Comparative Biochemistry and Physiology, Part B 159 (2011) 1–9

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

A selenium-dependent glutathione peroxidase in the Japanese scallop, *Mizuhopecten yessoensis*: cDNA cloning, promoter sequence analysis and mRNA expression

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ARTICLE INFO

Article history:
Received 2 November 2010
Received in revised form 7 January 2011
Accepted 17 January 2011
Available online 26 January 2011

Keywords:
Mizuhopecten yessoensis
Selenium-dependent glutathione peroxidase
mRNA expression
Promoter analysis
SNP

ABSTRACT

Glutathione peroxidase (*GPx*) is an antioxidant enzyme that protects cells from oxidative damage in the innate immune responses against bacterial infections. *GPx* is also involved in immune defenses. In this study, we report cloning and characterization of a *GPx* (designated as *MyGPx*) coding sequences and promoter from Japanese scallop, *Mizuhopecten yessoensis*. The full-length 1081 nt *MyGPx* mRNA contained a 28 nt 5′ untranslated region (UTR), a 603 nt open reading frame and a 450 nt 3′ UTR containing a polyadenylation signal (AATAAA). Multiple sequence alignment revealed that amino acids essential to enzymatic function of *MyGPx* proteins were highly conserved. A 1628 nt 5′-flanking sequence of *MyGPx* was identified by genome walking. Here, several potential transcription factor binding sites were detected in the putative promoter region, and nine single nucleotide polymorphisms (SNPs) were found in the 5′ sequence flanking the promoter region. Quantitative Real time PCR (qRT-PCR) was employed to measure *GPx* mRNA expression in adult tissues and monitor mRNA expression patterns during embryonic development and following stimulation by the bacteria *Vibrillo anguillarum*. Collectively, the results suggest that *MyGPx* fulfills an important function during *M. yessoensis* development and may be an important immune effector in adult molluscs.

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1. Introduction

The Japanese scallop, *Mizuhopecten yessoensis,* is an economically important marine mollusc found in the cold seas along the coasts of the northern islands of Japan, the northern part of the Korean Peninsula, and the Sakhalin and Kuril islands (Nagashima et al., 2005). In 1982, *M. yessoensis* (332 individuals) was introduced into China from Japan for aquaculture production, and rapidly became a favored aquaculture species in northern China due to its large size and high market value. However, problems with high mortality, poor growth and seed production have heavily impacted on commercial *M. yessoensis* farming for more than 10 years, and have caused serious economic losses to the scallop aquaculture industry in China. Mass infections of cultivated scallops are thought to be related to seawater pollution, opportunistic pathogens and stock degeneration (Pipe and Coles, 1995).

An improved understanding of the immune system of *M. yessoensis* will facilitate the effective management of disease and the development of sustainable scallop cultures. Like most molluscs, *M. yessoensis* does not have an adaptive immune system and instead relies on the innate immune system to defend itself against a variety of pathogens (Medzhitov and Janeway, 2002; Loker et al., 2004).Former studies have indicated that mollusc hemocytes undergo phagocytosis when the organism is attacked by microorganisms or viruses (Marikovsky et al., 2003). Invading bacteria are killed by hemocytes through the production of large amounts of reactive oxygen species (ROS), such as superoxide ($O_2\cdot^-$), hydroxyl radicals ($HO\cdot^-$) and hydrogen peroxide (H_2O_2) (Bogdan et al., 2000). Although ROS plays an important role in host defense, the unimpeded production or inadequate clearance of ROS will have detrimental effects on surrounding cells. Oxidative stress caused by high levels of ROS can lead to lipid peroxidation, protein oxidation, DNA damage, membrane disruption, and mitochondrial dysfunction (Schreck et al., 1991; Mourente et al., 1999). Consequently, maintaining ROS at manageable levels is essential for normal cell function and organism survival. Living organisms have evolved complex antioxidant defense systems for protection against oxidative stress generated by ROS. These systems consist of enzymatic (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (*GPx*)) and non-enzymatic components (ascorbic acid, β-carotene, glutathione, and α-tocopherol) (McFarland et al., 1999; Wedderburn et al., 2000).

