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Molecular characterization of glutathione peroxidase gene from the liver of silver carp, bighead carp and grass carp

Guang-Zhao Li, Xu-Fang Liang, Wei Yao, Wan-Qin Liao & Wei-Feng Zhu*
College of Life Science and Technology, Jinan University, Shipai, Guangzhou, China

The cDNAs encoding glutathione peroxidase (GPx) were cloned and sequenced from the liver of three Chinese carps with different tolerance to hepatotoxic microcystins, phytoplanktivorous silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*), and herbivorous grass carp (*Ctenopharyngodon idellus*). Using genome walker method, a 750 bp 5′-flanking region of the silver carp GPx gene was obtained, and several potential regulatory elements were identified in the promoter region of the GPx gene. The silver carp GPx gene was widely expressed in all tissues examined. Despite phylogenetic analysis, assigning this newly described carp GPx to the group of mammalian GPx2, the carp GPx seems more similar to GPx1 from a physiological point of view. The constitutive expression pattern of the three carp liver GPx gene, shows a positive relationship with their tolerance to microcystins. [BMB reports 2008; 41(3): 204-209]

INTRODUCTION

Cyanotoxins produced by freshwater cyanobacteria are common worldwide, and have been reported to cause serious poisonings and deaths of wild and domestic animals (1), as well as significant hazards to human health (2). The reported incidences of animal and human exposure to microcystins (3, 4) emphasize the need for a better understanding of the detoxification mechanism of these compounds. It has been known that microcystins can cause an increase in reactive oxygen species (ROS) production (5). Although ROS play an important role in host defense, high concentrations of ROS lead to oxidative stress which can cause cellular damage (6). This stress can be counteracted by enzymatic and non-enzymatic antioxidant systems. Among enzymatic systems, the glutathione peroxidases (GPx) belong to the first line of defense against peroxides, superoxide anion and hydrogen peroxide, and assumes an important role in detoxifying lipid and hydrogen peroxide, with the concomitant oxidation of glutathione (7).

GPx is a generic name for a family of multiple isozymes. In general, the members in the family can be divided into two sorts. One sort of the isozymes have selenium-dependent glutathione peroxidase activity and contain selenocysteine (SeC) encoded by a TGA codon, whereas the other sort have no selenocysteine. Five selenium-dependent GPx (GPx1, GPx2, GPx3, GPx4 and GPx6) have been identified in mammals based on their primary sequence, substrate specificity and subcellular localization (8-12). Although GPx5 has the similarity in nucleotide/protein sequence with GPx3, it is not selenoenzymes because its active site contains cysteine instead of selenocysteine (13).

Silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*), the most commercially important phytoplanktivorous fish in China, are suggested to be able to suppress and graze out *Microcystis aeruginosa* blooms (14). Although report about tissue distribution and depuration of two microcystins (microcystin-LR and microcystin-RR) is available in silver carp (15), little is known about the molecular detoxification mechanism of the cyanotoxins in this fish. Recent studies suggest that fish GPx may play an important role in the detoxification of microcystins, mainly focused on the changes of enzyme activities (16-18). Our previous study observed the increased GPx gene expression level in tilapia exposed to a single 50 μg kg^{-1} body weight (bwt) dose of MC-LR (19). However, the GPx gene expression level in different fishes with different food habit and tolerance to microcystins has not been characterized (20). In this report, the GPx cDNA sequences were cloned from phytoplanktivorous silver carp and bighead carp and herbivorous grass carp (*Ctenopharyngodon idellus*), the promoter region of silver carp GPx gene was also obtained and characterized, and finally, the tissue expression pattern of silver carp GPx gene and the constitutive GPx gene expression level in the liver of these three Chinese carps were determined, aiming at a better understanding on the molecular mechanism of microcystin detoxification in freshwater fishes.

