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Cloning, identification and functional characterization of a pi-class glutathione-S-transferase from the freshwater mussel *Cristaria plicata*

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ABSTRACT

Glutathione-S-transferases (GSTs) are multifunctional phase II detoxification enzymes that catalyze the attachment of electrophilic substrates to glutathione and play an important role in protecting organisms against the toxicity of reactive oxygen species (ROS). The piGST cDNA was cloned and sequenced after rapid amplification of cDNA ends (RACE) from the freshwater mussel *Cristaria plicata*. The comparison of the deduced amino acid sequences with GSTs from other species showed that the enzymes belonged to the pi-class and the amino acids defining the binding sites of glutathione (G-site) and for xenobiotic substrates (H-site) were highly conserved. The Cp-piGST cDNA is 816 nucleotides (nt) in length and contained a 615 nt open reading frame (ORF) encoding 205 amino acid residues, and has 19 nt of 5′ untranslated region (UTR) and a 3′ UTR of 182 nt including a tailing signal (AATAAA) and a poly (A) tail. The molecular weight of the predicted piGST is 23.4 kDa, with the calculated PI being 5.2. The mRNA transcript of Cp-piGST could be detected in all the examined tissues with highest expression level in hepatopancreas. The expression level of Cp-piGST in hepatopancreas and gill showed similar trend that were significantly increased after bacterial challenge compared to the control group at 12 h. Furthermore, the recombinant Cp-piGST with high enzyme activity was induced to be expressed as a soluble form by IPTG at 20 °C for 8 h, and then was purified by using the native Ni^{2+} affinity chromatography. The specific activity of the purified soluble Cp-piGST enzyme into pET30 was 2.396 μmol/min/mg, and which into pET32 was 1.706 μmol/min/mg. The recombinant Cp-piGST had a maximum activity at approximately pH 8.0, and its optimum temperature was 37 °C. The recombinant Cp-piGST enzyme activity became lower gradually with the denaturant concentration increasing.

1. Introduction

Molluscan hemocytes eliminate intracellular pathogens and extra cellular parasites by phagocytosis or encapsulation. During these processes increased amounts of oxygen are consumed, the cells undergo an oxidative burst and produce large amounts of reactive oxygen intermediates (ROI_S) [1]. However, too much increase of superoxide anion and these other ROIs may pose a potential cytotoxic problem to the host [2]. Organisms have evolved different strategies for coping with the negative reactions of ROS by a group of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) and nonenzymatic antioxidant molecules [3].

Among the antioxidant enzymes, GSTs are members of a superfamily of multifunctional dimeric proteins involved in the cellular detoxification of reactive electrophilic compounds, including various intracellular metabolites, pollutants, drugs and pesticides [4], and in protecting tissues against oxidative damage [5]. Moreover, GSTs also play a prominent role in many other physiological processes, including transporting endogenous hydrophobic compounds, catalyzing biosynthetic reactions and acting as signaling molecules [6]. Three major families of GSTs have been identified in mammals, the cytosolic GSTs (namely alpha, mu, pi, theta, sigma, omega and zeta), the mitochondrial GST (kappa class) and microsomal GSTs [4]. In contrast to vertebrate GSTs, a few studies have been carried out in the invertebrates. In mollusks, several GST genes belonging to different classes have been isolated and characterized. The omega class GST has been identified in the oyster *Crassostrea gigas* [7] and *Haliotis discus discus* [8]. The mu-class has been described in *Thais clavigera* [9], *Chlamys islandica*

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(AM279651), *H. discus discus* [8] and *Cyphoma gibbosum* [10]. The sigma class GST was identified in *Haliotis diversicolor* [11], *H. discus discus* [8], and *Laternula elliptica* [12]. Moreover, some cDNA sequences of different class GSTs from *Crassostrea ariakensis*, *Pinctada fucata* and *Chlamys farreri* have been recorded in GenBank.

The pi-class GST is a major class of cytosolic GST in normal tissues, which can inactivate lipoperoxidation products, lipid hydroperoxides and their derivatives, and reactive oxygen species via their SH group [6]. So far, some pi-class GST have been cloned and described in mollusks, such as *Mytilus edulis* [13], *Unio tumidus* [6], *Mytilus galloprovincialis* [14], *Venerupis philippinarum* [15], *Dreissena polymorpha* [16], *L. elliptica* [17]. It is known that GSTs also play an important role in cellular defense against oxidative stress.

Cristaria plicata, a species of freshwater bivalve of the mollusca phylum, is widely distributed throughout eastern Asia. It is often used in the cultured pearl industry in china. At present, it has been suffering serious problems due to the outbreak of diseases, especially wound infection caused by nucleus inserting operation which leads to high mortality in the process of pearl production [18]. In order to prevent disease from pearl oyster, it is necessary to further research the innate immune mechanisms of bivalves.

Previously, an intracellular Cu–Zn SOD has been cloned and described from *C. plicata* [19]. In this study, a Cp-piGST gene was cloned from *C. plicata*. The expression patterns of Cp-piGST mRNA transcript in different tissues of normal bivalve and in *Aeromonas hydrophila* challenged bivalve hepatopancreas and gill tissues were analyzed by quantitative real-time PCR. The Cp-piGST gene was subcloned into the pET30a and pET32a vector and expressed in *Escherichia coli* BL21 (DE3), respectively. In addition, the properties of recombinant Cp-piGST was detected under the different temperature, pH, and denaturant.

2. Materials and methods

2.1. Experimental animal, bacteria challenge, RNA isolation and cDNA synthesis

The bivalve *C. plicata*, shell length 18–25 cm, were collected from Poyang Lake in Jiangxi province, China. They were maintained at room temperature in freshwater tanks under natural photoperiod for a week, and were fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana*, and acclimatized in water tanks for one week till further processing.

