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Comparative Biochemistry and Physiology, Part A 149 (2008) 330–337
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Molecular characterization and mRNA expression of glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder (*Paralichthys olivaceus*)

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Received 6 December 2007; received in revised form 14 January 2008; accepted 15 January 2008
Available online 19 January 2008

Abstract

Glutathione peroxidase (GPX) and glutathione S-transferase (GST) are key enzymes of cellular detoxification systems that defend cells against reactive oxygen species (ROS). In this study, we isolated the GPX and GST full-length cDNA and investigated the expression of these mRNAs from livers of olive flounder during salinity changes (35, 17.5, 8.75, 4 and 0 psu) by quantitative PCR (QPCR). GPX cDNA consists of 429 base pairs (bp) and encodes a protein of 142 amino acids. GST cDNA consists of 663 bp and encodes a protein of 220 amino acids. Both of GPX and GST mRNA expressions were the highest in 4 psu and then decreased in 0 psu. Also, the levels of Na^+ and Cl^- decreased, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) increased during the experimental period. These findings provide molecular characterization of GPX and GST in olive flounder and suggest that GPX and GST play important roles in detoxification of ROS, thereby these maybe indicators of oxidative stress responses by salinity changes in olive flounder.
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Keywords: Olive flounder; Glutathione peroxidase; Glutathione S-transferase; Salinity change; Osmotic stress

1. Introduction

In marine organisms, salinity changes in the water cause a variety of physiological stress responses such as changes in plasma hormones, energy metabolism, and electrolyte equilibrium. These changes modify the osmotic pressure on a fish body (Barton and Iwama, 1991; Fiess et al., 2006). Stress induced by salinity change has been associated with enhanced reactive oxygen species (ROS) generation, causing oxidative damage (Siers, 1985; Liu et al., 2007). ROS are produced naturally during oxidative metabolism and include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^-), and singlet oxygen ($^1O_2$) (Dalton et al., 1999). They can increase lipid peroxidation, protein oxidation, and DNA damage, and can affect cell viability by causing membrane damage and enzyme inactivation. All these then accelerate cell senescence and apoptosis (Kim and Phyllis, 1998).

To protect against ROS-generated oxidative stress, aerobic organisms have evolved complex anti-oxidant defense systems. These systems include both enzymatic and non-enzymatic components (McFarland et al., 1999; Wedderburn et al., 2000). Anti-oxidant enzymes include glutathione peroxidase (GPX), glutathione S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT). Low molecular weight anti-oxidant materials such as glutathione (GSH), ascorbic acid, metallothionein (MT), and α-tocopherol have been found in livers of marine organisms (Basha and Rani, 2003).

Among the anti-oxidant enzymes, GPX is an essential component of the cellular detoxification system. Various forms of GPX are found in vertebrates: the cellular and cytosolic GPX (GPX1), the extracellular plasma GPX (GPX2), the cytosolic gastrointestinal GPX (GPX3), the phospholipid hydroperoxide GPX (PHGPX, GPX4), and epididymis-specific GPX (GPX5). For the five distinct GPX genes identified in mammals to date, the

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doi:10.1016/j.cbpa.2008.01.013

internationally adopted nomenclature is GPX1 to GPX5 (Vernet et al., 1999). The GPXs are selenoenzymes and catalyze the reduction of hydrogen peroxide and hydroperoxides using GSH. In this process, hydrogen peroxide is reduced to water (H_2O_2 + 2 GSH → 2 H_2O + GSSG), and organic hydroperoxides are reduced to alcohols (ROOH + 2 GSH → ROH + H_2O + GSSG) (Wendel, 1980; Doyen et al., 2008). Previous studies on GPX mRNA expression and activity have investigated the goldfish (*Carassius auratus*) (Choi et al., 2007) and Mozambique tilapia (*Oreochromis mossambicus*) (Basha and Rani, 2003) exposed to cadmium (Cd), and the sea bass (*Lateolabrax japonicas*) (Jifa et al., 2006) exposed to benzo(a)pyrene and sodium dodecyl benzene sulfonate.

