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Mitochondrial thioredoxin-2 from Manila clam (*Ruditapes philippinarum*) is a potent antioxidant enzyme involved in antibacterial response

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ABSTRACT

Thioredoxin (TRx) is a ubiquitous protein involved in the regulation of multiple biological processes. The TRx-2 isoform is exclusively expressed in mitochondria, where it contributes to mitochondrial redox state maintenance. In the present study, a novel thioredoxin-2 gene was identified in the Manila clam, *Ruditapes philippinarum*. The full-length sequence of *RpTRx-2* (1561 bp) consists of a 498 bp coding region encoding a 166 amino acid protein. The N-terminal region of RpTRx-2 harbors a mitochondrial localization signal (56 amino acids), while the C-terminal portion contains the characteristic ^{89}WCGPC^{93} catalytic active site. Phylogenetic analysis revealed that RpTRx-2 is closest to its ortholog from abalone. The broad distribution pattern of *RpTRx-2* mRNA in healthy animal tissues implicates a generally significant function in normal clam physiology. The transcription level of *RpTRx-2*, however, is highest in hemocytes. Lipopolysaccharide and *Vibrio tapetis* bacterium caused up-regulation of the RpTrx-2 transcript levels in gill and hemocytes. Interestingly, clam manganese superoxide dismutase (*MnSOD*) mRNA levels in hemocytes elicited a corresponding response to these immune challenges. RpTRx-2 was recombinantly expressed in *Escherichia coli* BL21 (DE3) and used in insulin disulfide reduction assay as well as metal-catalyzed oxidation assay to elucidate its antioxidant property by reducing substrate and protecting super-coiled DNA from oxidative damage through free radical scavenging, respectively. Collectively, our data indicated that RpTRx-2, a mitochondrial TRx-2 family member, is an antioxidant enzyme that may be involved in antibacterial defense of clams.

1. Introduction

The evolutionarily ancient and simple organisms, such as mollusks, rely solely on the innate arm of the immune response to defend against invading microbes. Yet, the innate immune system is remarkably robust and dynamic enough that it remains a vital component of complex organisms, such as humans, who have evolved the adaptive arm of the immune response as a secondary line of defense. The innate response involves hemocyte-mediated phagocytosis, well known to be a major defense mechanism in invertebrates [1]. Phagocytotic uptake of foreign material stimulates mitochondrial generation of reactive oxygen species (ROS) [2], which are byproducts of molecular O_2 metabolization in aerobic life and may be cytotoxic at elevated levels [3]. Low concentrations of ROS are, however, beneficial in mediating various physiological processes [4], including acting as a hemocyte-mediated antimicrobial defense in bivalves [5]. Thus, the organism must maintain a balance between elevated ROS levels to clear pathogenic infection and reduced levels to protect against detrimental oxidative stress. Biological systems have evolved to maintain such redox homeostasis and include several antioxidant systems comprising enzymatic and non-enzymatic strategies [6]. Intracellular redox regulation depends mainly on glutathione (GSH) and thioredoxin (TRx) buffering systems [7,8]. The TRx antioxidant system, in particular, functions as a catalytic cycle in which peroxiredoxin (PRx) oxidizes the TRx and then TRx reductase (TRxR) reduces it using NADPH [9].

TRx (EC 1.8.4.8) is a 12 kDa multifunctional protein, ubiquitous in all forms of life and exists as either a cytosolic (TRx-1) or mitochondrial (TRx-2) isoform [10]. Catalytic activity of TRx reduces disulfide bridge of substrate via the vicinal Cys residues in its active motif of WCGPC. Apart from its antioxidant-redox regulatory role, TRx is also involved in regulation of growth [11], transcription

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factors [12], protein folding [13] and scavenging radicals [14]. Although many cytosolic thioredoxins have been characterized [15,16], including the RpTRx from *Ruditapes philippinarum* Manila clam [17] that we reported recently, only a few mitochondrial TRx-2s have been described, namely those from rat [18], human [19], chicken [20] and abalone [21].

To gain insight into the role of TRx isoforms in the immunity of the Manila clam, we cloned the full-length cDNA of *TRx-2* from *R. philippinarum*. This species is an important fishery bivalve with high economic value and in recent decades has suffered severe epidemics of infections with various pathogens. In this study, we characterized the newly discovered *RpTRx-2*, determining its basal tissue distribution and transcriptional responses to challenge with LPS and *Vibrio tapetis* bacterium. Using a recombinant protein, the biological activity and antioxidant property of RpTRx-2 were confirmed. Collectively, our data lay a foundation to understand the roles of RpTRx-2 against mitochondrial homeostasis and antibacterial defense in clam.

2. Materials and methods

2.1. Manila clam cDNA library and identification of RpTRx-2

We have established a normalized clam (*R. philippinarum*) cDNA library using RNA isolated from multiple tissues of healthy clams. The basic procedure of cDNA library construction, normalization and initial GS-FLX™ sequencing strategies has been described in our previous report [17]. BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast) of the clam cDNA shotgun sequence database identified a putative cDNA with high homology to known *TRx-2* members, designated as Manila clam *TRx-2* (*RpTRx-2*) and further characterized.