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doi:10.1016/j.cbpb.2011.01.003

GPx is one of the most important endogenous antioxidant proteins and has a wide distribution in every oxygen-consuming living organism (Liu et al., 2010). It mediates antioxidant defense reactions by catalyzing the reduction of organic hydroperoxides and hydrogen peroxide to water and oxygen using GSH as an electron donor (Kutlu and Susuz, 2004; Drevet, 2006). Currently, four major selenium-dependent *GPx* (Se-GPx) have been identified in mammals: cellular *GPx* (GPx1) (Rotruck et al., 1973), gastrointestinal *GPx* (GPx2) (Chu et al., 1993), extracellular *GPx* (GPx3) (Takahashi and Cohen, 1986), and phospholipid hydroperoxide *GPx* (GPx4) (Ursini et al., 1985). Four other selenium-independent *GPx*, GPx-5 GPx-6, GPx-7 and GPx-8, are also present in mammalian tissues. Interestingly, the active site of GPx-6 contains a selenocysteine residue in humans, whereas it contains a cysteine residue in the mouse and rat (Kryukov et al., 2003). A functional difference between Se-GPx and non-Se-GPx is that Se-GPx enzymes can catalyze the reduction of both organic and inorganic peroxides like H_2O_2, whereas non-Se-GPx enzymes react exclusively with organic peroxides (Chambers et al., 1986; Perry et al., 1992.

In previous studies, *GPx* mRNA expression was induced in Zhikong scallop (*Chlamys farreri*) (Mu et al., 2010) challenged by *Listonella anguillarum*, and in Chinese shrimp (*Fenneropenaeus chinensis*) (Ren et al., 2009) and White shrimp (*Litopenaeus vannamei*) (Liu et al., 2007) injected with *Vibrio anguillarum*, suggesting that *GPx* may play a significant role in the innate immune system of marine invertebrates. In addition to its immune function, *GPx* was also found to have primordial roles during vertebrate embryogenesis and sperm maturation (Bösl et al., 1997; Thisse et al., 2003; Ursini et al., 1999; Pfeifer et al., 2001).

Recently, *GPx* cDNA has been obtained from various species; however, little is known about the promoter region of these genes. In this study, we describe the cDNA cloning and characterize the promoter region of a *GPx* gene from *M. yessoensis*. QRT-PCR was employed to measure *GPx* mRNA expression level in adult tissues and to monitor mRNA expression pattern during embryonic development and in adult tissues, following stimulation by *V. anguillarum*. Furthermore, the structural organization of the *MyGPx* promoter was predicted using TRANSFAC and AliBaba 2.1 software. Nine putative SNPs identified in the regulatory region of the gene will serve as additional molecular markers in the development of improved aquaculture genetics and breeding.

2. Materials and methods

2.1. Scallops

Adult *M. yessoensis* (averaging 80 mm in shell length) were obtained from Bilong farm in Dalian, Liaoning province and were incubated in seawater until use in later experiments. Embryonic and larval stage specimens were obtained from the *M. yessoensis* culture base of Liaoning Ocean and Fisheries Science Research Institute, China.

2.2. Cloning and sequencing of MyGPx

Total RNA was isolated from *M. yessoensis* using the RNAprep pure Tissue Kit (Tiangen Biotech, Beijing). The quality and quantity of the isolated RNA was assessed by agarose gel electrophoresis and spectrophotometry, respectively. cDNA was synthesized by reverse transcription using ~500 ng mRNA, 2 μl PrimeScript™ Buffer, 0.5 μl PrimeScript™ RT Enzyme Mix I, and 0.5 μl Oligo(dT)-adaptor primer made up to a 10 μl reaction mixture, according to the manufacturer's instructions (Promega,USA). The mixture was incubated at 37 °C for 15 min and the reaction was stopped by heating at 85 °C for 5 s.