GST is the most important anti-oxidant enzyme in the detoxification system. There are nine forms of GST in vertebrates: alpha, pi, mu, sigma, zeta, theta, omega, kappa, and rho (Konishi et al., 2005). The GST subunits are classified on the basis of a combination of criteria, including sequence similarities, substrate specificity, and immunological identity (Sheehan et al., 2001). GST catalyzes the conjugation of lipophilic xenobiotics to reduced GSH, which typically inactivates toxic xenobiotic agents (Salinas and Wong, 1999). Thus, GST can inactivate lipoperoxidation products, lipid hydroperoxides, and their derivatives. GST also directly inactivates ROS via SH groups (Doyen et al., 2008). Previous studies of GST mRNA expression and activity have investigated the European flounder (*Platichthys flesus*) (Sheader et al., 2006) exposed to Cd, the gilthead sea bream (*Sparus aurata*) (Varó et al., 2007) exposed to dichlorvos, and the rainbow trout (*Oncorhynchus mykiss*) (Ferrari et al., 2007) exposed to carbaryl and azinphos methyl.

Earlier studies of GPX and GST mRNA expressions and activity have focused on marine organisms exposed to heavy metals and carbon compounds. However, there have been few studies on GPX and GST mRNA expressions and activity in response to salinity changes. In Korea, Olive flounder has been regarded as one of the most commercially important fish for aquaculture (Kang, 2006). But the amount of rainfall highly concentrated in rainy season of summer causes salinity to decrease. To examine the oxidative stress resulting from salinity changes, we investigated the expression patterns of GPX and GST mRNA in the livers of olive flounders and measured the levels of Na^+, Cl^-, AST, and ALT in the plasma, as potential biomarkers of oxidative stress attributable to salinity changes.

2. Materials and methods

2.1. Experimental fish

Olive flounder (*P. olivaceus*; average length: 17 ± 1.7 cm, mass: 52.9 ± 14.5 g) were collected from a commercial fish farm and reared in four semi-recirculating tanks (50 L), 10 fish per tank, in a laboratory. Water temperature and photoperiod were maintained at 20 ± 1 °C and a 12L/12D cycle, respectively.

2.2. Salinity change

The flounder were kept in seawater (35 psu) for 48 h and then transferred sequentially to tanks with salinities of 17.5, 8.75, 4 and 0 psu by adding the underground water. The fish were maintained at each salinity for 48 h and not fed during the experiments.

2.3. Sampling

Five fish from each salinity (17.5, 8.75, 4 and 0 psu) were randomly selected for blood and liver sampling, and then anesthetized with tricaine methane sulfonate (200 mg L^{-1}, Sigma-Aldrich, USA) prior to blood and tissues collection. Blood was collected from the caudal vasculature with a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 12,000 g, 5 min) and stored at −80 °C. The liver was sampled from four different fish at each salinity. Immediately after collection, the tissue samples were stored at −80 °C until total RNA was extracted.

2.4. Identification of GPX and cDNA

Mixed primers for GPX were designed using highly conserved regions of rock bream (*Oplegnathus fasciatus*) GPX (GenBank accession no. AAU44619) and rainbow trout (*O. mykiss*) GPX (GenBank accession no. AAG30013): GPX forward primer (5′-ACT CAG ATG AAC GAG CTG CA-3′) and GPX reverse primer (5′-AYT TMR CCC TCT TMA GWA GCT C-3′).

Total RNA was extracted from livers using a TRIzol kit (Gibco/BRL, USA). Reverse transcription (RT) was conducted using M-MLV reverse transcriptase (Bioneer, Korea) and polymerase chain reaction (PCR) amplification was performed using 2× Taq Premix I (Solgent, Korea) according to the manufacturer's instructions. RT was conducted at 42 °C for 50 min and PCR was subsequently performed for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 2 min, denaturation at 94 °C for 20 s, annealing at 54 °C for 40 s, and extension at 72 °C for 1 min, followed by 1 cycle of 7 min at 72 °C for the final extension. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide (0.5 μg μL^{-1}). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Korea), and then plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoRI (Fermentas, USA). Based on the plasmid DNA, GPX cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

2.5. Identification of GST and cDNA

Mixed primers for GST were designed using highly conserved regions of red sea bream (*Pagrus major*) GST (GenBank accession no. BAE06150) and rock bream GST (GenBank accession no. AAU44618): GST forward primer (5′-ATG GCT GGA ARA GTT GTG CTG-3′) and GST reverse primer (5′-TTC CTC TTG CTG CCT GGC T-3′).