RESULTS AND DISCUSSION

Cloning and phylogenetic analysis of three Chinese carp GPx cDNA

The full-length GPx cDNA clones were isolated from silver carp

*Corresponding author. Tel: 86-20-8522-1497; Fax: 86-20-8522-6262; E-mail: tliangxf@jnu.edu.cn

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liver by RT-PCR and RACE. The silver carp GPx clones were 892 bp in length, contained an open reading frame (ORF) of 576 bp (encoding a polypeptide of 191 amino acids), flanked by 25 bp 5′-untranslated region (5′UTR) and 291 bp 3′-untranslated region (3′UTR). The theoretical molecular weight (Mw) of the putative peptide was estimated to be 21.59 kDa and the isoelectric point (pI) to be 6.37. The 40th amino acid corresponds to a selenocysteine encoded by a TGA codon. Comparison of the deduced amino acid sequence of silver carp GPx with other animal GPx sequences showed that catalytic residues of Gln[75] and Trp[153] which interact with selenocysteine were conserved in silver carp GPx (Fig. 1). Partial GPx cDNA sequences obtained from the liver of bighead carp and grass carp were 653 bp and 666 bp in length respectively (data not shown) and both encoding 119 amino acids (Fig. 2). The coding regions deduced from bighead carp and grass carp partial GPx cDNA sequences were both corresponded to the position 73-191 of the silver carp GPx. Studies with vertebrate species have identified crucial amino acids for the enzymatic mechanism of GPx. Some of these amino acids are conserved in the three Chinese carp GPx gene. Two catalytic residues Gln[75] and Trp[153] are conserved to hydrogen bonded to the Selenium/Sulfur moiety of GPxs (21). Meanwhile, the residues Arg[52, 98, 179,180] and Lys[86] which play a significant roles in directing the substrate toward the catalytic center in mammals (22) were conserved in silver carp, bighead carp and grass carp. GPx has three loop structures that stabilize the structure of the enzyme in mammal. The first loop is Asn[42] - Tyr[54] (NVASLUGTTVRDYT), the second is Leu[72] - Gln[82] (LGFPCNQF GHQ) and the third is Trp[160] - Phe[162] (WNF) (23). In the present study, the amino acids formed these three loop structures were all well conserved in GPx from silver carp, bighead carp, and grass carp.

The GPx sequences identified in the three Chinese carps were compared to those of other fishes and mammals available in GenBank with BLAST program. Both the nucleotide sequences and the deduced amino acid sequence analyses showed that the three Chinese carp GPx gene had a high sequence identity with GPx1 and GPx2 of mammals. To reveal the molecular phylogenetic position of silver carp, bighead carp, and grass carp GPx, a phylogenetic tree was constructed by the Mega 3.1 software. Basically, GPx1 to GPx4 can be classified into four subgroups, with GPx5 and GPx6 of mammals in the same cluster of GPx3 (Fig. 3).