2.2. In silico characterization of RpTRx-2

The full-length nucleotide and amino acid sequence of RpTRx-2 was analyzed using the NCBI BLAST program and TRx-2 homologs were retrieved. DNAssist (2.2) was used to determine the open reading frame (ORF) and encoded amino acid sequence of RpTRx-2, which was further examined using ExPASy proteomics server. The conserved domain (CD) was determined by using prosite profile analysis [22], motif scan Pfam hidden Markov models (HMMs) (Local models) (http://hits.isb-sib.ch/cgi-bin/PFSCAN), and the CD-Database (CDD) protein annotation resource (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The mitochondrial localization signal (MLS) was deduced by using the MitoProt II Server (v1.101) (http://www.cbs.dtu.dk/services/TargetP/) [23]. Disulfide connectivity was predicted using the DiANNA server [24]. Identity and similarity percentages between RpTRx-2 and orthologues were calculated utilizing EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). The amino acid sequence of RpTRx-2 was submitted to I-TASSER server [25] to generate a 3D model, based on multiple-threading alignments with potential templates including *Rhodobacter capsulatus* TRx-2 (PDB no, 2pptA) and human TRx-2 (PDB no, 1w4vA) and the predicted model was analyzed by RasMol 2.7.5.2 [26]. Pair-wise and multiple sequence alignments were generated using the ClustalW, version 2.0 [27]. To deduce the phylogenetic relationship, known TRx-2 and TRx-1 sequences were aligned in ClustalW and then the neighbor-joining (NJ) method was applied to construct a phylogenetic tree with 1000 bootstrap replicates using the MEGA 5 program [28].

2.3. Clams, immune challenges and tissue isolation

Clams with an average size of 35 ± 5 mm were collected from the eastern coastal area of Jeju Island (Republic of Korea) and maintained in 80 L tanks of aerated sand-filtered seawater with $34 \pm 1‰$ salinity and at 21 ± 1 °C. Clams were acclimatized to laboratory conditions for seven days prior to experiment.

In order to evaluate the tissue specific distribution of the *RpTRx-2* mRNA, tissues from adductor muscle, mantle, siphon, gill and foot were isolated from three healthy, unchallenged individuals. Hemolymph (1–2 mL/clam) was also collected from each animal using a sterile syringe; hemocytes were immediately harvested from the biological fluid by centrifugation at $3000 \times g$ for 10 min at 4 °C. In order to determine the immune response of *RpTRx-2*, two *in vivo* challenges were devised using lipopolysaccharide (LPS) and intact Gram-negative bacteria *V. tapetis*. LPS and *V. tapetis* were suspended in phosphate buffered saline (PBS). Two groups of clams were randomly chosen for intramuscular (i.m.) injection of either 100 μL LPS (50 ng/μL, *Escherichia coli* 055:B5; Sigma–Aldrich, USA) or 100 μL of *V. tapetis* (1.9×10^9 cells/mL). A third group of uninjected clams was used as control.

Hemocyte and gill samples were collected at 3, 6, 12, 24, 48, and 96 h post-injection (p.i.) from at least three clams of the LPS-, bacterial-injected and control groups. Tissues and hemocytes were snap frozen in liquid nitrogen and stored at −80 °C until processing for RNA extraction.

2.4. Total RNA isolation and cDNA synthesis

The total RNA was extracted from 50 mg of tissue sample and hemocyte samples using the Tri Reagent™ (Sigma–Aldrich). Purified RNA concentration was determined by measuring the absorbance at 260 nm in a UV-spectrophotometer (Bio-Rad). The total RNA was stored at −80 °C until further use.

RNA samples were thawed and diluted to 1 μg/μL concentration and used as template in cDNA synthesis using PrimeScript™ first-strand cDNA synthesis kit (Takara). The cDNA synthesis reaction was carried out according to the manufacturer's instructions. The synthesized cDNA product was diluted appropriately and used in quantitative real-time PCR (qRT-PCR).

2.5. RpTRx-2 transcriptional profiling by qRT-PCR

The *RpTRx-2* mRNA expression profile was determined by qRT-PCR, along with that for the gene of manganese superoxide dismutase (MnSOD) *RpMnSOD*. *RpTRx-2* gene-specific primers (F1, 5′-GTCCTTGTAAGCTGTTAGGGCCAA-3′ and R1, 5′-ATCACATCGTCGTCTTGAAGCCCT-3′) generated a 199 bp fragment of *RpTRx-2*. The *RpMnSOD* gene-specific primers (F, 5′-AAGGACATGTTGACACAGGCTTCG-3′ and R, 5′-AAAGCCTGTTGTTGGTTGCAGAGG-3′) generated a 135 bp fragment of *RpMnSOD*. The gene coding for clam *β-actin* was amplified by using *Rp-β-actin* gene-specific primers (F, 5′-CTCCCTTGAGAAGAGCTACGA-3′ and R, 5′-GATACCAGCAGATTCCATACCC-3′) and used as an invariant control [29]. Tissue-specific distribution of *RpTRx-2* was determined by measuring mRNA expression levels in adductor muscle, mantle, siphon, gill, foot and hemocytes. Pathogen inducibility was demonstrated in hemocytes and gill by using a time course experiment. qRT-PCR assays were performed in 20 μL reaction system containing 4 μL of diluted cDNA from different tissues, 10 μL of 2× Takara SYBR premix Ex *Taq*™, 0.8 μL of each primer (10 pmol/μL), and 5 μL dH_2O. A Thermal Cycler Dice™ Real-Time System (Takara) was used with the following cycling profile: a single denaturation cycle of 95 °C for 10 s; 45 amplification cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s; and a final single cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The relative *RpTRx-2* mRNA expression was determined by the Livak $2^{-\Delta\Delta CT}$ method [30], using the clam *β-actin* as a reference gene. To quantify the tissue-specific expression, the expression level of *RpTRx-2* in adductor muscle was used as the