Total RNA extraction and RT reaction were conducted using the same methods as for GPX. PCR was subsequently performed for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 2 min, denaturation at 94 °C for 20 s, annealing at 54 °C for 40 s, and

extension at 72 °C for 1 min, followed by 1 cycle of 7 min at 72 °C for the final extension. After PCR, the transformation and sequencing were the same as for GPX.

2.6. Rapid amplification of GPX and GST cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the RACE reaction, total RNA was extracted from the liver using a TRIzol kit (Gibco/BRL, USA). Using 3 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo (dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene, Korea).

GPX and GST-specific primers were selected from the PCR product obtained by RT-PCR. For 3′ RACE, the 50 μL PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′) and 1 μL of 10 mM 3′ RACE GPX-specific primer (5′-CTC GAG AAG GTG GAT GTG AAT GGG AAG-3′) and 1 μL of 10 mM 3′ RACE GST-specific primer (5′-GAC GGC ATG AAG CTC ATT CAG ACC AAG-3′), and 25 μL SeeAmp Taq Plus master mix (Seegene, Korea). PCR was performed for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by 1 cycle of 5 min at 72 °C for the final extension.

For 5′ RACE, the 50 μL PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mM 5′ RACE GPX-specific primer (5′-CTT CCC ATT CAC ATC CAC CTT CTC GAG-3′) and 1 μL of 10 mM 5′ RACE GST-specific primer (5′-CTT GGT CTG AAT GAG CTT CAT GCC GTC-3′), and 25 μL SeeAmp Taq Plus master mix (Seegene, Korea). PCR was performed for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 60 s, followed by 1 final extension cycle of 5 min at 72 °C. Amplified PCR products were processed by electrophoresis using 1% agarose gels containing ethidium bromide (0.5 μg μL^{-1}). The transformation and sequencing were the same as described above.

2.7. Quantitative PCR (QPCR)

QPCR was conducted to determine the normalized fold expression of GPX and GST mRNA using total RNA extracted from the liver of olive flounder during salinity changes. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows: GPX forward primer (5′-CGC ACC CCT TGT TTG TCT ATG TG-3′), GPX reverse primer (5′-CAT CGT TCC TAC TCA CTG GAC TCC-3′) and GST forward primer (5′-TCT GTC TGG ACC TGT GTA CCT G-3′), GST reverse primer (5′-GTT GGG AAA GTC TGA GAG GAT GC-3′) and β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′), β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was carried out as follows: denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data are expressed as the change with respect to the corresponding β-actin calculated threshold cycle (Ct) levels.

2.8. Phylogenetic analysis of GPX

GPX phylogenetic analysis was conducted using known vertebrate GPX amino acid sequences aligned using BioEdit software (Hall, 1999). The sequences compared to olive flounder GPX were as follows: GenBank: human (*Homo sapiens*): GPX1 (NM_000581), GPX2 (BC022820), GPX3 (NM_002084), GPX4 (NM_002085), GPX5 (NM_001509); cow (*Bos taurus*): GPX1 (NM_174076), GPX3 (NM_174007), GPX4 (NM_174770), GPX5 (NM_001025335); mouse (*Mus musculus*): GPX1 (NM_008160), GPX2 (NM_030677), GPX3 (NM_001083929), GPX4 (NM_001037741), GPX5 (NM_010343); zebrafish (*Danio rerio*): GPX1 (AAO86703), GPX4 (BC075964); goldfish (*C. auratus*): GPX1 (ABJ09418), GPX4 (EF116921); rainbow trout (*O. mykiss*): GPX1 (AF281338), GPX2 (AAV32968); rock bream (*O. fasciatus*): GPX (AAU44619); olive flounder: GPX (EU095498). The phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.9. Plasma parameters analysis

Plasma Na^{+}, Cl^{-}, AST and ALT were examined using a biochemistry autoanalyzer (Hitachi, Japan).

2.10. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by Tukey post hoc test was used to compare differences in the data ($P<0.05$). Values are expressed as mean ± S.D.

