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Selenium-dependent glutathione peroxidase gene expression during gonad development and its response to LPS and H_2O_2 challenge in *Scylla paramamosain*

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ABSTRACT

A selenium-dependent glutathione peroxidase cDNA was obtained from green mud crab *Scylla paramamosain* (*SpGPx*) by homology PCR technique and rapid amplification of cDNA ends (RACE) methods. The 1135 bp full-length cDNA contains a 9 bp 5′-untranslated region (UTR), an open reading frame (ORF) of 564 bp encoded a deduced protein of 187 amino acids (aa), and a 562 bp 3′-UTR with a 100 bp conserved eukaryotic selenocysteine insertion sequence (SECIS). It involves a putative selenocysteine (Sec^{40}, or U^{40}) residue which is encoded by an opal codon, $^{127}TGA^{129}$, and forms an active site with residues Q^{74} and W^{142}. Sequence characterization revealed that SpGPx contain a characteristic GPx signature motif 2 ($^{64}LAFPCNQF^{71}$), an active site motif ($^{152}WNFEKF^{157}$), a potential *N*-glycosylation site ($^{76}NTT^{78}$), and two residues (R^{90} and R^{168}) which contribute to the electrostatic architecture by directing the glutathione donor substrate. Multiple sequence alignment and phylogenetic analysis showed that SpGPx share a high level of identities and closer relationship with other selected invertebrate GPxs and vertebrate GPx1 and GPx2. Molecular modelling analysis results also supported these observations. Real time quantitative PCR analysis revealed that *SpGPx* was constitutively expressed in 10 selected tissues, and its expression level in gill and testis was higher than that in the other tissues ($p < 0.05$). The *SpGPx* expression increased and then declined during ovarian and testicular development implying thatnscrpits yowed that SpGPx might play an important role in gonad development by protecting them from oxidative stress. The expression of SpGPx mRNA was induced by lipopolysaccharide (LPS) and hydrogen peroxide (H_2O_2) in hepatopancreas and haemocytes. The results suggested that *SpGPx* was implicated in the immune response induced by LPS and H_2O_2.

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1. Introduction

During aerobic cellular metabolism, respiration, stress and inflammation, reactive oxygen species (ROS) are generated, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•). ROS are well recognized for playing a dual role as both deleterious and beneficial species: at low concentrations, they can serve the key signal transduction pathways including those that regulate reproductive system [1], and kill some types of bacteria that they engulf by phagocytosis; on the other hand, the overexpression of ROS will cause tissues damage and macromolecule peroxide, such as DNA damage, lipid peroxidation, loss of cellular function and even apoptosis [2–5]. It is well-known that ROS have been implicated in the aetiology of a variety of diseases. The increased ROS level might damage oocyte and spermatozoa, as a result infertility happened [6,7]. To defend the toxicity of ROS, organisms have evolved protective systems, including non-enzymatic scavengers (ascorbic acid, vitamin E, β-carotene, glutathione, and bilirubin) and antioxidant enzymes [superoxide dismutase (SOD, EC1.15.1.1), catalase (CAT, EC1.11.1.6) and glutathione peroxidase (GPx, EC1.11.1.9 and EC1.11.1.12)] [8–11].

Four major selenium-dependent GPx isozymes: classical GPx (GPx1), gastrointestinal GPx (GPx2), plasma GPx (GPx3), phospholipid hydroperoxide GPx (GPx4) have been characterized in mammals. Another two GPx isozymes, GPx5 and GPx6 also were identified in mammals and considered to be as the homologue of GPx3. GPx5 lacks the selenocysteine (Sec) at the active site and is a selenium-independent GPx. GPx6 was identified in humans and pigs as a selenium-dependent GPx, but in mouse and rat the orthologs in which Sec is replaced by Cys and lacked the eukaryotic selenocysteine insertion sequence (SECIS) element, belongs to selenium-independent GPx [11–13]. Recently, a new phospholipid hydroperoxide GPx (GPx7), which contains Cys instead of Sec in the active site, has been cloned in mammals [14,15]. GPxs catalyze the reduction of

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H_2O_2 or organic hydroperoxides to H_2O or corresponding alcohols, to balance the redox status of physiology [11,13]. They play important roles in immune and antioxidative defence systems to protect the organism against pathogens: bacteria, fungi and viruses [16–18].

GPxs have been cloned from several crustaceans, including white shrimp *Litopenaeus vannamei* [19], Chinese shrimp *Fenneropenaeus chinensis* [17], tiger shrimp *Penaeus monodon* [16], penaeid shrimp *Metapenaeus ensis* [20], giant freshwater prawn *Macrobrachium rosenbergii* [21], and salmon louse *Lepeophtheirus salmonis* [22]. GPx expression or/and activity were significantly up-regulated when shrimp encounter pathogen invasion, such as bacteria, virus and biomacromolecule [16,17,19,21]. It is reported that GPx gene expression of Chinese shrimp *F. chinensis* could be directly induced after injection of hydrogen peroxide (H_2O_2) [23]. Increased GPx expression and activity can protect organism against damage from ROS. But GPx gene expression on crab has not been reported when it subjected to pathogen invasion or oxidative stressor.

The green mud crab *Scylla paramamosain* is an important commercial species distributed along the southeaster coastal region of China and Indo-West Pacific regions [24–26]. In 2008, more than 3.33×10^8 m^2 and more than 100,000 tons of green mud crab were cultured in China [27]. But the current mainstay of mud crab seed supply depended on wild collection or wild caught seedstock as seed rearing. The quality gonad of cultured broodstocks declined and the survival rate of larval became low due to the reduced disease resistance and increasing stresses. Therefore, one of aim of the present study will focus on immune function of *S. paramamosain* GPx (SpGPx) after LPS and H_2O_2 challenge.