Fifty bivalves were employed for expression patterns experiment. The bivalves were randomly divided into 2 groups and each group contained 25 animals. The groups were injected into the adductor muscle 0.1 mL of phosphate buffer solution (PBS, pH 7.0) as control group or 0.1 mL bacterial suspension (*A. hydrophila*, dissolved in PBS, 10^9 cell mL^{-1}) as challenged group. Hemocytes, gill, hepatopancreas, muscle and mantle from five animals of each experiment group and control group were collected at 0, 6, 12, 24 and 48 h post-injection and immediately stored in liquid nitrogen until used. Total RNA samples were extracted using the TRIzol reagent (Invitrogen) according to the manufacture's instructions. The amount and the integrity of each RNA were checked by electrophoresis on 1% agarose gel in TAE (Tris 40 mM, Acetic acid 1 mM, EDTA 40 mM) buffer. The gel was stained with ethidium bromide and RNAs were visualized under UV light. The Smart cDNA was synthesized and amplified using a Clontech SMART PCR cDNA Synthesis Kit (Clontech) by following the supplier's protocol. The synthesis reactions were performed at 65 °C for 5 min, 42 °C for 1 h, and terminated by heating at 70 °C for 15 min, the cDNA mix was stored at −80 °C for PCR and quantitative real-time PCR.

2.2. Amplification and cloning of an internal Cp-piGST cDNA fragment

PCR amplification was done by using Smart cDNA as template, and two degenerated primers (GSTF1d and GSTR1d) designed on the basis of the cDNA conserved regions of piGST coding sequences from different species, *U. tumidus* (AAX20373.1), *M. edulis* (AAS60226.1), *M. galloprovincialis* (AAM91994.1). The primers were obtained from Generay Biotechnology (shanghai China). PCR was done in a 25 μL reaction containing H_2O 18.5 μL, 10 × PCR buffer 2.5 μL, dNTPs 2.0 μL, GSTF1d 0.6 μL, GSTR1d 0.6 μL, cDNA 0.6 μL, ExTaq (Takara, Japan) 0.2 μL and the cycling parameters included a 5 min denaturation at 94 °C, 35 cycles of heat denaturation at 94 °C for 30 s, annealing at 38 °C for 30 s, polymerization at 72 °C for 1 min, and a 10 min final extension at 72 °C. The PCR product was then cloned into the pGEM-T vector (Promega) and was sequenced in both directions.

2.3. Rapid amplification of cDNA ends (RACE)

The 5-end and 3-end of the Cp-piGST were cloned by using SMART-RACE technique. Four gene-specific primers GST3F1, GST3F2, GST5R1 and GST5R2 (Table 1) were designed in accordance with the sequenced partial sequence of Cp-piGST fragment. The 5-end of the cDNA was amplified using primers GST5R1 and 10 × Universal Primer A Mix (UPM) under the following conditions: H_2O 18.5 μL, 10 × PCR buffer 2.5 μL, dNTPs 2.0 μL, UPM 0.6 μL, GST5R1 0.6 μL, cDNA 0.6 μL, ExTaq (Takara, Japan) 0.2 μL, and the cycling parameters included a 5 min denaturation at 94 °C, 30 cycles of heat denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, polymerization at 72 °C for 1 min, and a 10 min final extension at 72 °C. Then a Nested PCR was performed using the GST5R2 and UPM primer under the same annealing conditions. the 3-end of the cDNA, primer GST3F1 and UPM (Table 1) in the first round PCR and GST3F2 and UPM in the second round were used under the following conditions: H_2O 18.5 μL, 10 × PCR buffer 2.5 μL, dNTPs 2.0 μL, UPM 0.6 μL, GST3F2 0.6 μL, cDNA 0.6 μL, ExTaq (Takara, Japan) 0.2 μL, and the cycling parameters included a 5 min denaturation at 94 °C, 35 cycles of heat denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, polymerization at 72 °C for 1 min, and a 10 min final extension at 72 °C. After all of the PCR products were cloned into the pGEM-T vector (Promega), respectively and sequenced as previously described. The full-length cDNA of Cp-PiGST was obtained by splicing the mid-fragment, 5-end and 3-end.

Table 1
Primers used in this present study.

Primers	Sequence (5′ → 3′)[a,b]	Sequence information
GST.F1d	ASMMTSATCTAYMMWAACTATGA	Degenerate primer
GST.R1d	YTAYTGTTTDCCRTTKCCRTT	Degenerate primer
GST-3F1	CCTGCCTGGATTC CTTCCCAACCCT	3′-RACE
GST-3F2	GAGGAGATTGCCAACCGACCAAACAT	3′-RACE
GST-5R1	CGGTGCTTCTGTATTTCTTGATGTTTG	5′-RACE
GST-5R2	TATTTCTTGATGTTTGGTCGGTTGGC	5′-RACE
Long	CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT	3′-RACE
Short	CTAATACGACTCACTATAGGGC	5′-RACE
PGSTF	AAGGGTACCATGAAGGCATACAAGCTCGT	ORF cloning
PGSTR	GGAGAATTCTCACTGTTTTCCATTGCCATT	ORF cloning
GST.F	GCCCTATGAAGATGTGAACTGC	Real-time PCR
GST.R	GTCGGTAGTCCTCCACTCCATC	Real-time PCR
ACT.F	TGTGCTGTCTGGCGGTTCA	Real-time PCR
ACT.R	TCCTCTCTGGTGGAGCGATG	Real-time PCR

[a] M = A/C; Y = C/T; D = A/G/T; R = A/G; S = C/G. W = A/C/T; K = G/T.
[b] The restriction enzyme sites are underlined.