3. Results

3.1. Identification of GPX full-length cDNA

RT-PCR was used to clone a fragment of GPX cDNA using total RNA extracted from the liver of olive flounder. A single PCR product of the expected size (296 base pairs) was obtained. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone a full-length cDNA encoding a GPX from the liver of olive flounder. GPX full-length cDNA contained 429 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 142 amino acids. The amino acid sequence of olive flounder GPX was compared to those deduced from the cDNA of other teleost species. The amino acid identity of GPX with those of other fish

Fig. 1. Comparison of the deduced amino acid sequences GPX of olive flounder with several teleost. The GPX sequences used for alignment were olive flounder GPX (ofGPX, ABU49600), rock bream (rbGPX, AAU44619), rainbow trout (rtGPX, AF281338), zebra fish (zfGPX, AAO86703), goldfish (gfGPX, ABJ09418). Identical amino acids are indicated by shade. Dashes indicate the amino acid gaps to align these sequences. Selenium-binding residues are indicated by asterisks. Catalytic active sites are indicated by boxes. Residues which are important for the activity of GPX are indicated by circles.

species is: 87% with rock bream GPX (GenBank accession no. AAU44619), 85% with rainbow trout GPX (GenBank accession no. AAG30013), 78% with goldfish GPX (GenBank accession no. ABJ09418), 78% with zebrafish (*D. rerio*) GPX (GenBank accession no. AAO86703) (Fig. 1).

3.2. Phylogenetic analysis of GPX

To reveal the molecular phylogenetic position of olive flounder GPX, a phylogenetic tree was constructed. As shown in Fig. 3, the sequences of GPX grouped into five clusters, including GPX1, GPX2, GPX3, GPX4 and GPX5. Phylogenetic analysis showed that the olive flounder GPX was closely related to GPX1 of other teleost. Zebrafish GPX4 and goldfish GPX4 were related to GPX4 of mammals. But teleost GPX1 seem more similar to mammals GPX2 than mammals GPX1 in phylogenetic tree.

3.3. Identification of GST full-length cDNA

RT-PCR was used to clone a fragment of GST cDNA using total RNA extracted from the liver of olive flounder. A single PCR product of the expected size (602 bp) was obtained. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone a full-length cDNA encoding a GST from the liver of olive flounder. GST full-length cDNA contained 663 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 220 amino acids. The amino acid sequence of olive flounder GST was compared to those deduced from the cDNA of other teleost species. The amino acid identity of GST with those of other fish species is: 77% with red sea bream GST (GenBank accession no. BAE06150), 76% with rock bream GST (GenBank accession no. AAU44618), 65% with zebrafish GST (GenBank accession no. AAH60914) (Fig. 2).

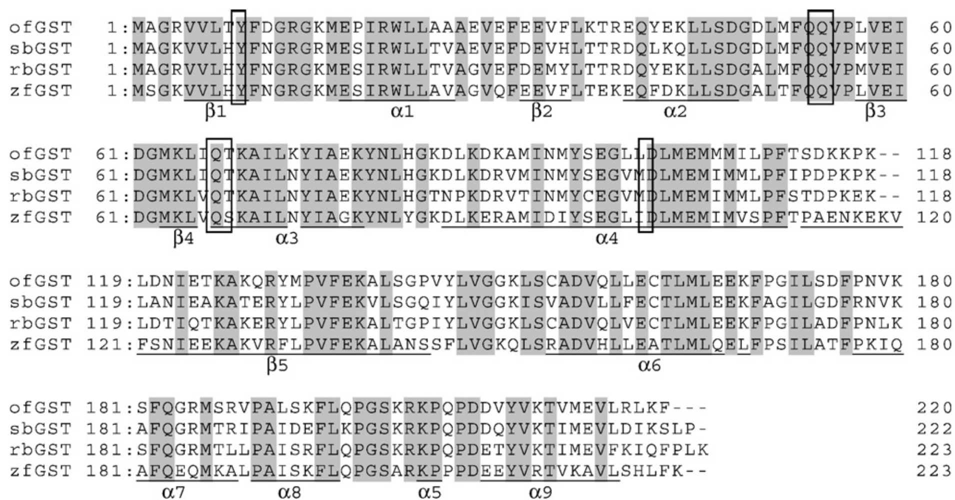

Fig. 2. Comparison of the deduced amino acid sequences GST of olive flounder with several teleost. The GST sequences used for alignment were olive flounder GST (ofGST, EU182592), red sea bream (sbGST, BAE06152), rock bream (rbGPX, AAU44618), zebra fish (zfGPX, AAH60914). Identical amino acids are indicated by shade. Dashes indicate the amino acid gaps to align these sequences. Glutathione-binding residues are indicated by boxes. Residues that make secondary structure elements are underlined. α refers to an α-helix, and β refers to a β-sheet.

