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Characterization of a 2-Cys peroxiredoxin IV in *Marsupenaeus japonicus* (kuruma shrimp) and its role in the anti-viral immunity

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ABSTRACT

Accumulating evidence suggests that peroxiredoxins (Prx) are key molecules in the pathogenesis of various infectious diseases and are potential therapeutic targets for major diseases such as cancers. In this study, we report a peroxiredoxin IV (Prx IV) in *Marsupenaeus japonicus*, designated as MjPrx IV, which exhibited peroxidase activity and participated in the anti-white spot syndrome virus (WSSV) immune response. MjPrx IV is a 245-amino acid polypeptide with a predicted 19-amino acid signal peptide, an Ahpc-TSA domain, and a 1-Cys PrxC domain. Phylogenetic analysis revealed that the protein belongs to the Prx IV subfamily. MjPrx IV transcripts were detected in the gills, hepatopancreas, heart, stomach, ovaries, spermary, and intestine tissues, and are upregulated in the gonads, gills and hemocytes of shrimp after WSSV challenge. The mature MjPrx IV peptide was recombinantly expressed in an *Escherichia coli* system. The protein exhibited peroxidase activity. Furthermore, dsRNA suppression of *MjPrx IV* increased WSSV replication in shrimp, whereas rMjPrx IV injection into shrimp decreased WSSV replication. These data suggest that MjPrx IV has an important role in shrimp antiviral immunity. To our knowledge, this study is the first to report a shrimp Prx IV that has anti-WSSV activity.

1. Introduction

Living organisms have ingenious mechanisms for restoring homeostasis after disruption by physical, chemical, or biological stimuli. Viral infections disrupt many biological processes, including redox homeostasis, which is maintained by cellular oxidants and antioxidant systems and is considered as a new therapeutic target for novel antiviral strategies [1–4].

Cellular oxidants consists of free radicals (superoxide anion radicals, hydroxyl radicals, peroxy radicals, alkoxy radicals, and nitric oxide) and their reactive metabolites, which are called reactive oxygen species (ROS), including hydrogen peroxide, singlet oxygen, and nitrogen dioxide. ROS cause oxidative stress to cells, resulting in direct or indirect damage to biological macromolecules, namely, proteins, lipids, nucleic acids, and carbohydrates [5]. Cellular antioxidant systems, including enzymes such as superoxide dismutase, catalase, glutathione (GSH) peroxidase, and peroxiredoxins (Prx), directly destroy ROS and proteins indirectly by preserving highly reductive environments in the cytosol (e.g., the thioredoxin and glutaredoxin systems) [6–8].

Hydrogen peroxide (H_2O_2), one of the important ROS, has a dual role (positive and negative) in organisms. Hydrogen peroxide functions as secondary messenger for intracellular signaling at physiologically low levels, but it induces the oxidative modification of cellular macromolecules, inhibits protein function, and promotes cell death at excessive concentrations [5]. The cells remove redundant H_2O_2 via several routes, including breakdown by peroxidases or reaction with non-enzymatic oxidants, such as vitamins and GSH [3,9]. Enzymes that eliminate H_2O_2 include Prx and the conventional enzymes catalase and glutathione peroxidase (GPx). Among these enzymes, Prx is considered to be the most efficient peroxidase because catalase exclusively eliminates H_2O_2 in peroxisomes, whereas GPx mainly eliminate H_2O_2 in the mitochondria [5].

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Prx is a novel family of non-selenium-dependent peroxidases that catalyze the reduction of H_2O_2 and various organic hydroperoxides to form water and alcohols, respectively. The first Prx was discovered in yeast by Kim et al. in the late 1980s [10]. The enzyme was designated as thiol-specific antioxidant (TSA), thioredoxin peroxidase before finally being called peroxiredoxin [11]. Prx occurs in all kingdoms with multiple isoforms, which are well studied in mammals. To date, six Prx isoforms (PrxI–PrxVI) have been identified in mammals and classified into three subgroups, designated as typical 2-Cys Prx, atypical 2-Cys Prx, and 1-Cys Prx according to the number and position of conserved cysteine residues. The functions, cellular localization, and reaction mechanisms of mammalian Prx have been fully studied and reviewed in several articles [11–15]. Prxs share the same basic catalytic mechanism, wherein the active site cysteine (the peroxidatic cysteine) is oxidized into sulfenic acid by a wide range of peroxide substrates. The peroxidatic cysteine of 2-Cys Prx could be hyperoxidized into sulfinic or sulfonic derivatives, thereby losing its peroxidase activity, but acquiring molecular chaperone activity. Various reversible modifications are also involved in Prx regulation, such as glutathionylation, phosphorylation, nitrosylation, hyperoxidation, and acetylation. These characteristics contribute to the complexity of the diverse activities of Prxs, from signal modulators, tumor suppressors, apoptotic regulators to therapeutic targets and broad-spectrum antioxidants [9].

