CloneTech) and 3′ Full RACE Amplification Kit (Takara). The primers used for the RACE contained several gene specific primers (5-MT-R: 5′-GTGTCACAAGTCTTCCCTTTGC-3′ and 3-MT-F: 5′-TTGTTGTCCATCTGGGTGC-3′) designed according to the obtained MT cDNA fragment and several primers provided in the RACE Kit. The PCR program was properly modified, based on the instructions of the kit. The PCR program of 5′ RACE was run as follows: 94 °C for 5 min; 94 °C 30 s, 59 °C 30 s, 72 °C 30 s, for 35 cycles; then 10 min at 72 °C for the ending extension. The PCR program of 3′ RACE was run as follows: 94 °C for 5 min, 35 cycles was running (94 °C 30 s, 56 °C 30 s, 72 °C 30 s); then 10 min at 72 °C for the last extension. Extracting, purifying and sequencing of the expected fragments were performed as mentioned above.

2.3. Sequence analysis

Sequences similarity analyses of MT were performed using the Blast program at the National Center for Biotechnology Information. The open reading frame (ORF) of the MT was determined using the Primer Premier 5 and translated into amino acid sequence. The multiple sequence alignments of *B. pectinirostris* MT and other fish species were produced by Vector NTI 10 software. Their and their Genebank accession numbers were as follows: *Lithognathus mormyrus* MT (AAL37187.1), *Sparus aurata* MT (AAC32738.1), *Perca fluviatilis* MT (CAA65927.1), *Gobiomorphus cotidianus* MT (AAO89258.1), *Pagras major* MT (BAA92364.1), *Ictalurus punctatus* MT (AAC36348.1), *Misgurnus anguillicaudatus* MT (ADY68769.1), *Danio rerio* MT (AAS00513.1), *Cyprinus carpio* MT-II (AAF64232.1), *Paralichthys olivaceus* MT (ABN50351.1), *Oncorhynchus mykiss* MT (AAA49566.1), *Pelteobagrus fulvidraco* MT (ABW88898.1) and *B. pectinirostris* MT (KJ786447).

2.4. RT-PCR analysis of MT mRNA expression in different tissues

Total RNA from testis, liver, gill, spleen, kidney and muscle was extracted with Trizol reagent. Reverse transcription was performed with BioRT cDNA First Strand Synthesis Kit (Bioer). Two primers (MT-real-F: 5′-CAAAGACTGGAAACTGCACC TG-3′; MT-real-R: 5′-AGCCAGTGTCACAAGTATTCCCT-3′) were used for analyzing MT mRNA expression in different tissues. PCR was run as follows: 94 °C for 5 min; 30 cycles performed (30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C) with 10 min at 72 °C for the ending extension. Two primers (β-actinF: 5′-TCCACGAAACCACCTACAACAG-3′, β-actinR: 5′-CAGAGTATTTACGCTCA GGTGGG-3′) (*B. pectinirostris* beita-actin GeneBank accession: KC622028) were used to obtain a β-actin cDNA fragment as control. The PCR was performed as follows: 94 °C for 5 min; 94 °C 30 s, 60 °C 30 s and 72 °C 30 s, for 30 cycles; with 10 min at 72 °C for the ending extension. All PCR products were affirmed via electrophoresis on 1% agarose gel, DNA gel green was added for the visualization of the bands.