3.4. QPCR for GPX and GST mRNA expression

We measured the levels of GPX and GST mRNA expressions in the liver using the QPCR, from olive flounder during salinity changes (35, 17.5, 8.75, 4 and 0 psu). The expression of GPX mRNA in the liver was increased as salinity changes, and the expression was the highest in 4 psu (14.6 fold) ($P<0.05$). The expression of GPX mRNA was decreased in 0 psu comparing to 4 psu (8.6 fold) ($P<0.05$).

Similarly, the expression of GST mRNA in the liver was also increased as salinity changed. The expression was the highest in 4 psu (17.7 fold) ($P<0.05$) and then decreased in 0 psu comparing to 4 psu (10.2 fold) ($P<0.05$) (Fig. 4).

3.5. Plasma parameters

The plasma Na^+, Cl^-, AST and ALT during salinity changes were shown in Fig. 5. The plasma Na^+ was 170±2 mM L^{-1} in 35 psu and no significant changes until in 4 psu ($P>0.05$). In 0 psu, the level of Na^+ decreased and its level was 34.3±3.2 IU L^{-1} ($P<0.05$). The plasma Cl^- was 145±2 mM L^{-1} in 35 psu and no significant changes until in 4 psu ($P>0.05$). In 0 psu, the level of Cl^- decreased and its level was 5.0±0.5 IU L^{-1} ($P<0.05$).

The plasma AST was 13.0±2.8 IU L^{-1}, and ALT was 2.5±0.5 IU L^{-1} in 35 psu and no significant changes until 4 psu ($P>0.05$). In 0 psu, the level of plasma AST increased and its level was 34.3±3.2 IU L^{-1} ($P<0.05$). Similarly, the level of plasma ALT increased and its level was 5.0±0.5 IU L^{-1} ($P<0.05$).

4. Discussion

In this study, we isolated the full-length cDNAs for GPX and GST from the livers of olive flounders exposed to salinity changes and investigated the expression patterns of GPX and GST mRNA. To obtain fundamental data regarding the physiological response to ROS-generated oxidative stress, we also analyzed changes in the plasma levels of Na^+, Cl^-, AST, and ALT.

The GPX cDNA of the olive flounder encodes 142 amino acids in one ORF and has a total of 429 nucleotides. According to the BLAST algorithm (Blastp; NCBI), the GPX amino acid sequence displays high identity with GPX of other species (rock

