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Molecular characterization and expression analysis of interferon-γ-inducible lysosomal thiol reductase (GILT) gene from pearl oyster *Pinctada fucata*

Dianchang Zhang[a,1], Dequan Pan[a,b,1], Shuge Cui[a,c], Tianfeng Su[a], Lihua Qiu[a], Caiyan Zhu[a], Shigui Jiang[a,*]

[a] *Division of Aquaculture and Biotechnology, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China*
[b] *College of Animal Science, South China Agricultural University, Guangzhou 510642, China*
[c] *School of Life Science and Technology, Jinan University, Guangzhou 510632, China*

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ABSTRACT

Interferon-γ-inducible lysosomal thiol reductase (GILT) is an important thiol reductase, involved in class, MHC-restricted antigen processing by catalyzing disulfide bond reduction in mammals. Herein, we describe the identification and characterization of pearl oyster *Pinctada fucata* GILT (designated as poGILT). The poGILT cDNA was 1273 bp long and consisted of a 5′-untranslated region (UTR) of 24 bp, a 3′-UTR of 484 bp with two cytokine RNA instability motifs (ATTTA), and an open reading frame (ORF) of 765 bp encoding a polypeptide of 254 amino acids with an estimated molecular mass of 28.9 kDa and a theoretical isoelectric point of 7.4. The N-terminus of the poGILT was found to have a putative signal peptide with a cleavage site amino acid position at 19–20. SMART analysis showed that the poGILT contained a GILT active-site $C^{69}PDC^{72}$ motif and a GILT signature motif $C^{115}QHGKEECIGNLIETC^{130}$. Homology analysis of the deduced amino acid sequence of the poGILT with other known GILT sequences by MatGAT software revealed that the poGILT shared 42.9–67.3% similarity and 22.9–49.8% identity to the other known GILT sequences. The expression level of poGILT mRNA was higher in digestive gland, moderate in adductor muscle, gills, gonad, intestine and mantle, and lower in hemocytes. The poGILT mRNA expression was significantly up-regulated in gill and digestive gland after LPS or *V. alginolyticus* stimulation, respectively. These results suggested that the poGILT was a constitutively expressed acute-phase protein, the expression of which can be enhanced after LPS or *V. algrinolyticus* stimulation, perhaps involved in the innate immune response of pearl oyster.

1. Introduction

Pearl oyster *Pinctada fucata* is distributed over coastal area of South China and is the most important bivalve mollusk for seawater pearl production in China. However, since the mid-1990s, pearl oyster has suffered serious diseases caused mainly by bacteria (Lau et al., 2006), rickettsia-like organism (Wu and Pan, 1999), parasites (Hine and Thorne, 2000; Spiers et al., 2008) and viruses (Suzuki et al., 1998; Kitamura et al., 2000, 2002), which could be related to the dramatic decline in South China seawater pearl production. In order to control diseases and enhance the yields and quality of seawater pearl, it is necessary to understand the innate immune defense mechanisms of pearl oyster, which lacks the adaptive immune system. Recently, we characterized some immune-related genes of pearl oyster, such as Inhibitor of NF-κB (IκB) (Zhang et al., 2009a), a clip-domain serine protease (Zhang et al., 2009b) and a putative lipopolysaccharide-induced TNF-α factor (LITAF) (Zhang et al., 2009c), and demonstrated that they played an important role in innate immune responses of pearl oyster.

