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Molecular cloning, characterization, expression and enzyme activity of catalase from planarian *Dugesia japonica* in response to environmental pollutants

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ABSTRACT

Catalase (CAT) is an important antioxidant enzyme that protects aerobic organisms against oxidative damage by degrading hydrogen peroxide to oxygen and water. *CAT* mRNAs have been cloned from many species and employed as useful biomarkers of oxidative stress. In the present study, we cloned the cDNA sequence of *CAT* gene from freshwater planarian *Dugesia japonica* (designated as *DjCAT*) by means of RACE method. Sequence analysis and multiple alignment jointly showed that the full-length cDNA sequence consists of 1734 nucleotides, encoding 506 amino acids. Three catalytic amino acid residues of His71, Asn144 and Tyr354, two CAT family signature sequences of a proximal active site signature (^{66}FDRERIPERVVHAKGGGA^{77}) and a heme-ligand signature motif (^{350}RLFSYRDTQ^{358}) are highly conserved, suggesting that the DjCAT belongs to the NADPH and heme-binding CAT family and has similar functions. In addition, the transcriptional level of *CAT* gene and activity of CAT enzyme upon acute exposure of environmental pollutants glyphosate and 1-decyl-3-methylimidazolium bromide ([C_{10}mim]Br) were investigated systematically. The variation of *CAT* mRNA expression in *D. japonica* was quantified by real-time PCR and the results indicated that it was up-regulated after exposure to glyphosate or [C_{10}mim]Br with a dose-dependent manner but not linearly. Even though the variation trend of CAT activity upon glyphosate stress was not monotonously increased and inconsistent with that after [C_{10}mim]Br exposure on day 1 and 3 sampling time, with the duration prolonged to day 5 they both presented a dose-dependent increase and the differences achieved extreme significance in all treated groups compared to the control. These findings suggested that DjCAT plays an important role in antioxidant defense in *D. japonica*, and the mRNA expression of *CAT* would also be used as an effective biomarker to monitor the pollution in aquatic environment just like its corresponding enzyme.

1. Introduction

Environmental pollutants released from anthropogenic resources enter aquatic animals via food-uptake, epidermis etc., and adversely affect development, reproduction, and health of them at molecular, individual, and eventually population levels. It is well-studied that environmental pollutants including industrial chemicals, pesticides and other xenobiotics can induce oxidative stress by generating reactive oxygen species (ROS) (Dazy et al., 2009; Zhang et al., 2016a; Wu et al., 2017). Under normal conditions, a certain amount of ROS, such as superoxide anions ($O_2^{\cdot-}$), hydrogen peroxides (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) are formed in aerobic organisms as by-products of normal metabolic process and a balance is maintained between the production and clearance of ROS (Nordberg and Arner, 2001; Ken et al., 2003). However, overproduction of ROS can cause harmful damages to cellular macromolecules such as proteins, lipids and DNA, and subsequently lead to the cytotoxic effects and functional disorders in organism. Fortunately, to overcome oxidative stress aerobic organisms have evolved complex enzymatic and non-enzymatic system as the antioxidant defense mechanism (Zhang et al., 2016b). The enzyme system is mainly composed of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). SOD catalyzes the dismutation of superoxide into molecular oxygen and H_2O_2, while H_2O_2 can be further quenched by GPX and CAT.

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CAT is a key antioxidant enzyme that exists in all aerobic organisms from bacteria to mammals (Klotz and Loewen, 2003). It is a hemoprotein formed by four identical subunits of approximately 50–60 kDa (Garcia et al., 2000). By decomposing H_2O_2, one main type of ROS being responsible for the oxidative damage and disease processes, CAT is involved in various biological processes including inflammation, mutagenesis, prevention of apoptosis, and stimulation of a wide spectrum of tumors (Bandyopadhyay et al., 1999). Besides, CAT is also essential in the innate host defense against pathogen infection and protective responses of animals to environmental stress (Zhang et al., 2008, 2011; Li et al., 2013; Xia et al., 2016; Xu et al., 2017; Yu et al., 2017). Considering its importance, the *CAT* homologs have been cloned in fish *Onychostoma macrolepis* (Yu et al., 2017) and *Carassius auratus* var. Pengze (Li et al., 2013), arthropod *Daphnia magna* (Kim et al., 2010), *Locusta migratoria* (Zhang et al., 2016b) and *Chilo suppressalis* (Lu et al., 2017; Xu et al., 2017), mollusk *Chlamys farreri* (Li et al., 2008), *Crassostrea hongkongensis* (Zhang et al., 2011) and *Anodonta woodiana* (Xia et al., 2016) and annelid *Eisenia fetida* (Xiong et al., 2013), and their dynamic expressions of mRNA and/or enzymatic activities in response to pathogens or environmental contaminants were explored. However, to the best of our knowledge the full-length cDNA sequence of *CAT* gene has not been cloned and functionally studied in lower invertebrates, such as the planarian flatworm.