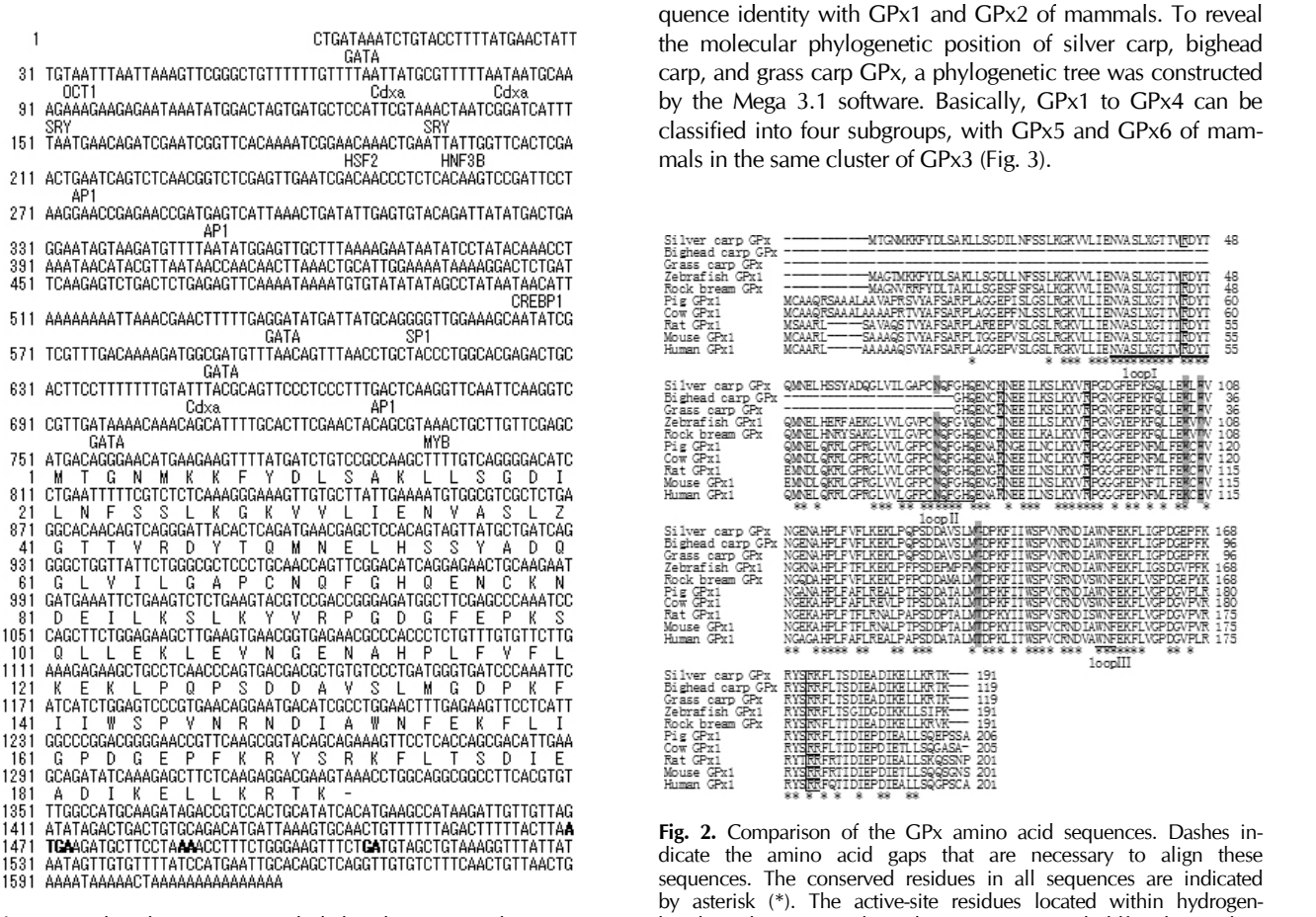

Fig. 1. Nucleotide sequence and deduced amino acid sequence of silver carp selenium-dependent GPx. The SECIS element is located in the 3' UTR and the ATGA_AA_GA motif is showed in bold. Putative regulatory elements were predicted with TFBIND (http://tfbind.ims.u-tokyo.ac.jp/).

Fig. 2. Comparison of the GPx amino acid sequences. Dashes indicate the amino acid gaps that are necessary to align these sequences. The conserved residues in all sequences are indicated by asterisk (*). The active-site residues located within hydrogen-bonding distance to the selenium atom are boldfaced. Residues directing the substrate toward the catalytic center are boxed. Residues that are important for the activity of the enzyme are darkened. Three loop structures that stabilize the structure of the enzyme are underlined.