Prx IV is the only secretory Prx protein that possesses an N-terminal signal peptide and is uniquely present in the plasma and the endoplasmic reticulum of cells. Since the identification of Prx IV (AOE372) in human cells in 1997 [12], many of the functions of Prx IV in mammals had been reported, such as enhancing tumor growth and metastasis [16,17], suppressing ROS production by stimulating epidermal growth factor in the p53 pathway [18], preventing respiratory syncytial virus (RSV)-induced oxidative damage in human respiratory epithelial cells [19], and participating in the regulation of NF-κB, granulocyte colony-stimulating factor receptor (G-CSFR), and the thromboxane A2 receptor signaling pathway, which are important for innate immunity, acute myeloid leukemia (AML), and G protein-coupled signal transduction, respectively [9,20–22]. Most of these reports come from research on vertebrates, but little is known about the function of Prx IV in invertebrates.

White spot syndrome virus (WSSV), which belongs to the Whispovirus genus, Nimaviridae family, is a double-stranded circle DNA virus that has a wide range of host and seriously affects crustacean cultures [23]. Recent reports have shown an imbalance in redox homeostasis, including hydrogen peroxide changes, during WSSV infections in shrimp [24,25]. In this study, we report the cloning and functional identification of a Prx IV peroxidase in *Marsupenaeus japonicus* (kuruma shrimp), designated as MjPrx IV. The MjPrx IV transcripts in normal and WSSV-infected shrimp tissues were investigated using RT-PCR and real-time PCR. MjPRX IV protein was recombinantly expressed with *in vitro* peroxidase activity, and the antiviral activity of MjPRX IV was investigated using the augmentation assay and dsRNA interference.

2. Material and methods

2.1. Immune challenge and tissues preparation

Adult shrimp *M. japonicus* were purchased from a market in Jinan, Shandong Province, China and cultured temporarily in laboratory tanks filled with air-pumped seawater. Before use, the shrimp were acclimatized for 24 h at room temperature (25 °C) under laboratory conditions. The shrimps (20 for each group) in the experimental group were injected with WSSV (3×10^7 copies per shrimp) for the immune challenge, whereas the shrimps in the control group were injected the same volume of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The preparation and quantification of the viral inocula were based on a previous paper [26]. Hemolymph was extracted from the ventral sinus of the shrimp using 1/10 volume of anticoagulant buffer (10% sodium citrate, pH 7.0) at 0, 6, 12, 24, and 48 h after the challenge, then immediately centrifuged at 800 × g for 15 min at 4 °C to isolate the hemocytes. Afterward, tissues from the gills, hepatopancreas, heart, stomach, ovaries, spermary, and intestines were dissected for RNA extraction. Three shrimps were used for every sample to eliminate individual differences.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted from various shrimp tissues using Unizol reagent (Biostar, Shanghai, China). The first strand cDNA was reverse transcribed according to the instructions of the SMART PCR cDNA synthesis kit (BD Biosciences Clontech).

2.3. Gene cloning and sequence analysis

The full-length cDNA sequence was obtained by random sequencing of a normalized cDNA library from kuruma shrimp ovaries, which was constructed in our lab beforehand. The cDNA sequence was confirmed by sequencing the open reading frame (ORF) fragment amplified with the gene specific primers MjSF (5′-CTTGGCCTGGACTAACACACC-3′) and Oligo anchor R primer (5′-GACCACGCGTATCGATGTCGACT$_{16}$(A/C/G) −3′) from another ovary cDNA template. The PCR conditions were as follows: 1 cycle at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 50 s; and 1 cycle at 72 °C for 10 min. The online program BLAST (http://blast.ncbi.nlm.nih.gov/) was used to analyze nucleotide sequence homology and amino acid sequence. cDNA translation and deduced protein prediction were achieved using ExPASy (http://www.au.expasy.org/), whereas signal sequencing and domain prediction were carried out using SMART (http://www.smart.embl-heidelberg). Phylogenetic analysis was conducted using MEGA 4.0 via the neighbor-joining method, and multiple alignments was performed using the GenDoc software [27].

2.4. Tissue distribution and expression pattern of Prx IV upon WSSV challenge

The tissue distribution of Prx IV at the mRNA level in the hemocytes, gills, hepatopancreas, stomach, heart, intestines, spermary, and ovaries was analyzed using RT-PCR using the primers Prx IVrtF (5′-CTATGGGGTCTACCTGGAG-3′) and Prx IVrtR (5′-TGCTGGTCTGTGAACTGG-3′). Quantitative real-time PCR (Q-PCR) was used to determine the *MjPrx IV* expression patterns in the gonads, stomach, hemocytes and gills of the WSSV-challenged shrimp using the primers used in the tissue distribution assay. Q-PCR was performed according to the manufacturer's instructions using an SYBR Premix ExTaq kit (Bioteke, Beijing, China) with real-time thermal cycler (Bio-Rad, Hercules, CA). *β-actin* was amplified for internal standardization with the primers actinF (5′-CAGCCTTCCTTCCTGGGTATGG-3′) and actinR (5′-GAGGGAGCG AGGGCAGTGATT-3′). The methods used were in accordance with the paper published previously [28]. All samples for real-time PCR analysis were repeated in triplicate. The *MjPrx IV* expression levels in response to viral challenge was calculated with $2^{-\Delta\Delta CT}$ methods [29], and subjected to the statistical analysis, followed by an unpaired sample *t*-test. Differences were considered significant at $P < 0.05$ [26].