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Characterization and transcriptional regulation of thioredoxin reductase 1 on exposure to oxidative stress inducing environmental pollutants in *Chironomus riparius*

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ABSTRACT

We characterized thioredoxin reductase 1 (TrxR1) from *Chironomus riparius* (CrTrxR1) and studied its expression under oxidative stress. The full-length cDNA is 1820 bp long and contains an open reading frame (ORF) of 1488 bp. The deduced CrTrxR1 protein has 495 amino acids and a calculated molecular mass of 54.41 kDa and an isoelectric point of 6.15. There was a 71 bp 5' and a 261 bp 3' untranslated region with a polyadenylation signal site (AATAAA). Homologous alignments showed the presence of conserved catalytic domain Cys–Val–Asn–Val–Gly–Cys (CVNVGC), the C-terminal amino acids 'CCS' and conserved amino acids required in catalysis. The expression of CrTrxR1 is measured using quantitative real-time PCR after exposure to 50 and 100 mg/L of paraquat (PQ) and 2, 10 and 20 mg/L of cadmium chloride (Cd). CrTrxR1 mRNA was upregulated after PQ exposure at all conditions tested. The highest level of CrTrxR1 expression was observed after exposure to 10 mg/L of Cd for 24 h followed by 20 mg/L for 48 h. Significant downregulation of CrTrxR1 was observed after exposure to 10 and 20 mg/L of Cd for 72 h. This study shows that the CrTrxR1 could be potentially used as a biomarker of oxidative stress inducing environmental contaminants.

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1. Introduction

The larvae of the aquatic midge *Chironomus riparius* is widely used as a test organism in aquatic ecotoxicological studies because it represents an important link in the aquatic food web and its association with benthic sediments (Lucan-Bouché et al., 2000; OECD, 2001). Traditionally whole-organism and biochemical level end points are used to evaluate potential ecotoxicological impacts of environmental pollutants in *Chironomus* spp. (De Bisthoven et al., 2001; Martinez et al., 2003). However, recently it has been widely accepted that the use of the expression of the genes involved in many biological processes offer high sensitivity and mechanistic value in the diagnosis of environmental contamination, as the mRNA levels represent a snapshot of the cells activity at a given time (Snell et al., 2003; Ankley et al., 2006). Measurement of single gene mRNA expressions quantified using real-time PCR is also used as a useful biomarker of stress in animals (Bustin, 2002). However, due to limited sequence information molecular level studies are still very limited in *C. riparius* and recently we have developed an Expressed Sequence Tags (ESTs) database using 454 pyrosequencing for this ecotoxicologically important species (Nair et al., 2011b) and several antioxidant genes have been characterized and expression analysis has been done (Nair and Choi, 2011a; Nair et al., 2011a).

The thioredoxin reductases (TrxRs) are homodimeric proteins, belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases, with each monomer containing an FAD prosthetic group, a NADPH-binding site and an active site that contains a redox-active disulfide (Holmgren, 1985; Williams, 1995; Tamura and Stadtman, 1996; Arne´r and Holmgren, 2000; Mustacich and Powis, 2000). In mammals three isozymes of TrxRs has been identified; the cytosolic form (TrxR1), mitochondrial form (TrxR2), and testis-specific form (TrxR3) (Gasdaska et al., 1995; Gladyshev et al., 1996). Model insects belonging to the dipteran order such as *D.melanogaster* and *A. gambiae*, have no glutathione reductase and the Trx system is well studied (Kanzok et al., 2001; Huang et al., 2008a,b).

Aquatic organisms are exposed to various environmental pollutants including oxidative stress inducing chemicals. Cadmium (Cd) is one such highly toxic heavy metal contaminant, with no physiological function, released to the aquatic environments from industrial effluents (Korte, 1983; Nriagu et al., 1998; Jung et al., 2005) and has been demonstrated to be toxic to many organisms (Sastry and Subhadra, 1982; Pratap and Wendelaar Bonga, 1990; Ahel et al., 1993; Gillesby and Zacharewski, 1998; Satarug et al., 2003; Henson and Chedrese, 2004; Lee and Choi, 2006; Sandhu and Vijayan, 2011). Cadmium is known to induce the production of reactive oxygen species (ROS) which could cause multiple toxic effects such as DNA damage, lipid peroxidation,

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and induction of apoptosis (Stohs and Bagchi, 1995; Stohs et al., 2000; Risso-de Faverney et al., 2001; Waisberg et al., 2003; Yalin et al., 2006; Liu et al., 2008). Induction of antioxidant genes on exposure to Cd has been reported in many studies (Shaikh et al., 1999; Wang et al., 2004; Murugavel et al., 2007; Soares et al., 2008; Kim et al., 2010).

Several antioxidant enzyme genes like glutathione S-transferases (EC 2.5.1.18) and catalase (EC 1.11.1.6), are induced to protect the cells from oxidative stress causing chemicals in *C. riparius* (Nair and Choi, 2011a; Nair et al., 2011a). Thioredoxin reductase (TrxR) is also an antioxidant enzyme playing an important role in the cellular defense to scavenge ROS (Mustacich and Powis, 2000). Thioredoxin reductase 1 is induced under stress conditions including oxidative stress and is the critical component of the Trx system in the cytosol (Sakurai et al., 2005). Since TrxR play important role in the oxidative stress response, studying the regulation of *C. riparius* TrxR1 gene under oxidative stress and its expression on exposure to environmental pollutants will be useful in understanding its role in defense responses. Moreover, the expression profiles CrTrxR1 to a specific environmental pollutant could be used as a potential biomarker for future environmental biomonitoring studies. However, as far as we know, no sequence information is available about TrxR genes in *C. riparius* and thus, its response to oxidative stress causing environmental pollutants remains unknown. Therefore, in the present study, the full length cDNA of CrTrxR1 is identified from *C. riparius* ESTs database and the mRNA expression profile of CrTrxR1 is studied in different developmental stages (egg, larva, pupa, male and female), as well as in response to a known oxidative stress inducer, Paraquat (1, 14-dimethyl-4, 44-bipyridinium) dichloride (Suntres, 2002) and subsequently to Cd exposure is investigated. Phylogenetic analysis and secondary structure predictions were done using free internet based software.