Isolation and characterization of silver carp GPx gene 5′-flanking region

To understand the regulation of silver carp GPx gene, a 750 bp fragment upstream of the start codon was amplified using genome walker method. Analysis of the upstream 750 bp sequence of silver carp GPx gene, revealed that there was no classical TATA box or CCAAT box in the 5′ flanking region and that the region was not GC rich (<50% G + C). The ubiquitous transcription element sites, one SP1 binding site and three AP binding sites, located at 191 bp, 76 bp, 455 bp and 455 bp upstream of the start codon respectively. One liver-specific site (HNF-3b) and one heat shock factor 2 site (HSF2) as well as four GATA sites were identified at 406 bp, 419 bp, 46 bp, 154 bp, 207 bp and 738 bp upstream of the start codon respectively. Three homologous chicken CdxA caudal type homeobox protein binding sites and one octomer transcription factor 1 (OCT1) were also identified at 98 bp, 663 bp, 677 bp, 704 bp upstream of the start codon respectively. Furthermore, there were two homologous sex-determining region Y sites (SRY) and one MYB recognition element found in the region at 611 bp, 655 bp, 9 bp upstream of the start codon respectively (Fig. 1).

Cis-acting DNA elements in promoters are responsible for interacting with corresponding transcription factors to control transcription of related genes in response to a variety of environmental and developmental signals. Both human GPx1 and GPx2 promoter regions are capable of responding to exogenous agents such as paraquat and t-butyl hydroperoxide (24, 25). This transcriptional response of oxidative stress is likely to be more physiologically relevant *in vivo* than the post-transcriptional regulation mechanism which occurs *in vitro* in the presence of selenium deficiency (26, 27). Extensive further studies are required to determine what element(s) affect expression of silver carp GPx gene and what element(s) respond to cellular stress induced by exogenous agents.

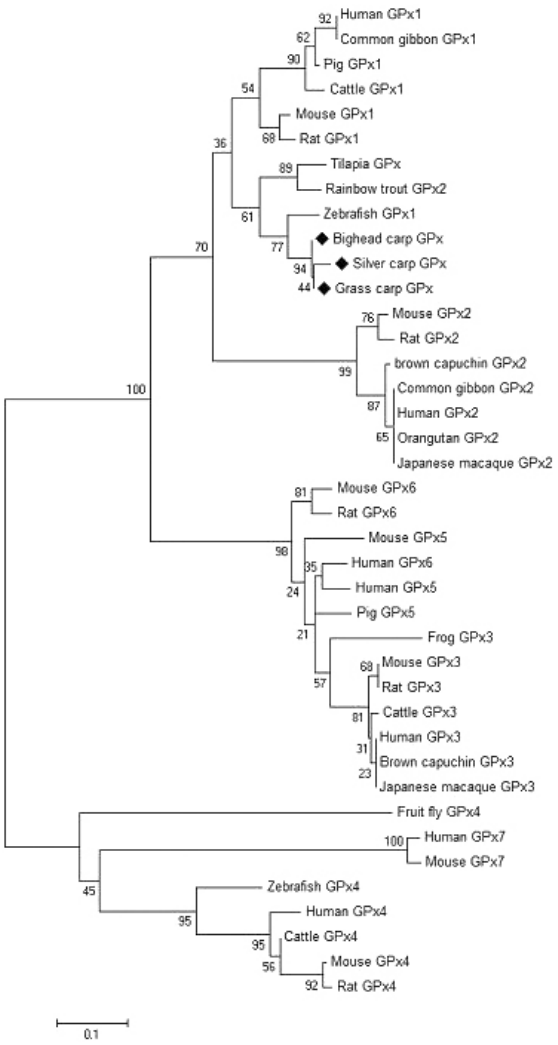

Fig. 3. A molecular phylogenetic tree of GPx based on the Neighbour-joining method with values for each internal branch determined by bootstrap analysis with 1000 replications. Values indicate percentages along the branch.

Tissue expression pattern of silver carp GPx gene and constitutional liver GPx mRNA level among the three Chinese carps

The silver carp GPx was widely expressed in all tissues examined including liver, adipose tissue, intestine, muscle, and brain. The highest expression of silver carp GPx was observed in liver, followed by adipose tissue and intestine, whereas the lowest expression was observed in the muscle and brain (Fig. 4).