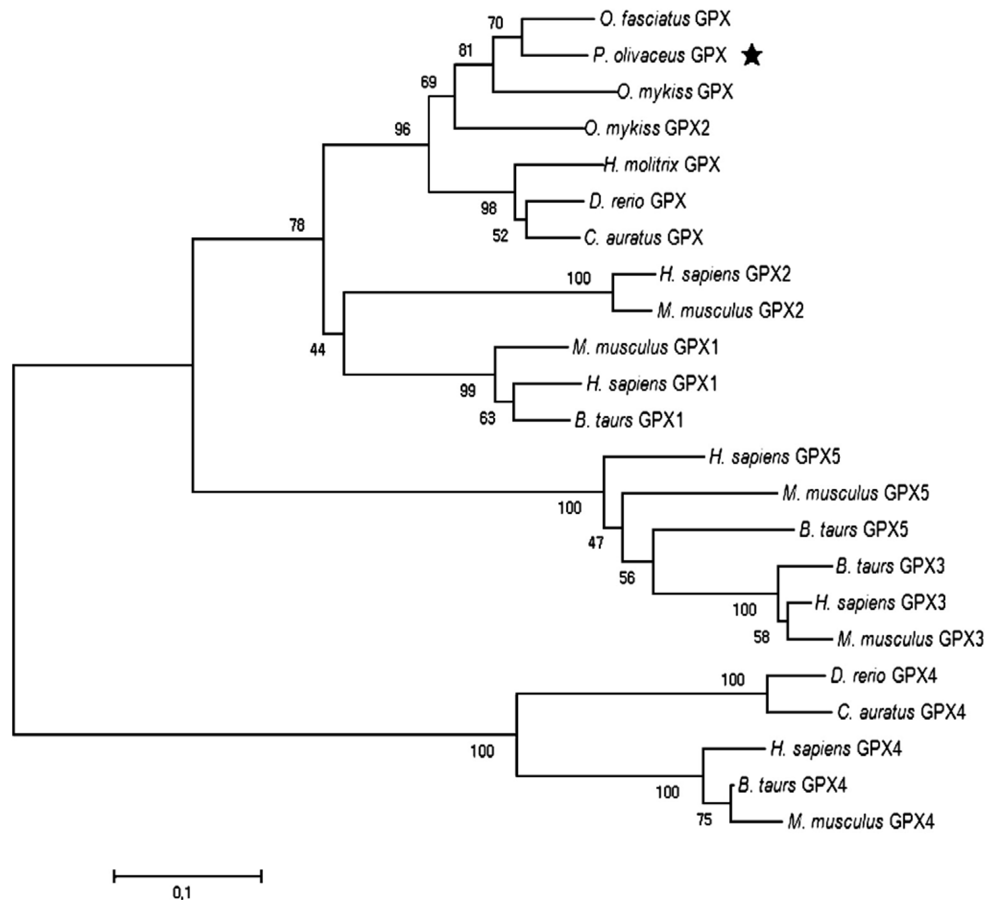

Fig. 3. The phylogenetic tree showing the relationship of olive flounder GPX with other classes of GPXs. The amino acid sequences were taken from the GenBank: human (*Homo sapiens*): GPX 1 (NM_000581), GPX 2 (BC022820), GPX 3 (NM_002084), GPX 4 (NM_002085), GPX 5 (NM_001509); cow (*Bos taurus*): GPX 1 (NM_174076GPX 3 (NM_174007GPX 4 (NM_174770GPX 5 (NM_001025335mouse (*Mus musculus*): GPX 1 (NM_008160), GPX 2 (NM_030677), GPX 3 (NM_001083929), GPX 4 (NM_001037741), GPX 5 (NM_010343); zebrafish (*Danio rerio*): GPX 1 (AAO86703), GPX 4 (BC075964); goldfish (*Carassius auratus*): GPX 1 (ABJ09418), GPX 4 (EF116921); rainbow trout (*Oncorhynchus mykiss*): GPX 1 (AF281338), GPX 2 (AAV32968); rock bream (*Oplegnathus fasciatus*): GPX (AAU44619); olive flounder: GPX (EU095498). The olive flounder GPX is indicated by asterisk. The scale bar indicates the evolutionary distance between the groups.

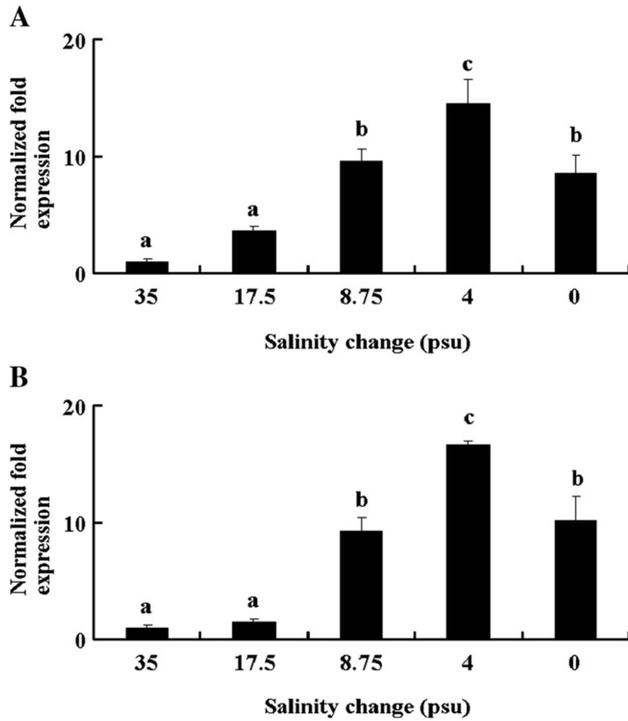

Fig. 4. GPX (A) and GST (B) mRNA levels relative to β-actin mRNA levels are analyzed by real-time PCR in olive flounder during salinity changes. The mean value of the control was set to 1. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are expressed as means ± S.D. ($n=5$).

bream, 87%; rainbow trout, 85%; goldfish, 78%; and zebrafish, 78%) (Fig. 1). And phylogenetic tree indicates that our cloned olive flounder GPX cDNA is similar to mammals GPX2 than GPX1 (Fig. 3). But GPX1 and GPX2 are both cytosolic enzymes and highly conserved (Vernet et al., 1999). Despite of phylogenetic analysis, it is difficult to clearly separate GPX1 and GPX2 in teleost.

