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Short communication

Molecular characterization and functional analysis of two phospholipid hydroperoxide isoforms from *Larimichthys crocea* under *Vibrio parahaemolyticus* challenge

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ABSTRACT

Glutathione peroxidases family is a key role in the antioxidant system in oxybiotic organisms for cell redox homeostasis. One of their members, phospholipid hydroperoxide glutathione peroxidase (GPx4) have unique monomeric structure and can directly react with complex lipid and membrane-bound peroxides under the presence of glutathione(GSH). In this paper, two complete GPx4 cDNAs (designated as LcGPx4a and LcGPx4b) from *Larimichthys crocea* are identified by rapid amplification of cDNA ends. The cDNA of LcGPx4a was consisted of a 5′-untranslated region (UTR) of 258 bp, a 3′-UTR of 330 bp, and an open reading frame (ORF) of 561 bp encoding 186 amino acid (aa) polypeptides. And the full-length sequence of LcGPx4b was 1164 bp with a 5′-UTR of 34 bp, a 3′-UTR of 551 bp and an ORF of 576 bp encoding a polypeptide of 191 aa residues with a predicted signal peptide of 15 aa. The characteristic selenocysteine insertion (SECIS) sequence was detected in the 3′UTR of the two sequences with 78 bp in length. The conserved active site of selenocysteine (Sec) encoded by TGA was also identified and formed a tetrad functional structure with glutamine, tryptophan, and asparagine in LcGPx4a and LcGPx4b. Two signature site motifs ("LRILAFPSNQFGNQEPG" and "LRILGFPCNQFGGQEPG") were both conserved in the deduced amino acid of LcGPx4a and LcGPx4b. The genomic structure analysis revealed that the two sequences both had 7 exons and 6 introns, and the Sec opal codon and SECIS element were located at the third and seventh exons, respectively. LcGPx4a and LcGPx4b both have a wide distribution in 9 tissues with various relative expression levels and a highest expression pattern in the liver. Under *Vibrio parahaemolyticus* challenge, their relative expression levels were altered in the liver, spleen, kidney, and head kidney but with different magnitudes and response time. LcGPx4a and LcGPx4b showed a significantly up-regulated trend in the spleen during experimental period. Above results suggested that LcGPx4a and LcGPx4b were two conserved immune molecules and might play a role in the immune response of fish with a tissue-depemdent manners.

1. Introduction

Glutathione peroxidases (GPxs) are a key component of antioxidant enzyme family that have been discovered in various forms of life [1]. They reduce hydrogen peroxide (H2O2) or organic hydroperoxides to water or corresponding alcohols typically utilizing glutathione(GSH), which acts as a reductant and is simultaneously oxidized to glutathione disulfide(GSSG). Nowadays, 8 members of GPxs have been identified in mammal on the basis of their structure, cellular expression and specific function [2,3]. These members can be classified into two types: selenium-dependent and selenium-independent forms [4]. Selenium-dependent GPxs (Se-GPxs), including GPx1, GPx2, GPx3, GPx4 and GPx6, have a vital enzymatic site: selenocysteine(Sec), whereas selenium-independent GPxs (GPx5, GPx7 and GPx8) use cysteine as the enzymatic site instead of Sec [5]. Notable is, Sec is encoded by an opal (UGA) codon which typically means stop and causes termination of translation [6]. Therefore, organisms had developed a mechanism that UGA meaning Sec recognized by a specific $tRNA^{(Ser)Sec}$ when a particular stem loop, called selenocysteine insertion sequence (SECIS), forms in downstream or the 3′untranslated region (UTR) during the transcript [5]. In organisms development, Se-GPxs take a fundamental part in maintaining the homeostasis of reactive oxygen species(ROS), whereas selenium-independent GPxs function as a backup system [7].

