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Cloning and functional analysis of glutathione peroxidase gene in red swamp crayfish *Procambarus clarkii*

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ABSTRACT

Glutathione peroxidases (GPxs) are key enzymes in the antioxidant defense systems of living organisms, including crustaceans. The red swamp crayfish *Procambarus clarkii* is the most commonly farmed freshwater crayfish in Chinese inland nowadays due to its commercial value. However, high stocking density has resulted in adverse effects in growth performance and health. To investigate the function of GPxs in immune defense of the crayfish, we cloned and characterized a full length *GPx* (*PcGPx*) from *P. clarkii* by a reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The 931 bp *PcGPx* cDNA contains a 38 bp 5′-untranslated region (UTR), a 519 bp coding sequence (CDS) and a 375 bp 3′-UTR with a selenocysteine insertion sequence (SECIS). The *PcGPx* was predicted to encode 172 amino acids, and its putative molecular mass was 20.9 kDa with a *pI* of 4.37. A selenocysteine (Sec) encoded by the unusual stop codon, TGA, was in the protein coding region. Phylogenetic analysis showed that PcGPx clustered with the GPxs from the penaeid shrimp *Metapenaeus ensis* and *Caenorhabditis elegans*, sharing much higher similarity with vertebrate GPx1 and GPx2 than with GPx3 and GPx5. Quantitative RT-PCR revealed that *PcGPx* was extremely highly expressed in ovary and early embryos. In addition, the levels of *PcGPx* mRNA and reactive oxygen species (ROS) significantly increased after challenge with gram-negative *Vibrio harveyi*, gram-positive *Staphyloccocus aureus* or white spot syndrome virus (WSSV). These results suggest that PcGPx may play important roles not only in immune defense, but also in oogenesis in the crayfish.

1. Introduction

Reactive oxygen species (ROS) are generated as by-products of aerobic metabolism in cells. Particularly large amounts of ROS are produced in inflamed tissues as an essential constituent of the host immune defense system for removing various pathogens [1]. Although they perform an important role in host's defense system, overabundance of ROS can oxidize and damage cell membrane, proteins, and nucleic acids [1,2]. To reduce the oxidative stress resulted from ROS, cells have developed a series of enzymatic scavenger systems including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx).

GPxs play a crucial role in detoxifying lipids and hydrogen peroxide, which are rapidly produced during phagocytosis and physiological metabolism, with the concomitant oxidation of glutathione [1]. GPxs are usually selenoproteins, and have been identified in both animals and plants [3]. In mammals, there are eight GPx families that have been described based on the primary structure, specific substrate accessibility, and spatial expression [3,4]. They formed two clusters, representing the classical GPx (GPx1, GPx2, GPx3, GPx5 and GPx6) and phospholipid hydroperoxide GPx (GPx4, GPx7 and GPx8). The classical GPx (cGPx) is multimeric (commonly tetrameric) and soluble, while the phospholipid hydroperoxide (PHGPx) is monomeric and often membrane-associated [5]. They are appeared in almost every animal cell, though the tissue distribution of the isoforms is different. GPx1 is the most prominent GPx isoform and it is able to lower hydrogen peroxide and many of organic peroxides by using glutathione as the reducing substrate [3,6]. GPx4 is expressed in a variety of tissues. The main substrate for GPx4 is phospholipid hydroperoxides, indicating its critical role in resisting lipid peroxidation [7]. Recent studies have shown that GPx4 might play a role in the neurotoxicity induced by MeHg in mice [8].

In decapod crustaceans, GPx activities were detected in the giant freshwater prawn, *Macrobrachium rosenbergii* [9] and white shrimp, *Litopenaeus vannamei* [10]. Full-length GPx complementary

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DNAs (cDNAs) were also cloned and characterized in *L. vannamei* [10], *Penaeus monodon* [1] and *Metapenaeus ensis* [2], respectively. GPxs expression and activity were demonstrated to be significantly upregulated when shrimps were injected with microbes, indicating a function in eliminating the excessive ROS caused by microbe infection [1,11]. Additionally, in *M. ensis*, GPx was found to be specifically expressed in early ovaries, suggesting that the GPx might play a pivotal role in preventing oocytes from oxidative damage and thus in crustacean reproduction.

The red swamp crayfish *Procambarus clarkii* is the most commonly farmed freshwater crayfish in Chinese inland nowadays owing to its high market value and consumer demand. However, with the increasing of stocking density, the adverse effects on growth performance and health have been evident [12,13]. Disease outbreaks were reported frequently in recent years, and the pathogenetic microorganisms have been isolated from the diseased crayfish [13,14]. Therefore, understanding the immune ability of the crayfish and their defense mechanisms has become a primary concern. We have previously investigated the effect of environment and diet on the growth and immunology in the crayfish [15–17]. The aim of the current study was to explore the function of *GPx* gene in *P. clarkii* by cloning and analyzing the sequence of the gene and investigating its spatial and temporal expression patterns. Furthermore, to provide insights into the role of *GPx* in counteracting pathogens, we also assayed its molecular responses after *P. clarkii* was injected with bacteria and virus.

2. Materials and methods

2.1. Animals

The crayfish *P. clarkii* were purchased from Baishazhou aquatic market in Wuhan, China. Prior to the experiment, these crayfish were reared in aquaria with water volume of around 68 × 46 × 25 cm and with continuous aeration for one week for acclimatization to the new conditions as described by Wang et al. [17].