GPX are selenium-dependent enzymes. In olive flounder GPX, the Gln26 and Trp104 residues are involved in binding selenium. Several other amino acids (Lys30, Lys119, Arg42, and Arg120) contribute to the electrostatic architecture, which reacts with the selenium. Residues known to be important for the activity of GPX (Asn21, Lys56, Glu58, and Ser87) are found in olive flounder GPX (Wang et al., 2006) (Fig. 1).

The olive flounder GST cDNA encodes 220 amino acids in one ORF and has a total of 663 nucleotides. With Blastp, the GST amino acid sequence displays high identity with alpha-class GSTs of other species (red sea bream, 77%; rock bream, 76%; and zebrafish, 65%), indicating that the olive flounder GST cDNA we cloned belongs to the alpha-class GSTs.

Two conserved domains are present in the olive flounder alpha-class GST: domain I (the G-site) in the C-terminal region of the protein, and domain II (the H-site) in the N-terminal region. Domain I (residues 1–81), which is highly specific for glutathione, consists of four β-sheets with three α-helices, arranged in a βαβαββα motif; the major amino acid residues reported to be the key in GSH binding (Tyr9 and Glu67) are located in domain I. Domain II (residues 87–222) interacts with electrophilic xenobiotic substrates and consists of six α-helices with one β-sheet; most of the residues in domain II contribute to interactions with the second hydrophobic substrate. Among the domain II α-helices, helix-α9, which is an alpha class-specific signature, is thought to be important in the binding of non-substrate ligands to alpha-class GST; it affects both the rate of GSH binding and the ionization state of the catalytically

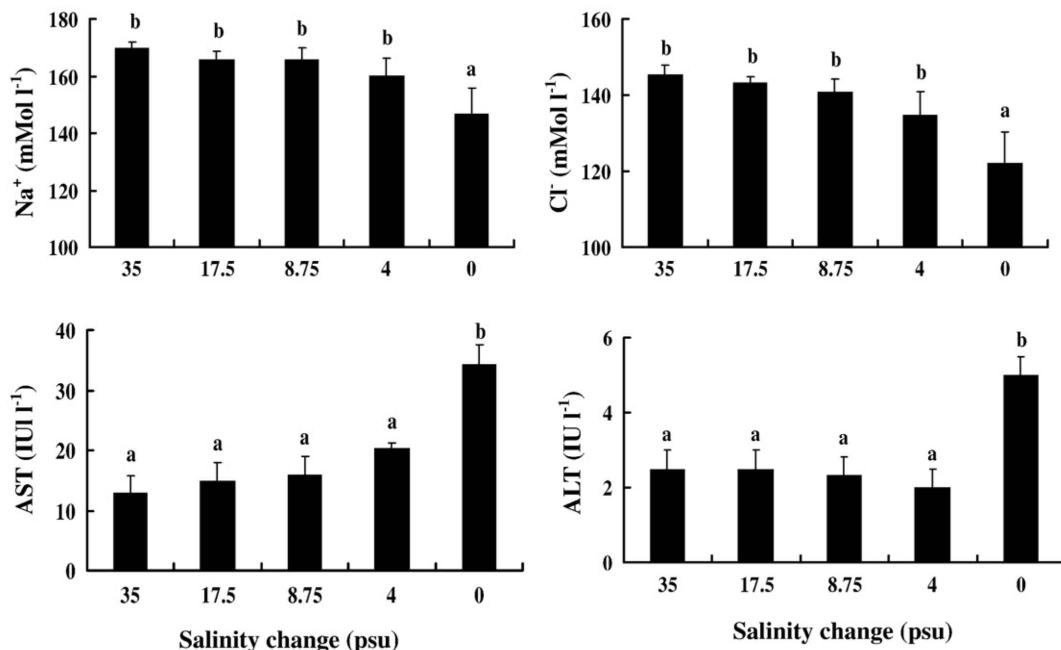

Fig. 5. Levels of plasma Na^+, Cl^-, AST, and ALT in olive flounder during salinity changes. Values with dissimilar letters are significantly different ($P<0.05$). Value represents the means ± S.D. ($n=5$).

essential residue Tyr9 (Gustafsson et al., 1999). Helix-α9 was also identified in domain II of the olive flounder GST (Fig. 2).

