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Identification and cloning of a selenium dependent glutathione peroxidase from giant freshwater prawn, *Macrobrachium rosenbergii*

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ABSTRACT

A selenium dependent glutathione peroxidase (Se-GPx) cDNA was cloned from haemocyte by a reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA (RACE). The 913 bp cDNA contained an open reading frame (ORF) of 558 bp encoded a deduced amino acid sequence of 186 amino acids. The prawn Se-GPx sequence contains a selenocysteine (Sec) residue which is encoded by the unusual stop codon, ^{115}TGA^{117}. According to the molecular modeling analysis, the active site Sec residue, located in the loop between β3 and α2 in a pocket on the protein surface, and hydrogen bonded to Gln_{73} and Trp_{141}. A GPx signature motif 2, ^{63}LAFPCNQF^{70} and active site motif, ^{151}WNFEKF^{156}, two arginine (R) residues, R_{89} and R_{167} contribute to the electrostatic architecture that directs the glutathione donor substrate, and two putative *N*-glycosylation site, ^{75}NNT^{77} and ^{107}NGS^{109} were observed in the prawn Se-GPx sequence. In addition, the eukaryotic selenocysteine insertion sequence element is conserved in the 3′-UTR. Comparison of amino acid sequences showed that prawn Se-GPx is more closely related to vertebrate GPx 1. The prawn Se-GPx was synthesized in haemocyte, hepatopancreas, muscle, stomach, gill, intestine, eyestalk, heart, epidermis, lymph organ, ventral nerve cord, testis and ovary. The increase of respiratory burst in haemocyte was observed in pathogen, *Debaryomyces hansenii*-injected prawn in order to kill the pathogen, and the up-regulation in SOD and GPx acitivity, and prawn Se-GPx mRNA transcription were involved with the protection against damage from oxidation.

1. Introduction

It is well known that phagocytic cells are the first line of defence against pathogenic infection. The cells generate reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, that can damage all biomolecules and destroy phagocytosed pathogens [1,2]. Although ROS play an important role in host defense, their overexpression and residual ROS can cause cellular damage. In order to prevent the damage caused by ROS, aerobic organisms are able to maintain very low levels of ROS. A variety of small antioxidant molecules, such as glutathione and thioredoxin, are synthesized to scavenge ROS, and even tyrosin has been demonstrated to have a protective role against oxidative stress [3]. In addition, several enzymatic antioxidant systems, such as superoxide dismutase, catalase, and glutathione peroxidase have been well characterized to be involved with scavenge ROS [4].

Glutathione peroxidase (GPx) (EC 1.11.1.9) is an important antioxidant molecule, which protects biomembranes and other cellular components from oxidative damage by catalyzing the reduction of a variety of hydroperoxides (ROOH), using glutathione (GSH) as the reducing substrate during phagocytosis and/or physiological metabolism [5–8]). Today six isoforms are known; therefore it is called more like an enzyme family than single enzyme. They are present in almost every cell of animals, but the tissue distribution of the isoforms show high variation [6,9–13]. According to the presence of selenocysteine encoded by a TGA, there are two subgroups of GPxs, selenium dependent glutathione peroxidase (Se-GPx) and non-selenium glutathione peroxidase (non-Se-GPx).

In decapod crustaceans, GPx has been characterized by enzyme activity determination in giant freshwater prawn, *Macrobrachium rosenbergii* [14,15], crayfish, *Procambarus clarkia* [16] and white shrimp, *Litopenaeus vannamei* [17,18], and the cDNA sequence of GPx has been identified in *L. vannamei* [18] and mud crab, *Scylla serrata* (GeneBank accession number: FJ429110). Liu et al. [18] shown that the sequence of *L. vannamei* GPx is a Se-GPx which contains a putative selenocysteine residue encoded by the unusual stop codon, TGA, and forms the active site with residues Glu^{75} and

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Trp[153]. The eukaryotic selenocysteine insertion sequence (SECIS) is conserved in the 3' untranslated region of *L. vannamei* Se-GPx cDNA. In addition, the expression of GPx and its activity in haemocyte of *L. vannamei* was significantly increased after being injected with pathogen *Vibrio alginolyticus* in order to protect cells against damage from oxidation.

In the present study, a Se-GPx cDNA was isolated from haemocyte of *M. rosenbergii*, and compared with other known isoforms from other animals, and evaluated its transcription when *M. rosenbergii* was infected by *Debaryomyces hansenii*.

2. Materials and methods

2.1. Prawn

Giant freshwater prawn, *M. rosenbergii*, were obtained from the farm at the Department of Aquaculture, National Pingtung University of Science and Technology, and allowed to acclimate at 26 ± 1 °C in 2 ton cylinder tank (diameter: 200 cm and water deep: 65 cm) for 1 week. During the acclimation period, the prawn were fed a formulated shrimp diet twice daily. The prawn with an average weight of 25.3 ± 2.9 g were used for this study. Only prawn at intermoult stage were used.

2.2. Haemocyte collection and total RNA isolation

Haemolymph (0.50 ml) was withdrawn from the ventral sinus cavity of prawn into a 1 ml sterile syringe (25 gauge) containing 0.5 ml of a pre-cooled (4 °C) anticoagulant solution (0.80 g sodium citrate, 0.34 g EDTA, 10 ml Tween 80 in 1 L distilled water, then the osmolality was adjusted to 490 mOsm kg^{-1} with glucose). The haemocyte was isolated by centrifugation at 500 × g and 4 °C for 20 min, thereafter the haemocyte pellet was used for the total RNA isolation using the ULTRASPEC™ RNA, Total RNA Isolation Reagent (Biotecx, Houston, TX, USA) following the manufacturer's instructions.

