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Molecular cloning of four glutathione peroxidase (GPx) homologs and expression analysis during stress exposure of the marine teleost *Sparus aurata*

Emmanouil E. Malandrakis *, Athanasios Exadactylos, Olga Dadali, Eleni Golomazou, Spiridon Klaoudatos, Panagiota Panagiotaki

Department of Ichthyology and Aquatic Environment, School of Agricultural Sciences, University of Thessaly, Fytokou str. 38445, Volos, Greece

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ABSTRACT

Glutathione peroxidase (GPx; EC 1.11.1.9) is an important family of enzymes that protects organisms from oxidative damage. Four full-length GPx cDNAs were cloned and characterized by rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) from the liver of gilthead sea bream (*Sparus aurata*), an economically important species for Mediterranean aquaculture. Structural and functional annotations were performed for all paralogs, which suggested possible differences in function and subcellular localization. The phylogenetic analysis, based on the amino acid sequences, revealed four groups corresponding to teleostean GPx1a, GPx1b, GPx4a, and GPx4b and three groups for mammalian GPx1, GPx2 and GPx4. The tree topology indicated past duplication events for fish genes, unlike their mammalian homologs. Transcriptional analysis in ten tissues by reverse transcription quantitative polymerase chain reaction (RT-qPCR) evidenced a tissue-specific pattern for each GPx homolog. Fish experimental groups were exposed to stress factors such as fasting and confinement. Relative expression analysis in fish liver demonstrated that GPx1 genes were not regulated by dietary restriction; GPx4b was differentially expressed opposed to regularly fed fish. On the other hand, both GPx1 and GPx4 genes were up-regulated in fish post exposed to confinement, considered as a response to acute stress. The results underline the role of GPx genes as indicators of stress and welfare status in gilthead sea bream aquaculture.

1. Introduction

Reactive oxygen species (ROS) are chemically active compounds containing oxygen and occur naturally as by-products of metabolism. Oxidative stress is caused by a disequilibrium between the production of ROS and the ability of a cell, a tissue or an organism to detoxify ROS and repair the resultant damage. Glutathione peroxidases (GPx; EC 1.11.1.9) utilize glutathione (GSH) as a cofactor and reduce peroxides, such as hydrogen peroxide or organic hydroperoxides. These are reduced to water and alcohols, in the presence of GSH, which acts as electron donor and is simultaneously oxidized to glutathione disulfide (GSSG). GSSG is converted back to GSH in the presence of NADPH by glutathione reductase. GPx genes have been characterized in various forms of life including bacteria, fungi, plants and animals (Missall et al., 2005; Navrot et al., 2006; Mariotti et al., 2012).

GPx isoforms in mammalian cells differ in their cellular expression and substrate specificity (Flohe and Brigelius-Flohe, 2012). GPx enzymes are characterized by the presence of a selenocysteine active site, which is actually a selenol encoded by an opal (UGA) codon. GPx transcripts bear a selenocysteine insertion sequence (SECIS) element, which occurs in the 3′UTR of the mRNA molecule. Naturally occurring GPx isozymes, which incorporate cysteine instead of selenocysteine, have been described in mammals (Ghyselinck et al., 1991). On the other hand, when selenium-dependent GPx mutants where engineered such selenocysteine to cysteine substitution reduced dramatically enzyme activity (Maiorino et al., 1995).

GPx1 and GPx4 genes have been recently characterized in fish, including commercially important species, such as silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*) and grass carp (*Ctenopharyngodon idellus*) (Li et al., 2008), southern bluefin tuna (*Thunnus maccoyii*) (Thompson et al., 2010), Pacific bluefin tuna (*Thunnus orientalis*) (Yamashita et al., 2012) and rainbow trout (*Oncorhynchus mykiss*) (Pacitti et al., 2013). Cloning of GPx1 in yellowtail kingfish (*Seriola lalandi*) (Bain and Schuller, 2012b) was carried out in order to study antioxidant gene expression induction by tert-butyl hydroquinone (TBHQ) in cultured cells. TBHQ is a known phenolic metabolite of a fish feed preservative. Down-regulation of GPx4a in coho salmon (*Oncorhynchus kisutch*) olfactory system was associated with cadmium (Cd) exposure *in vivo* (Wang et al., 2012).