2.2. cDNA cloning and sequence analysis

Total RNA was isolated from the ovary using Trizol reagents (Invitrogen). The first-strand cDNA synthesis was performed in 20 μl reaction volume containing 1 μg RNA, 200 U M-MLV reverse transcriptase (Invitrogen), 1 μl dNTP mixture (10 mM each), and Smart F1 and Anchor R1 primers. Sequences of the primers are listed in Table 1. The first strand cDNA was used as the template for amplification of the crayfish *GPx*.

Table 1
Degenerate and specific primers used in the experiment.

Primers	Sequence
Smart F1	5′-TACGGCTGCGAGAAGACGACAGAAGGG-3′
Anchor R1	5′-GACCACGCGTATCGATGTCGACT(16)V-3′
PcGPx F1	5′-AACKTDGCNACVTACTGAGG-3′
PcGPx R1	5′-GRAACTTCTCRWAGTTCCAC-3′
Smart F2	5′-TACGGCTGCGAGAAGACGACAGAA-3′
5′SP R	5′-ACTCGCAGGCGCTCTTCAAGAAGG-3′
5′NSP R	5′-CCGGCTCCAGCATTTCAAACTGAT-3′
3′SP F	5′-ATGAATGCACTCGCGGAGTTCTAC-3′
Anchor R2	5′-GACCACGCGTATCGATGTCGAC-3′
3′NSP F	5′-CCGAGGACCCGCTCTTCACCTTCT-3′
PcGPx F2	5′-GCTCACCGTCCCTTCATATACCCA-3′
PcGPx R2	5′-TAGCGGATCCCGTTCATGATCTCT-3′
β-actin F	5′-AAATCACGGCTCTGGCTCCCT-3′
β-actin R	5′-GAAGCATTTGCGGTGGACGAT-3′

Based on the conserved sequences of other animal's *GPx*, two degenerate primers (PcGPx F1 and PcGPx R1) were designed as shown in Table 1. The middle partial fragment was first amplified from the above cDNA and sequenced. Based on this sequence, four specific primers (3′SP F, 5′SP R, 3′NSP F and 5′NSP R) were designed (Table 1). Two PCRs were performed for amplifying the 3′ and 5′ ends of GPx from the crayfish by rapid amplification of cDNA ends (RACE). Smart F2 and 5′SP R were used for the first run, and Smart F2 and 5′NSP R for the second run (nested PCR) to amplify the 5′end of this gene. The primers 3′SP F and Anchor R2 were used for the first run, and 3′NSP F and Anchor R2 for the second run to amplify the 3′end of this gene (Fig. 1).

PCR products were purified and cloned into pMD19-T vector (TaKaRa). Positive clones were sequenced in BGI-Wuhan. Nucleic acid homology searches were performed by BLAST at web servers of National Center for Biotechnology Information (NCBI). The amino acids were deduced using DNAstar software. Signal sequence and selenocysteine insertion sequence (SECIS) were predicted through SingalP3.0 and SECISearch2.19 (http://genome.unl.edu/SECISearch.html), respectively. Alignments were performed with ClustalX and the phylogenetic tree was constructed with MEGA4.0 software using BIO neighbor-joining algorithm (BioNJ) with 1000 bootstrap replicates [18,19]. Tertiary structure was analyzed by ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred).

2.3. Spatial and temporal expression analysis by quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from various tissues using Trizol reagents (Invitrogen). RNA was extracted from ovary, hepatopancreas, epidermis, central nerve system (brain and thoracic ganglia), heart, gill, gut, muscle and testis of the crayfish in the reproduction season, or from embryos at various stages as described in Dai et al. [20]. For qRT-PCR detection, approximately 5 μg of total RNA treated by DNase (RNase-free DNase I, Takara, Japan) was reverse transcribed with M-MLV (Invitrogen) at 42 °C with anchor R1 primer according to manufacture's instruction. The cDNA was diluted (1:10) for use as a template and qPCR was performed on a Miniopticon system (Bio-Rad). *β-actin* was used as a reference gene. The PCR amplification was carried out in EU Thin-wall 8-tube strip in a 20 μl reaction volume, which contained 10 μl SYBR Premix Ex Taq (2×) (TaKaRa), 1 μM each forward and reverse primers (PcGPx F2 and PcGPx R2 for *PcGPx* gene, β-actin F and β-actin R for *β-actin* gene, Table 1) and 2 μl of diluted cDNA. The qPCR procedure was consisted of 95 °C for 2 min, followed by 95 °C for 5 s, 58 °C for 20 s and 72 °C for 10 s per cycle for 40 cycles, then a melting curve analysis was carried out by a slow increase (0.2 °C/s) from 55 °C to 98 °C, in purpose of examining if there were primer-dimers or nonspecific amplification. The relative expression ratio of *PcGPx* gene was calibrated against *β-actin* gene using the $2^{-\Delta CT}$ calculation method: $\Delta CT = (CT_{PcGPx} - CT_{actin})$.

2.4. Expression profiles of PcGPx in response to bacteria and virus challenges

Twenty adult crayfishes (approximately 20 g) were injected individually with 2×10^6 cfu *Vibrio harveyi*, or *Staphyloccocus aureus*, or 2×10^6 copies of white spot syndrome virus (WSSV) into the abdominal segment. PBS was used to inject as control. The hepatopancreas was sampled at different time points (2, 6, 12, 24, and 48 h) after microbe injection from the challenged group or the control group. The total RNA of hepatopancreas was extracted and qRT-PCR was performed as described above. The relative expression ratio of *PcGPx* gene for samples injected with microbes (A) to samples injected with PBS (B) was calibrated against *β-actin* gene