The expressed sequence tag (EST) sequence for glutathione peroxidase was obtained from the *M. yessoensis* mantle cDNA library (GenBank accession no. **GR867007-GR867987)**, recently constructed using the creator SMART cDNA library construction kit (Clontech, Palo Alto, CA, USA) (Liu et al., 2010). Subsequently, 3′-RACE (rapid amplification of cDNA ends) was carried out using the 3′-Full RACE Core Set Kit (TaKaRa, Dalian, China). For 3′-RACE, two gene-specific primers, QMyGPx-F and MyGPx3′-F2, (Table 1) were designed according to the EST sequence. The 3′-end of the *GPx* gene was cloned by nested PCR using the QMyGPx-F primer and the 3′ RACE Outer Primer in the first round of PCR, and the MyGPx3′-F2 primer and the 3′ RACE Inner Primer in the second round of PCR. 3′-RACE PCR reactions were carried out at 94 °C for 3 min; followed by 35 cycles at 94 °C for 30 s, then at 55 °C for 30 s, and 72 °C for 2 min; followed by an extension step at 72 °C for 10 min. Specific PCR products were cloned into pMD19-T Simple Vectors (TaKaRa, Dalian, China) and sequenced in two directions.

2.3. Analysis of MyGPx amino acid sequences

The predicted amino acid sequences of the *MyGPx* gene were analyzed using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequences of *GPx* from different organisms were aligned using the Clustal 1.81 program (Thompson et al., 1997) using default settings. A phylogenetic tree of *GPx*, constructed by the maximum likelihood (ML) method, was drawn using PhyML 3.0 software at http://www.atgc-montpellier.fr/phyml/. The reliability of the branching was tested by 1000 bootstrap replications. The presence and location of the signal peptide was predicted at http://www.cbs.dtu.dk/services/SignalP/. The molecular mass and theoretical isoelectric point of the protein was predicted using a Protein MolWt and AA Composition Calculator (http://www.proteomics.com.cn/proteomics/pi_tool.asp).

2.4. Cloning and analysis of the promoter sequence

Genomic DNA isolated from the adductor muscle was purified using the traditional phenol–chloroform method (Li et al., 2007). The Genome Walking Kit (TaKaRa, Japan) was used to isolate the promoter region of the *GPx* gene. Genome walkings were carried out using three gene-specific primers (Table 1) based on the *MyGPx* cDNA sequences as well as four shorter arbitrary degenerates (AP1,

Table 1
Oligonucleotide primers used in this study.

Primer	Sequence (5′-3′)	Sequence information
QMyGPx-F	AACCACGAGATCCTGACAGCC	3′ RACE and Real-time MyGPx primer
MyGPx3′-F2	ACAGGTCTTACTGAAGCCGTAG	3′ RACE primer
3′ RACE outer primer	TACCGTCGTTCCACTAGTGATTT	3′ RACE primer
3′ RACE inner primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG	3′ RACE primer
QMyGPx-R	AAGGAGGTTGGATCGTCACTCG	Real-time MyGPx primer
Qβ-actin-F	AGTCCCAATCTACGAAGGTTATG	Real-time β-actin primer
Qβ-actin-R	CCAGTGATGAGGAGGAAGCAG	Real-time β-actin primer
M13-47	CGCCAGGGTT TTCCCAGTCACGAC	Vector primer
RV-M	GAGCGGATAACAATTTCACACAGG	Vector primer
MyGPx-GSP-1	GTGTAATCCCGTACAGTCGTGTCTCAG	Genome walking primer
MyGPx-GSP-2	TTCCACGCCAAGACATTGGATGGAGA	Genome walking primer
MyGPx-GSP-3	AGGACTCCGGACACTGACAACCGTCGC	Genome walking primer
MyGPx-SNP-F	CCATATACAACTGTTATCCGATTACCG	Locating MyGPx SNP primer
MyGPx-SNP-R	AATGTCTTGGCGTGGAAGTCG	Locating MyGPx SNP primer

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