Oxidative stress during food deprivation has been extensively studied in mammals (Robinson et al., 1997; Gomi and Matsuo, 1998; Lee et al., 2006; Sorensen et al., 2006; Tucci et al., 2010). Unlike mammals, fish may survive longer periods of food restriction. Thus, growth

* Corresponding author.
E-mail address: emalandrak@uth.gr (E.E. Malandrakis).

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compensation and proximate composition are well studied in food deprived fish (for review see Ali et al., 2003). On the other hand, little is known in respect to oxidative stress induction (Pascual et al., 2003; Bayir et al., 2011). For example, growth reduction in food deprived salmon was associated with down-regulation of genes involved in oxidative stress response, such as catalase (CAT) and hemopexin (HPX) (Skugor et al., 2011).

While extensive studies on enzyme activity of GPx have been carried out in respect to acute stress in fish, studies on gene expression of GPx homologs are scarce. Castro et al. (2012) demonstrated that GPx enzyme activity was increased in response to heat shock in fish fed with a diet containing 55% protein and decreased in fish fed with 45% protein. GPx enzyme activity was shown elevated in response to heat shock in goldfish (Bagnyukova et al., 2007). Calduch-Giner et al. (2010) recognized a set of genes implicated in intracellular trafficking and scavenging of ROS, which was up-regulated in gilthead sea bream following confinement exposure. These genes play a cytoprotective role against oxidative insults, promoting the synthesis of cysteine and reducing NADPH equivalents. Thus, the repertoire of genes involved in glutathione synthesis and recycling was highlighted. However, transcriptional orientation and activation of either GPx1 or GPx4 paralogous genes (a and b forms) are considered, in some cases, as entities whenever their antioxidant functionality is studied (e.g. Thompson et al., 2010).

Therefore, the present study was focused on four full-length GPx cDNAs (GPx1a, GPx1b, GPx4a and GPx4b), which were isolated from the liver of gilthead sea bream (*Sparus aurata*) and compared with other known homologs available in public databases. Moreover, tissue specific expression of these four homologs was studied. Also, a null hypothesis was posed whether period of fasting, or time of confinement affects GPx gene expression patterns. Liver was chosen as being the major organ of enzymatic transformation of ROS (Schwabe and Brenner, 2006). Both these treatments (fasting and confinement) are considered to be quite important stress factors during intensive rearing of fish. Handling stress is introduced in several cases, as netting is a common practice in aquaculture enterprises e.g. grading, vaccination etc., while on the other hand, food restriction is experimentally applied for evaluation of growth compensation phenomena.

2. Material and methods

2.1. Fish rearing and sampling

Two hundred fifty gilthead sea bream (*Sparus aurata*) specimens were transferred from a commercial farm (Dias SA, Fthiotida, Greece) and randomly allocated, equally, into six polyester tanks of 500 L each, connected with a re-circulating system. Rearing conditions followed previously described protocols (Papoutsoglou et al., 1999) with minor modifications. Fish groups, sorted evenly in all tanks, were hand-fed twice a day (09:00 and 14:00 h) with 4.5 mm extruded pellets according to the provided feeding regime (1.8% w/w) of the manufacturer (Biomar, Volos, Greece). At the day of the experiment initialization, fish had an overall average initial mass of 63.5 ± 4.4 g and an average standard length of 13.9 ± 0.3 cm. The experimental design included a fish starving group, a group implemented to confinement and a control group, along with their corresponding duplicates.