Differ from GPx1, GPx2, GPx3 and GPx6, which are homotetramer, phospholipid hydroperoxide (GPx4) is monomeric and membrane-associated [8]. GPx4 had been initially characterized as lipid peroxidation inhibiting protein because it could react with, besides H_2O_2 and small hydroperoxides in general, hydroperoxides in complex lipids, including

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phospholipid, cholesterol and cholesterol ester hydroperoxides, even when they are inserted into bio-membranes or lipoproteins [9,10]. In particular, its substrate is that protein thiols can replace the function of GSH as reductants when the latter becomes limited [11,12]. Thus, compared to other members, GPx4 can either act as a GSH or a thiol peroxidase according to the availability of GSH. In mammals, GPx4 exists in three isoforms: cytosolic, mitochondrial and sperm nuclear GPx4, which are derived from the same gene [13]. Cytosolic GPx4 is widespread in cells and play a vital role in life, which had been proved that the knockout of it could cause mice death [14]. And mitochondrial and sperm nuclear GPx4 are mainly expressed in testis with less amounts in other tissues, which take a crucial structural role in spermatogenesis [15–17].

To date, GPx4 has been characterized in some fishes, such as *Danio rerio* [18], *Thunnus maccoyii* [19], *Seriola lalandi* [20], *Cyprinus carpio* [21], *Oncorhynchus mykiss* [22], *Oncorhynchus kisutch* [23] and *Sparus aurata* [24]. It was well-known that GPxs members could be used as an indicator, which evaluate the effect of different environmental factors to fishes [25]. For example, the GPx4 expression pattern of *C. carpio* and *O. kisutch* have occurred significantly varied under cadmium exposure [21,23]. Also, several physical factors could lead to the variant of GPx4 expression pattern in fish, like confinement and cold stress [21,24]. In microbial infected-organism, GPxs also exhibited an up-regulated expression patterns [26,27]. In fact, abundant ROS production could benefit to resist pathogens due to the raised activity of macrophage [28–30]. And the excessive ROS generation would damage to organisms, and GPxs acted as a regulator to maintain the ROS balance in organism [31]. Studies on the function of GPx4 suggested that it was not merely prevents lipid peroxidation, but participated in cellular inflammation, apoptosis and signal transduction [32]. The loss of GPx4 activity have a strong association with some diseases, such as male infertility and arteriosclerosis [33]. Thus, it was necessary to understand the role of GPx4 in the immune response of fish under pathogen challenge.

Larimichthys crocea, previous known as the large yellow croaker, belongs to Sciaenidae under Osteichthyes. It has a large economic marine market and been widely cultured in southeast China with good nutritional quality and palatability [34]. However, the production of *L. crocea* is now decreasing due to overfishing and diseases caused by pathogens such as vibrio, parasite and virus [35–38]. Therefore, what the correlation between the antioxidative and immunity system against the pathogens need to be studied. In this study, two complete cDNA sequences of phospholipid hydroperoxide from *L. crocea* (designated as LcGPx4a and LcGPx4b, respectively) were cloned. Their molecular features, gene structures and motif regions were characterized compared to other known GPx4 genes. Also, their expression patterns of different tissues and temporal expression profiles at mRNA level after being challenged by *Vibrio parahaemolyticus* are investigated by real-time reverse transcriptase PCR. Above results can provide new perspectives into the role of two GPx4 isoforms in the potential regulatory mechanism of the antioxidative and immunity system.

2. Materials and methods

2.1. Fish rearing and bacterial infection

Healthy *L. crocea* (fish length 21–23 cm, weight 107–175 g) were collected from the Fujian Shacheng Harbor Cultivation Base in Fujian Province, P.R.China. In this experiment, each tank stored in 1000 L of aerated seawater at 19.01 ± 1.25 °C with a temperature control equipment. Seventy-five individuals were reared in six tanks a week before *V. parahaemolyticus* infections and fed commercial feed during acclimatization. *V. parahaemolyticus* was provided by Professor Chenghua Li from Ningbo University, P.R.China. It was firstly revived and cultured in thiosulfate citrate bile salt sucrose agar culture medium at 28 °C with the scraping line method. Then, one single colony was selected and cultured in lysogeny broth liquid medium, and the bacterial numbers were counted using the plate count method.