2.3. Reverse transcription (RT) PCR and subcloning of Se-GPx cDNA

First-strand cDNA synthesis in RT was accomplished using Super-Script II RNase H^- reverse transcriptase (Promega, Madison, WI, USA) to transcribe poly $(A)^+$ RNA with oligo-$d(T)_{18}$ as the primer. Reaction conditions recommended by the manufacturer were followed.

Full-length selenium dependent glutathione peroxidase (Se-GPx) cDNA of *M. rosenbergii* was obtained by RT-PCR, and 3′ and 5′ rapid amplification of cDNA (RACE) methods. Degenerate primers were designed based on the highly conserved GPx amino acid sequence of *L. vannamei* [18], bluefine tuna, *Thunnus maccoyii* (GeneBank accession number: EF452497) and human, *Homo sapiens* (GeneBank accession number: NM_002083) in GeneBank and another database [19] and by using the Clustalw program (http://align.genome.jp/). The degenerate primer pair of MGPXF (5′-CAGTTYGGVCATCAGGARAA-3′) and MGPXR (5′- ATVAGGAARTTCTCRAARTT-3′) was used to amplify the first prawn GPx cDNA fragment. The PCR reaction was carried out in a 50 μl reaction volume containing 2 μl of cDNA, 5 μl of 10× Pro Taq buffer (PROTECH, Taiwan), 0.5 μl of Pro Taq Plus DNA polymerase (5U/μl, PROTECH, Taiwan), 1 μl of 10 mM dNTPs, and 1 μl of each primer. PCR reactions were performed as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min, and elongation at 72 °C for 2 min, followed by a 10 min extension at 72 °C and cooling to 4 °C.

For 5′-RACE and 3′-RACE, the primer pairs of M-GPX-5′-I (5′-ATTGGGTGAGCTTCTGAC-3′) and M-GPX-5′-II (5′-TTCAACAGCTCTTCATCAG-3′), and M-GPX-3′-I (5′-CAATTTTGTGCCTAAGAT-3′) and M-GPX-3′-II (5′-TCCACTGCCTTCTGATGA-3′) were used, respectively. First-strand cDNA was used in the 5′-RACE system (cat. no. 18374-058, Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was terminated at the 5′-end by the terminal transferases, TdT and dCTP. The primer set consisted of M-GPX-5′-I and Abridged Anchor Primer (AAP) (50-GGCCACGCGTCGACTAGTACGGIIGGGIIGGGIIGG-30) for the first-run PCR, and M-GPX-5′-II and Abridged Universal Amplification Primer (AUAP) (50-GGCCACGCGTCGACTAGTAC-30) for the second-run PCR. The PCR reaction conditions were the same as that described above, except that the annealing temperature of the second run PCR was changed to 55 °C. For 3′-RACE, reverse-transcription was performed using the PT1 primer (50-GTTGCCGAC GACGAGCCTACTTTTTTTT-30), and then the first-strand cDNA was used as the template for amplification of the 30 fragment of shrimp GPx. Two PCRs were performed with primers M-GPX-30-I and PT2 (50-GTTGCCGACGACGAGCCTAC-30) for the first run, and M-GPX-30-II and PT2 for the second run. The PCR reaction conditions were the same as those described above.

The PCR fragments were subjected to electrophoresis on a 1.5% agarose gel for length difference, and all PCR-amplified cDNA fragments were cloned into the PCRII TOPO vector of the TOPO TA cloning system (Invitrogen), and transferred into *Escherichia coli* cells according to the manufacturer's protocol. Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing.

2.4. Nucleotide sequence analysis

Nucleotide sequence analysis was performed using the dideoxynucleotide chain termination method [20] on a DNA sequencer (Model 373A, Applied Biosystems, Lincoln, NE, USA). Plasmid DNA at 1 mg was used for sequencing with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and was subjected to electrophoresis on 6% denaturing gels. Clones were sequenced with the M13 forward and reverse primers. The GPx gene sequence was analyzed and compared using the BLASTX and BLASTP search programs (http://blast.genome.ad.jp) with a GenBank database search. The multiple sequence alignment of the prawn GPx gene was created using the Clustalw program (http://align.genome.jp/). In addition, the SECIS element of prawn Se-GPx was analyzed using the SECISearch 2.19 program (http://genome.unl.edu/SECISearch.html).

2.5. Phylogenetic analysis

Phylogenetic trees were constructed on the basis of the proportion of amino acid differences (p-distances) by the Neighbour-joining method [21] using MEGA 4 software [22]. For the construction of the phylogenetic tree, indels were removed from the multiple alignments. The reliability of the tree obtained was assessed by bootstrapping using 1000 bootstrap replications [23].

2.6. Molecular modeling analysis of prawn Se-GPx

The three-dimensional structure of prawn Se-GPx was obtained by subjecting its amino acid sequence to RCSB PDB website (http://www.rcsb.org/pdb/home/home.do) for searching a homologue template (PDB ID: 2F8A, human GPx 1). Molecular modeling analysis of prawn Se-GPx was carried out by using Swii-pdbviewer software. Briefly, the raw sequence of prawn Se-GPx was aligned to the homologue template and created the modeling project. Thereafter, the project was calculated and compared in the server of SWISS-MODEL workspace (http://swissmodel.expasy.org/workspace/index.php) [24–27].