The GPxs of three Chinese carps in this study, together with zebrafish GPx1 and rainbow trout GPx2, are more closely related to those of GPx2 than to those of GPx1 of mammals. GPx1 and GPx2 are both cytosolic enzymes. Tissue dis-

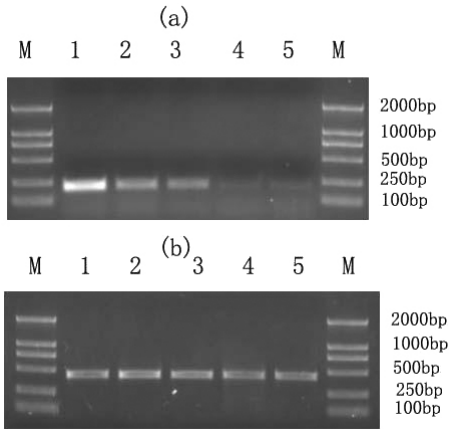

Fig. 4. GPx (a) and beta-actin (b) mRNA expression in different tissues of silver carp by RT-PCR. M, marker; 1, liver; 2, adipose tissue; 3, intestine; 4, muscle; 5, brain.

tribution in mammalian species showed that GPx1 is ubiquitous, whereas GPx2 is mainly restricted to the gastrointestinal tract and human liver (but not rat liver) (8, 28). However, in the present study, silver carp GPx was widely expressed in all major tissues examined including liver, adipose tissue, intestine, muscle, and brain. It should be pointed out that despite phylogenetic analysis, assigning this newly described carp GPx to the group of mammalian GPx2, the carp GPx seems more similar to GPx1 from a physiological point of view. In fact, both tissue distribution and absence of antioxidant response elements (ARE) typical of the GPx2 mammalian gene in the 5′ flanking region, which make GPx2 part of the adaptive response in mammals (29), suggest that this newly discovered carp gene shares the physiological role with mammalian GPx1 enzymes. On the other hand, phylogenetic analysis may be complicated by the high degree of conservation beween GPx1 and GPx2.

GPx gene expression was detected in the liver of the silver carp, bighead carp and grass carp by RT-PCR. The ratio GPx/beta-actin mRNA (%) was determined to be 200.4 ± 30.5 (silver carp), 188.1 ± 16.6 (bighead carp) and 102.0 ± 13.2 (grass carp).

In aquatic ecosystems, fish stand at the top of the aquatic food chain, and are possibly affected by exposure to toxic cyanobacteria. Liver is the major site in fish and other vertebrates for the detoxification of ingested toxic materials by oxidation, and ROS is known to be involved in the process (5, 30). As one of primary antioxidant enzymes, GPx plays an important role in protecting membrane from being oxidated. By catalyzing through reduced glutathione, GPx protects the cell and hypersensitive molecules from the attack of free oxygen radical (31). The important role that GPx plays in detoxifying process of microcystins is supported by many experiments. The activitiy of GPx was enhanced in liver of *Corydoras paleatus* exposed to 2 μg/L microcystin-RR (16). The activities of GPx increased significantly after 6 h exposure in hepatocytes of common carp exposed to 10 μg/L MC-LR, in loach after oral exposure to MCs (18, 32), and in tilapia fed with cyanobacterial cells (17). Our previous study also showed that the gene expression level of GPx tended to increase in the liver of tilapia after exposure to a single 50 μg kg^{-1} body weight (bwt) dose of MC-LR (19).

It has been known that the amount of cyanotoxin is much higher in phytoplankton than the aquatic plants (33). In the present study, semi-quantitative RT-PCR was conducted to determine the constitutive expression level of GPx gene among three Chinese carps, including phytoplanktivorous silver carp and bighead carp and herbivorous grass carp, under natural environment. The constitutive expression pattern of the three carp liver GPx gene, shows a positive relationship with their tolerance to microcystins (20): resistent fish (planktivorious silver carp and bighead carp) is notably higher than sensitive fish (herbivorous grass carp). Since oxidation and ROS generation is necessarily involved in the detoxification process of cyanotoxins in hepatocytes, the high expression of liver GPx in phytoplanktivorous fish (silver carp and bighead carp), might be important to restrain over production of ROS and protect the fish liver from injury. The speculated cyanotoxin content in the food of these three Chinese carps, coincides well with their liver GPx expression level. We suggest that liver GPx might be important for Chinese carps to detoxify microcystins in phytoplankton food.