Interferon-γ-inducible lysosomal thiol reductase (GILT) was initially described as IP30 by Luster et al. (1988). GILT is synthesized as a 35-kDa precursor and targeted to the endocytotic compartments by the mannose-6-phosphate receptor (Arunachalam et al., 2000; Maric et al., 2001). In the endocytic pathway, GILT is processed into mature form by proteolytic removal of N- and C-terminal peptides and to generate a 28–30 kDa mature peptide (Li et al., 2002). The cDNA encoding GILT has been identified in a variety of species including human (Luster et al., 1988), mouse (Maric et al., 2001), rat (XM_214298), cow (XM_607840), pig (Dan et al., 2008), dog (XM_533874), frog (NM_214298), zebrafish (Woods et al., 2005), pufferfish (CR697192) and large yellow croaker (Zheng and Chen, 2006), amphioxus (Liu et al., 2007), sea urchin (XM_786456), fruit fly (AE003744), nematode (NM_063996) and disk abalone (De Zoysa and Lee, 2007). All deduced GILT proteins contained a signature sequence $CQHGX_2ECX_2NX_4C$, a CXXC active-site motif, more than

* Corresponding author. Tel.: +86 20 89108338; fax: +86 20 84451442.
E-mail address: jiangsg@21cn.com (S. Jiang).
[1] These authors contributed equally to this work.

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one putative Asn-linked glycosylation site and 10–11 conserved cysteines (Zheng and Chen, 2006; Liu et al., 2007; Phan et al., 2001).

In vertebrates, GILT has been identified as the only thiol reductase involved in class II MHC-restricted antigen processing, which functions to catalyze the reduction of disulfide bond, thus unfolding native protein antigen and facilitating their subsequent cleavage by proteases (Arunachalam et al., 2000; Maric et al., 2001; Li et al., 2002). Recent studies have shown that, in addition to involvement in antigen processing, GILT may have additional roles including negative regulation of T cell activation and neutralization of extracellular pathogen and clearance of cell debris resulting from infection (Barjaktarevic et al., 2006; Lackman and Cresswel, 2006). However, the functions of GILT were poorly known in invertebrates, only recent studies showed that GILT was involved in innate immune response in amphioxus (Maric et al., 2001) and disk abalone (Woods et al., 2005). Therefore, to further understand the functions of GILT in innate immune responses of invertebrates, it is necessary to isolate and characterize more GILT genes from invertebrates. In the present study, pearl oyster GILT cDNA (designated as poGILT) was cloned and characterized, and its spatial and temporal expression was also investigated. The results showed the poGILT was a constitutively expressed acute-phase protein, the expression of which can be enhanced after LPS or *V. algrinolyticus*, perhaps involved in innate immune response of pearl oyster.

2. Materials and methods

2.1. cDNA library construction and EST analysis

A cDNA library was constructed from the body of pearl oyster injected with *V. alginolyticus*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 6741 successful sequencing reactions. BLAST analysis of all the EST sequences revealed that an EST of 425 bp (EST no. pmpca 0_009306) was homologous to the GILTs of amphioxus (AAQ83892) and sablefish (ACQ58865). This EST was selected for further cloning of the GILT gene of pearl oyster.

2.2. Pearl oyster and immune challenge

Pearl oysters *P. fucata* (shell length 5.4 ± 0.4 cm, body weight 37.3 ± 7.7 g) were obtained from pearl oyster culture base of South China Sea Fisheries Research Institute in Xincun village, Hainan province, China and maintained at 26–28 °C in tanks with the recirculating seawater for one week before experiment. The pearl oysters were fed twice a day on *Tetraselmis suecica* and *Isochrysis galbana*. To take account of individual variability, 15 individual pearl oysters, which were divided into three replicates, were used for each time point (2, 4, 8, 12, 24, 36 and 72) in the lipopolysaccharide (LPS) and bacterial challenge experiments.

The LPS challenge experiment was performed by injecting with 100 μl LPS (*Escherichia coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany, LPS 10 μg ml^{-1}) dissolved in phosphate-buffer saline (PBS) into the adductor muscles of each pearl oyster. The bacterial challenge experiment was performed by injecting with 100 μl of *V. alginolyticus* resuspended in PBS to OD_{600} = 0.4 (1 OD = 5×10^8 bacteria ml^{-1}) into the adductor muscles of each pearl oyster. The untreated pearl oysters were used as the blank, while the pearl oysters that were injected with 100 μl PBS were used as the control in the challenge experiments. The injected pearl oysters were returned into seawater tanks and 15 individuals were randomly sampled at 2, 4, 8, 12, 24, 36 and 72 h into the experiments. The gills and digestive glands were collected from the blank, the control and injected groups, respectively, and immediately stored in liquid nitrogen until used. For tissue distribution experiment, hemolymph, gills, digestive glands, mantle, gonads, adductor muscles and intestines were collected from three untreated pearl oysters and stored in liquid nitrogen until use. Total RNA samples were extracted using the TRIzol reagent (Invitrogen) according to the manufacture's instructions.