It has been found that crab and shrimp broodstocks were fed with non-enzymatic scavengers, such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E), which could facilitate the reproductive performance [28–34]. Up to date, only the GPx of *M. ensis*, which belongs to GPx3, was found to be specifically expressed in ovaries and thus may play an important role in oocytes maturation and penaeid shrimp reproduction [20]. But the function of other GPx isoforms in crustacean reproduction has not been elucidated. Although some antioxidant enzyme genes have been cloned in *S. paramamosain* [35,36], the function of antioxidant enzymes in reproduction of green mud crab has not been investigated. Another important aim of the study will focus on antioxidant function of SpGPx in gonad development.

In this study, we cloned the nucleotide sequence of GPx from haemocytes of *S. paramamosain*, characterized and compared with known selenium-dependent glutathione peroxidase (Se-GPx) isozymes in other species, and analysed SpGPx mRNA expression profiling in different tissues, at different development stage of gonad and after crab being injected with LPS and H_2O_2. The data will provide great help to understand antioxidant function of GPx from crustacean in both gonad development and innate immunity.

2. Materials and methods

2.1. Animals

For gene cloning, green mud crabs *S. paramamosain* were purchased from local market at Xiamen city, China. The weight of body and gonad were measured respectively. For gene expression profile analysis of gonad development, according to the gonadosomatic index (GSI = gonads weight/body weigh × 100), the female crabs were grouped into six stages: stage 1 – stage 6, and the male crabs were grouped into three stages: stage 1 – stage 3 [37]. Each developmental stage contains 5 crabs ($n = 5$). For tissue expression analysis, eyestalks, gills, heart, intestines, hepatopancreas, haemocytes, muscles, ovaries, stomach and testis were dissected from five adult female and five male crabs and stored at −80 °C until used.

For challenge experiments, green mud crabs (220–320 g) were obtained from a commercial crab farm in Zhangzhou city, China, and animals were acclimatized in filtered seawater at 25 ± 2 °C for 7 days before the experiments were carried out. The seawater was changed 100% daily and fresh clams were fed to crabs. LPS from *Escherichia coli* (L2880, Sigma, USA) was dissolved with modified crab saline solution (MCSS: NaCl, 496 mM; KCl, 9.52 mM; MgSO4, 12.8 mM; CaCl2, 16.2 mM; MgCl2, 0.84 mM; NaHCO3, 5.95 mM; HEPES, 20 mM; pH 7.4 [38]) to 5 mg ml^{-1} as stock solution. Hydrogen peroxide (H_2O_2) was dissolved with MCSS to 0.3‰. Crabs were injected with a dose of 0.5 mg kg^{-1} LPS [36] or 100 μl kg^{-1} of 0.3‰ H_2O_2 and an equal volume of MCSS as control treatments. The crabs were challenged at the left fifth leg base, and were dissected at 0 h, 3 h, 6 h, 12 h and 24 h (except H_2O_2 group) after treatment. At each time course, at least three crabs were analysed.

Haemolymph of crabs was taken from the left fifth leg and stored with an equal volume of anti-coagulant solution (NaCl 510 mM; glucose 100 mM; citric acid 200 mM; Na-citrate 30 mM; EDTA-Na_2 10 mM; pH 7.3 [39]) followed by centrifugation at 800 × g at 4 °C for 20 min [36]. The haemocytes pellets, gills and hepatopancreas from challenged crabs were quick-frozen in liquid nitrogen and store at −80 °C refrigerator until used.

2.2. Total RNA isolation and reverse transcription

Total RNA was isolated from different tissues using Trizol agent (Invitrogen, USA) referring to the manufacturer's protocol. The integrity of RNA was checked by spectrophotometric analysis and then it was electrophoresed on a 1% agarose gel. Three micrograms of high-quality DNase treated total RNA from each tissue was separately reverse-transcribed by M-MLV reverse transcriptase (Promega, China) following the manufacturer's protocol.

2.3. Polymerase chain reaction (PCR) and subcloning of GPx cDNA

Full-length Se-GPX cDNA of *S. paramamosain* from haemocytes was obtained by homology PCR technique, 3′ and 5′ rapid amplification of cDNA ends (RACE) methods. Degenerate primers (DP-F/R, Table 1) were designed based on the highly conserved GPx amino acid sequences of *L. vannamei* (accession no. AAY41441), *M. rosenbergii* (accession no. ACM68948), *Homo sapiens* (accession no. AAP80181), *Bos taurus* (accession no. NP_776501), *Rattus norvegicus* (accession no. NP_110453) in GenBank database and employed the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). After the initial *denaturation at 94* °C for *3 min*, PCR was performed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s and elongation at 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR products were resolved by electrophoresis of ethidium bromide stained agarose gel. The target DNA fragments were excised and purified by Gel Extraction Kit (Generay Biotech, China), cloned into PMD19-T vectors (Takara, Japan) and then transformed into the competent cells of *E. coli* JM109. The positive clones were sequenced.

Based on the sequences of homology-based cloning in green mud crab, four gene-specific primers (GPx-F1, GPx-F2, GPx-R1, and GPx-R2) were designed (Table 1). 5′- and 3′- RACE were carried out by using an SMART™ RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's instructions. The PCR products of interest were obtained as above mentioned. One pair primers of head to toe full length cDNA (GPx-h, GPx-t) (Table 1) were designed to confirm the sequence.

2.4. Sequence characterization and phylogenetic analysis

The *SpGPx* sequence was analysed and compared by using the BLASTX and BLASTP search programs (http://www.ncbi.nlm.nih.

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