Nine tissues were selected from five healthy individuals for tissue expression patterns detection and stored in RNAstore (CWBIO, China) at −80 °C until use, including the gill, liver, spleen, kidney, muscle, heart, intestine, head kidney, and brain. The gene expression of muscle was considered as the control for the analysis of different tissues.

Totally, 70 individuals were randomly divided into two groups, as experimental animals for injection. One group of 35 individuals were syringed intraperitoneally with phosphate buffer saline (PBS) (500 μL per fish, pH 7.4), as the blank group. Another group of 35 individuals were syringed intraperitoneally with live *V. parahaemolyticus* (1×10^8 CFU/mL, 500 μL per fish, resuspended in PBS, pH 7.4), as the infectious group. The liver, spleen, kidney and head kidney of five individuals from the blank and infectious groups were randomly dissected at 0, 6,12, 24, 48 and 72 h post-injection of *V. parahaemolyticus*, and stored in RNAstore (CWBIO, China) at −80 °C until use. The 0 h post-injection was served as another control for the temporal expression analysis.

2.2. Total RNA, DNA isolation and cDNA synthesis

Total RNA was isolated from each tissue with RNAiso Plus (TaKaRa, Japan) following the manufacturer's instructions and then treated with RNase-free DNase I (Takara, Japan). The genomic DNA of *L. crocea* from muscle was isolated with Tianamp Marine Animals DNA Kit (Tiangen, China) following the instructions of the manufacturer. The first strand cDNA was synthesized from 1 μg of the total RNA with Reverse Transcriptase M-MLV (RNase H-) (TaKaRa, Japan) and oligo (dT18) primer following the manufacturer's instructions. Then, all DNA and cDNA samples were stored at −20 °C until use.

2.3. Cloning the full-length cDNA of LcGPx4a and LcGPx4b

Based on the *L. crocea* whole-genome data [37], the partial sequences of LcGPx4a and LcGPx4b were obtained with special primers (Table 1). PCR was performed in 50 μL volume, containing 5 μL of 10 × PCR Buffer, 4 μL of dNTP mixture, 2 μL of primer-F, 2 μL of primer-R, 0.5 μL of cDNA template from the healthy liver, and 0.5 μL of rTaq DNA polymerase (TaKaRa, Japan). The PCR amplification on a Thermal Cycler (Bio-Rad, USA) under the following conditions: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 52.5–54.4 °C for 30 s, and 72 °C for 45 s; and an extension at 72 °C for 7 min. At last, PCR production were gel-purified by MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa, Japan) after 1.2% agarose gel electrophoresis, ligated into the pMD18-T vector (TaKaRa, Japan), and then transformed into competent *Escherichia coli* DH5@ cells (TaKaRa, Japan). Three positive clones were sequenced in Beijing Genomics Institute (Shanghai, China).

Total RNA of the liver from healthy individuals was used to 3′ end rapid amplification of cDNA ends (RACE) and analyzed using the 3′-Full RACE Core Set with PrimeScript™ RTase (TaKaRa, Japan) following the manufacturer's instructions. Specific primers of LcGPx4a and LcGPx4b are listed in Table 1. The 3′RACE outer primer and the GPx4a-3F1 and GPx4b-3F1 primers were used in a first-round PCR reaction with the first-strand cDNA as the template. The 3′RACE inner primer and the GPx4a-3F2 and GPx4b-3F2 primers were used in a second-round PCR reaction with the diluted product of the first-round PCR reaction as the template. The first-round PCR conditions were as follows: denaturation at 94 °C for 3 min; 20 cycles of denaturation at 94 °C for 30 s, annealing at 55–58 °C for 30 s, and extension at 72 °C for 90s; and a final extension at 72 °C for 10 min. The second-round PCR conditions were the same, except the annealing temperatures were increased to 58–60 °C and the PCR cycle number was 30. All PCR products were gel-purified, ligated, and sequenced as described above.

In the 5′ end RACE, the liver template total RNA was transcribed by PrimeScript™ II Reverse Transcriptase (TaKaRa, Japan) with oligo