Table 1
Primers for gene amplification and characterization.

Primer name	Sequence (5′ to 3′)	Application
GILT-F1	AGTTCGCAGAACCACAAGAC	For RACE PCR
GILT-R1	TCTTGTGGTTCTGCGAACTG	
GILT-F	ACCATGGGTCACTCTGAACG	
GILT-R	ACCATGGGTCACTCTGAACG	For real-time RT-PCR
RPL37-F	CCAAGAAGGTTGGAATTGTG	
RPL37-R	TCCCTCAATCTTCTGACTGC	
Adaptor-dT	GGCCACGCGTCGACTAGTACT$_{17}$	For reverse transcription
T3	AATTAACCCTCACTAAAGGG	
T7	GTAATACGACTCACTATAGGGC	For sequencing and RACE PCR

2.3. Rapid amplification of poGILT cDNA ends

Based on the identified EST sequence, two gene-specific primers GILT-F1 and GILT-R1 (Table 1) were designed to amplify the full-length cDNA of poGILT by rapid amplification of cDNA ends (RACE) technique. To obtain 5′-end of the poGILT cDNA, PCR reaction was performed in a T-1 Thermocycler (Biometra) by using the T3 and GILT-R1 primers (Table 1) in a 25 μl of reaction volume, containing 2.5 μl of 10× PCR buffer, 1.5 μl of $MgCl_2$ (25 mmol l^{-1}), 2.0 μl of dNTP (2.5 mmol l^{-1}), 1 μl of each primer (10 μmol l^{-1}), 15.8 μl of double-distilled water, 0.2 μl (1.0 U) of *Ex* Taq (TaKaRa) and 1 μl of 100-fold diluted cDNA library as template. The cycle condition was one initial denaturation cycle of 94 °C for 2 min, then 35 PCR cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR amplification of 3′-end of the poGILT was carried out using the T7 and GILT-F1 primers (Table 1), the PCR temperature profile was 94 °C for 2 min, following by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and then an additional extension of 72 °C for 10 min. The PCR products were separated by agarose gel (1.2%) electrophoresis, and then the bands were excised and purified using a DNA Gel Extraction Kit (KeLi, China). Finally, the purified DNA fragments were cloned into the pMD18-T vector (TaKaRa) and sequenced.

2.4. Sequence analysis of poGILT

The poGILT amino acid sequence was deduced using DNAstar software. The percentage of similarity and identity was

Table 2
Homology analysis of poGILT amino acid sequence with other known GILT amino acid sequences determined by MatGat software.

Species	Accession number	Similarity (%)	Identity (%)	Amino acids
Amphioxus	ACQ58973	67.3	49.8	256
Sable fish	ACQ58973	61.3	41.1	256
Large yellow croaker	ABB87180	61.3	38.6	256
Zebrafish	AAH83267	60.8	43.3	255
Oranger-spotted grouper	ABS19625	58.1	39.3	260
Pig	NP_001124518	57.9	35.7	246
Atlantic salmon	ACI69367	56.3	40.4	253
Cattle	AAI49407	55.1	35.2	244
Human	AAF04618	54.4	33.2	261
Mouse	AAH54852	53.9	32.7	248
Disc abalone	ABQ24037	51.6	33.6	228
Salmon louse	ACO12961	46.9	29.1	224
Fruit fly	NP_650287	45.3	24.7	250
Mosquito	ABF18298	42.9	22.9	240