2. Materials and methods

2.1. Maintenance of *C. riparius* and chemical exposure

Chironimus riparius (Diptera: Chironomidae) larvae were reared in a 2 L glass chamber containing aerated, dechlorinated tap water and acid washed sand. The larvae were fed with fish flake food (Tetramin, Tetrawerke, Melle, Germany) and exposed to a 16 h light plus 8 h dark photoperiod at a temperature of 20 ± 2 °C. Fourth instar larvae were exposed to 50 and 100 mg/L of known oxidative stress inducer paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride; PQ) (Sigma-Aldrich Chemical, St. Louis, MO, USA) for 12 and 24 h and also to 0, 2, 10 and 20 mg/L of cadmium chloride (Sigma-Aldrich) for 0, 12, 24, 48 and 72 h. The exposure concentrations of PQ and Cd were selected based on the result from previously conducted acute and chronic toxicity tests (Lee and Choi, 2006; Choi and Ha, 2009). Three replicates of 15 fourth instar larvae in each exposure set were performed for all exposure conditions and for the controls in beakers containing 100 mL dechlorinated tap water. The controls were maintained without any exposure to chemicals for the different durations and concentrations along with the chemical exposed samples. After the exposure, the larvae were collected and immediately frozen in liquid nitrogen before being stored at −80 °C. Developmental expression of the CrTrxR1 transcript was investigated in egg (two egg masses), fourth instar larvae, pupae and male and female adults (five animals for each stage).

2.1.1. Sequence analysis and phylogenetic tree generation

An ESTs database was developed by pyrosequencing from fourth instar larvae of *C. riparius* using genome sequencer GSFLX system (Roche, Mannheim, Germany). The reads were assembled using the GS De Novo Assembler (http://www.454.com/products-solutions/analysis-tools/gs-de-novo-assembler.asp). *C. riparius* thioredoxin reductase 1 cDNA sequence was retrieved from the ESTs database using BlastX searches of the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/). Secondary structure was predicted using the PSIPRED protein structure prediction server (http://bioinf4.cs.ucl.ac.uk:3000/psipred/). Phylogenetic analysis of the predicted amino acid sequences of different classes of TrxRs along with CrTrxR1 were conducted using the neighbor joining method using MEGA software, version 4.0 and bootstrap values calculated with 1000 replicates (Tamura et al., 2007).

2.1.2. Expression analysis of CrTrxR1 by real-time polymerase chain reaction

Total RNA was isolated using Trizol™ (Invitrogen, USA) from chemical exposed and control larvae as per manufacturer's instructions and the quality of RNA preparation was verified. One microgram of total RNA was reverse transcribed to cDNA with oligo dT_{20} primer using iScript™ select cDNA synthesis kit (Bio-Rad, USA) as per the manufacturer's instructions in a total reaction volume of 20 μL. The primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) (Table 1) and were tested on a representative *C. riparius* cDNA preparation using reaction conditions with 94 °C for 4 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C or 30 s and 72 °C for 10 min using PTC 100 thermal cycler (MJ Research, Lincoln, MA, USA) with the PCR mix (Bioneer, South Korea) according to the manufacturers' manual. The RT-PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide to verify the amplicon length and to assure that only one product is amplified.

To study CrTrxR1gene transcript expression in each developmental stage and after exposure to different concentrations and durations of PQ and Cd quantitative real-time RT-PCR (qRT-PCR) was performed. Each reaction included 1 μL of template cDNA, 0.2 μM of corresponding forward and reverse primers, 10 μL of 2× IQ SYBR Green Super Mix (Bio-Rad, USA) in a final reaction volume of 20 μL. The RT-PCR reactions were run with an initial denaturing at 95 °C for 7 min followed by 44 cycles of 95 °C for 15 s, 55 °C for 1 min and 72 °C for 0.15 s and a melting curve analysis was done. Amplification and detection were performed using a CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA USA) and accompanying software (CFX Manager Software) according to the manufacturer's instructions. The qRT-PCR was done using samples from three independent exposure and control sets. The expression level of CrTrxR1 gene in different developmental stages and after exposure to different concentrations and time intervals of PQ and Cd was calculated relative to expression levels of the *Chironomus* β-actin mRNA used as an internal standard to normalize the expression levels.

2.2. Statistical analysis

Cycle threshold (C_t) values were converted to relative gene expression levels by $2^{-\Delta\Delta Ct}$ method using the gene expression analysis software in the CFX96 PCR-machine (Bio-Rad, USA). The data were checked for of homogeneity of variance before analysis. Statistical differences between the results obtained from different experiments in control and treated larvae were analyzed using one-way ANOVA with SPSS 12.0 KO (SPSS Inc., Chicago, IL, USA). Dunnett's post-doc

Table 1
Primers used in Real time PCR study.

Gene	Primer Name	Sequence of primer (5′–3′)	Amplified product length (bp)
CrTrxR1	*CrTrxR1-F*	GACATTTTCTCATTAGACCGTGAAC	120
	CrTrxR1-R	ACGAACCAAAATTGTTGACTCATAG	
β-actin	*CrActin*-F	GATGAAGATCCTCACCGAACG	145
	CrActin-R	TTCGAGTGAGGTTGATGCAG	

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