Fish exposed to confinement were trapped in a net inside the tank. Fish were sampled at 1, 3 and 5 min of confinement. Additional specimens were subjected to netting for 5 min and were released back to the tank in order to recover from stress and were re-sampled after 1 and 24 h (post exposure). Fish exposed to fasting remained unfed for 42 days and were sampled after 7, 14, 21 and 42 days of starvation initiation. For all treatments, feeding was denied for 24 h before sampling. Sampling occurred throughout the experiments between 8:00 and 10:00 h to avoid fluctuations on daily rhythms of antioxidant enzymes' expression.

Briefly, six fish from each experimental group and per time point were stunned (Directive, 2010/63/EU) and liver was extracted; in total 60 fish specimens were sampled during the 3 months' experimental period. Additionally, three fish were obtained for tissue specific gene expression (liver, brain, muscle, kidney, head kidney, heart, spleen, gills, stomach and mid-intestine).

Samples, immediately after stunning, were submerged in RNAlater (Life Technologies, Carlsbad, CA, USA) and stored in −20 °C until further processing. RNA was extracted from all tissues with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the standard protocol as described by the supplier. Quantity and quality of the extracted RNA were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., DE, USA) and gel electrophoresis, respectively. Finally, total RNA was treated with DNase I amplification grade (Invitrogen), in order to eliminate genomic DNA contamination.

2.2. cDNA isolation and subcloning

Zebrafish GPx1a (BC083461), GPx1b (BC081388), GPx4a (EU090067) and GPx4b (EU665237) were found in the NCBI database. Blast search ("somewhat similar sequences") of the EST database (http://www.ncbi.nlm.nih.gov/nucest/) at NCBI, using these zebrafish sequences, identified multiple sea bream ESTs of potential GPx homologs. To obtain the sea bream full-length GPxs, ESTs were aligned, specific primers were designed and used for 3′ and 5′ RNA ligase mediated rapid amplification of cDNA ends PCR (RLM-RACE) (GeneRacer, Invitrogen, Carlsbad, CA, USA). Primer sequences, annealing temperatures and primer regions are listed in Table 1. PCR was performed with KAPA Taq DNA Polymerase (KAPA Biosystems, Cape Town, South Africa) in an MJ Research PTC-200 thermal cycler. The PCR products were gel purified, ligated in a pcR®II-TOPO® vector, transformed in TOP10 chemically competent cells (Invitrogen) and sequenced with the M13 forward and reverse primers (DBS, Durham, UK).

2.3. Nucleotide analysis and phylogenetic reconstruction

The sequences obtained using 3′ and 5′ RACE-PCR were assembled by their overlapping sequences into full length cDNAs. SECISearch 2.19 program (http://genome.unl.edu/SECISearch.html) was used to identify the SECIS secondary structures (Kryukov et al., 2003). Amino acid sequences, active sites, other annotations and pairwise identities (%) were predicted using Geneious 4.8.5 software (Biomatters Ltd, Auckland, New Zealand). Sea bream GPx alignments were constructed using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2) (Andersen et al., 2004). Homologous sequences in public databases were obtained with the aid of blastn, blastp and tblastn algorithms at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) (Petersen et al., 2011) and TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP) (Emanuelsson et al., 2007) were used to predict signal peptides and subcellular localization of the GPx enzymes.

For phylogenetic analysis of amino acid sequences, sea bream GPx enzymes were used along with 31 isozymes of mammalian and teleostean GPxs. Amino acid sequences were aligned with the ClustalW algorithm and a dendrogram was created by the neighbor-joining method using the software MEGA 5.10 (http://www.megasoftware.net) (Tamura et al., 2011). Jones–Taylor–Thornton matrix (Jones et al., 1992) served as the substitution model for amino acids, gaps/missing data were pairwise deleted and the phylogeny test was replicated 10,000 times.

2.4. Gene expression analysis

cDNA was generated from 1 μg of total RNA using SuperScript® III First-Strand Synthesis Supermix (Invitrogen) up to a final volume of 20 μL. The generated cDNA was incubated for 20 min at 37 °C with