GPX provides a first-line defense against peroxides, superoxide anions, and hydrogen peroxide (Hughes and Gallagher, 2004). GST is part of the vitally important phase II detoxification system and catalyzes the conjugation of GSH to lipophilic xenobiotics to decrease their activity and facilitate excretion (Doyen et al., 2008). Salinity changes have been previously demonstrated to induce increased GPX activity in the whiteleg shrimp (*Litopenaeus vannamei*) and can also generate severe oxidative stress in the shrimp (Liu et al., 2007). An association between salinity changes and GST activity has also been reported, and salinity changes could be the abiotic factor causing the increased GST activity observed in Mediterranean mussels (*Mytilus galloprovincialis*) (Bebianno et al., 2006). Increased GST mRNA expression and activity were reported in Mozambique tilapia (Basha and Rani, 2003) and brown trout (Hansen et al., 2007) exposed to heavy metals, which generate ROS in vertebrates.

In the present study, a QPCR analysis of olive flounder revealed increased mRNA expression for both GPX and GST in the liver in response to salinity changes (Fig. 4), with the highest expression of each at 4 psu and decreased expression of each at 0 psu. For example, the GPX expression level at 4 psu was 14.6-fold higher than at 35 psu, and the expression level at 0 psu was 8.6-fold higher than at 35 psu. The increased GPX and GST expressions in olive flounder suggests that salinity changes caused oxidative stress and enhanced ROS-generated oxidative damage, inducing the expression of ROS-scavenging GPX and GST. However, in this study, mRNA expression for both GPX and GST decreased at 0 psu. In contrast, the plasma AST and ALT levels increased at 0 psu, indicating the presence of damaged and dysfunctional liver cells. Thus, the decrease of GPX and GST mRNA expressions at 0 psu was likely the result of liver damage. This is consistent with previous studies demonstrating decreased GPX and GST activity and increased plasma AST and ALT levels in gilthead sea bream exposed to Cd. As well as, silver carp (*Hypophthalmichthys molitrix*) exposed to hepatotoxic microcystins (Vaglio and Landriscina 1999; Li et al., 2007).

We also determined the plasma levels of Na^+, Cl^-, AST, and ALT in olive flounder at different salinities (Fig. 5). The Na^+ and Cl^- levels decreased with decreasing salinity, but not significantly so until 4 psu, after which they decreased significantly more at 0 psu. It seems that the balance of ions and water needed to adapt to the hypo-osmotic environment, and the osmoregulation was disrupted at 0 psu. These results are in agreement with those in gilthead sea bream (Mancera et al., 1993) and black porgy (*Acanthopagrus schlegeli*) (Chang et al., 2007).

The amino transfer enzymes AST and ALT are a general index of liver function in vertebrates. In fish, they are used to evaluate stress responses caused by temperature change, low oxygen, pH, ammonia, or heavy metals (Pan et al., 2003). In this study, the AST and ALT levels were not significantly changed until 4 psu, followed by a further increase at 0 psu. This suggests that salinity changes damage liver cells and degrade liver function in olive flounder, which is consistent with previous study in black porgy (Chang et al., 2007).

In summary, we isolated full-length GPX and GST cDNAs and determined the expression patterns of GPX and GST mRNAs in the liver of the olive flounder in response to salinity changes. With decreasing salinity to 4 psu, the mRNA expression levels for both GPX and GST were increased in the liver, presumably because of their roles as ROS scavengers, and the plasma levels of AST and ALT were increased at 0 psu. These results indicate that the liver cells of the olive flounder were damaged and their function was degraded by oxidative stress during salinity changes. Furthermore, decreases in the Na^+ and Cl^- levels suggest that the fish's ability to osmoregulate was disrupted in the hypo-osmotic environment. The results of this study will be used as basic data for determining the degree of oxidative stress induced by salinity changes.

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