Planarians are one of the most abundant predators in streams and upper reaches of rivers and play a key role in freshwater ecosystems. As the sentinel species, freshwater planarians are usually considered as ideal early warning indicators of aquatic ecosystem deterioration because of their cosmopolitan distribution and high sensitivity to environmental toxicants. More and more reports have confirmed that planarians is a favorable organism for assessing toxic effects of pollutants (Horvat et al., 2005; Kovačević et al., 2009; Alonso and Camargo, 2015; Zhang et al., 2016c; Hagstrom et al., 2017). *D. japonica* is a common freshwater species in East Asia and widely distributes in China. Considering its powerful regenerative capacity and highly chemical sensitivity, *D. japonica* is used as a model organism in the fields of regenerative medicine, stem cell biology, neurological disease and toxicology (Yuan et al., 2016). In our previous study, it was found that the ionic liquid (IL) 1-octyl-3-methylimidazolium bromide ([C_8mim]Br) induced the production of ROS in *D. japonica*, and CAT together with other antioxidant enzymes played critical roles in oxidative defense (Zhang et al., 2016a). However, the mRNA expression of *CAT* gene in *D. japonica* in response to pollutants stress has still been unclear. Therefore, the aims of the present study were to: (1) clone and characterize *CAT* gene from *D. japonica*, and (2) analyze the expression profiles at the mRNA level and enzyme activities respectively after exposure to different environmental pollutants.

2. Materials and methods

2.1. Planarian culture and maintenance

D. japonica was collected from Yu-quan stream in Qi County, China and maintained in autoclaved tap water without any addition at laboratory. Animals were fed with raw fish spleen once a week, and culture medium was renewed weekly after feeding. Planarians fasting for at least one week were used in the experiments.

2.2. Full-length cDNA cloning of DjCAT

Total RNA was extracted from five planarians using Trizol reagent (TaKaRa, China) and the first-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA using oligo(dT) primers and M-MLV reverse transcriptase (TaKaRa, China) based on the manufacturer's protocol.

A 418 bp EST fragment for *CAT* was screened out from our *D. japonica* transcriptome database. According to the EST sequence, the 5′-gene specific primers and 3′-gene specific primers were designed by Primer Premier 5.0 (Table 1). The corresponding full-length transcripts were amplified by rapid amplification of cDNA ends (RACE) using SMART 5′ and 3′ RACE kits (Clontech, USA) according to the manufacturer's instructions. The PCR products were purified and cloned into pMD-19T vector, which were then transferred into the *Escherichia coli* DH5α, and positive clones were submitted for sequencing. The full-length of *CAT* gene was assembled with the SeqMan software in DNAStar 5.0.

Table 1
Primers used in this study.

Gene	Primer	Sequence (5′→ 3′)	Use
DjCAT	3GSP1	TTC CTT CCT GGA CAT TCT GTA TTC	3′-RACE
	3GSP2	TTG ATG TGA CTA AAG TTT GGC CAC A	
	5GSP1	TCC ATC AGG GAT ACC ACG ATC AGC	5′-RACE
	5GSP2	GCA AAT TGG TGA GAT GTT TCA GGT C	
	CAT-F	AAC TTT CCT TCC TGG ACA TTC TG	qRT-PCR
	CAT-R	CAT TCT ATC TGG ACT AGC CTC AA	
Djef2	RT-F	TTA ATG ATG GGA AGA TAT GTT G	qRT-PCR
	RT-R	GTA CCA TAG GAT CTG ATT TTG C	

2.3. Bioinformatic analysis

The potential open reading frame (ORF) was identified with the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The physicochemical properties were analyzed by Protparam (http://web.expasy.org/protparam/). The signal peptide was predicted by the SignalP-4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Subcellular localization was predicted via PSORT II (http://psort.hgc.jp/form2.html), and potential glycosylation sites was forecasted by NetNGlyc 1.0 Server (http:// www.cbs.dtu.dk/services/NetNGlyc/). The three-dimensional (3D) structure prediction was performed by SWISS-MODEL (https://swissmodel.expasy.org/).

The similarity analysis of nucleotide and amino acid sequences were carried out by using BLASTn and BLASTp at web servers of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Protein sequence conservation analysis was performed via DNAMAN6.0, and the pairwise sequence identities of representative organisms (Table 2) were calculated using the MegAlign program in DNAStar 5.0. After multiple alignment via Clustal_X1.83, the phylogenetic tree was reconstructed based on neighbor-joining (NJ) method implemented in MEGA 7.0, and statistical support was provided by 10000 bootstrap pseudo-replications.

2.4. Chemical exposure and sampling

To study the effects of environmental toxicant exposure on *CAT* gene expression and CAT enzymatic activity, we exposed *D. japonica* to one commonly used IL 1-decyl-3-methylimidazolium bromide ([C_{10}mim]Br) in industry or glyphosate-based herbicide in agriculture. [C_{10}mim]Br with > 99% purity and glyphosate isopropylamine (IPA) salt aqueous solution (glyphosate 30%) were purchased from Hubei Hengshuo Chemical CO., LTD. (Wuhan, China) and Tianjin Huayu Pesticide CO., LTD. (Tianjin, China), respectively. Based on the pre-experiment results of the median lethal concentrations (LC_{50}, 96 h) of each pollutant on *D. japonica*, three concentrations of 1/4LC_{50}, 1/2LC_{50} and 3/4LC_{50} (6, 12, 17 mg L^{-1} for [C_{10}mim]Br and 32, 64, 96 mg L^{-1} for glyphosate) were designed for treatment groups, and the autoclaved tap water was used as the control. Stock solution of [C_{10}mim]Br or glyphosate was prepared on the day when the toxic exposure began and diluted to desired concentrations using autoclaved tap water. As for the exposure experiment of each toxicant, 160 planarians with normal morphology and ca. 10 mm in gliding length were divided into four groups averagely and exposed to three treatment groups and the