MATERIALS AND METHODS

Fish sampling

Silver carp, bighead carp and grass carp (average mass 500 g) were caught in Xiangang Reservoir (Boluo County, Guangdong Province, China). Randomly selected fish were killed, and livers were dissected immediately for RNA isolation. Five fishes for each species were sampled.

PCR cloning of GPx cDNA sequences of three Chinese carps

Total RNA was isolated using SV Total RNA Isolation System (Promega, USA). Reverse transcription was performed with oligo$(dT)_{18}$ primer using First Strand cDNA Synthesis Kit (Toyobo, Japan). Two degenerate primers GPx01F (5′-GGACATCAGGA GAACTGCAA(A/G)AA(T/C)GA(A/G)GA-3′) and GPx02R (5′-A CCAGGAACTT(C/T)TC(G/A)AA(G/A)TTCCA-3′) were designed to clone partial GPx cDNA sequences by PCR.

Gene-specific primers scGPx3′S1 (5′-AGTCTCTGAAGTAC GTCC-3′) (for silver carp and bighead carp), gcGPx3′S1 (5′-AA TCTCTGAAGTATGTCCG-3′) (for grass carp) and GPx3′S2 (5′-AAGAGAAGCTGCCTCAACC-3′) (for the three carps) were designed for 3′-RACE of three Chinese carp GPx cDNA. 3′-RACE was performed using a 3′-Full RACE Core Set (TaKaRa, Japan). Two Gene-specific primers scGPx5′S1 (5′-GATGTCATTCCTG TTCAC-3′) and scGPx5′S1 (5′-GGTCGGACGTACTTCAGAGA CT-3′) were designed in the cloned PCR fragments of silver carp GPx cDNA for 5′-RACE. 5′-RACE was performed using the SMART RACE Kit (TaKaRa, Japan).

Cloning of 5′-flanking region of silver carp GPx gene

Two Gene-specific primers GSP1 (5′-CTACTGTGGAGCTCGT TCATCTGAGTG-3′), GSP2 (5′-TGTTCCCTGTCATGCTCG-3′) were designed for cloning the sequence of 5′-flanking region of silver carp GPx gene. Genomic DNA was isolated from silver carp using Blood & Cell Culture DNA Kit (QIAGEN, USA) according to the manufacture's recommendations. Universal Genome Walker Kit (Clontech, USA) was used for cloning the sequence (34). Putative transcription regulatory regions were predicted with TFBIND (http://tfbind.ims.u-tokyo.ac.jp/) (35).

Phylogenetic analysis

Phylogenetic trees were constructed by the Neighbour-joining method using MEGA 3.1 software. The reliability of the tree obtained was assessed by bootstrapping using 1000 bootstrap replications.

Analysis of relative liver GPx expression among three Chinese carps and tissue expression pattern of silver carp GPx

The relative liver GPx mRNA abundance of three Chinese carps was determined by PCR amplification of liver cDNA samples within the exponential phase, using beta-actin as an external control. The relative liver GPx cDNA level of three Chinese carps was expressed as the ratio GPx/beta-actin cDNA (%). Values are expressed as means ± S.E. (n = 5) for each species. The tissue expression pattern of silver carp GPx mRNA among liver, adipose tissue, intestine, muscle and brain was also demonstrated by RT-PCR.

Statistical analysis

Statistical analyses of differences among treatment means of relative liver GPx cDNA level of three Chinese carps, was done using SPSS 10.0 by one-way analysis of variance (ANOVA) and the post hoc test. Differences were considered significant if $P \leq 0.05$.

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