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# Molecular cloning and expression pattern analysis of two novel disulfide isomerases in shrimp

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ABSTRACT

Protein disulfide isomerase (PDI) catalyzes formation and isomerization of disulfide bridges and has chaperone activity. Currently, increasing evidence suggests the significance of PDI in immune and stress responses. To clarify the role of PDIs in the innate immunity of shrimp, two PDI genes were isolated and identified from *Fenneropenaeus chinensis* (fleshy prawn). *FcPDI1* is 1878 bp in length and encodes a protein of 383 amino acids. It has 18-amino acid signal peptide, 3 thioredoxin domains with 3 active sites of CGHC, and KEDL retention signal at its C-end. FcPDI1 is an atypical PDI. The open reading frame of *FcPDI2* encodes a 497-amino acid protein and shows the classical domain organization a-b-b′-a′. Phylogenic analysis and multiple alignments show that FcPDI1 is similar to PDI that contains 3 thioredoxin domains from other species including invertebrates and vertebrates. FcPDI2, LvPDI, and insect PDIs are grouped into one cluster and are similar to PDIs having a-b-b′-a′ domain organization. Tissue distribution shows that *FcPDI1* and *FcPDI2* were expressed in all detected tissues at the mRNA level. Changes in *FcPDI1* and *FcPDI2* expression at the mRNA level in hemocytes, hepatopancreas, gills, and ovaries upon *Vibrio* or white spot syndrome virus challenge were also analyzed. The results suggest that *FcPDI1* and *FcPDI2* might have roles in the innate immunity of shrimp. FcPDI1 was also successfully expressed in *Escherichia coli* and the recombinant FcPDI1 showed insulin reductase activity. Results show that FcPDI might play an important role in the innate immunity of shrimp.


## 1. Introduction

Protein disulfide isomerase (PDI, EC5.3.4.1) has multifunctional roles and participates in protein folding, assembly, and posttranslational modification (Wilkinson and Gilbert, 2004). PDI catalyzes the formation of disulfide bridges and the isomerization of incorrect disulfide bridges (Noiva, 1999). It also functions as a chaperone protein and reportedly facilitates refolding of denatured lysozyme (Puig and Gilbert, 1994). In general, PDI has a classical domain arrangement of a-b-b′-a′ where the "a" and "a′" domains are thioredoxin domains with a CXXC active site, whereas the "b" and "b′" domains are catalytically inactive and have thioredoxin-like domains (Kemmink et al., 1997). PDI also has a signal peptide in the N-end and KEDL retention signal in the C-end (Ferrari and Soling, 1999; Noiva, 1999). Recently, an increasing number of atypical PDI such as ERp57, ERp72, and ERp28, different in the number and organization of thioredoxin domains, have been found (Ferrari and Soling, 1999). Both typical and atypical PDIs are found in parasites (Hsu et al., 1989; Knodler et al., 1999; McArthur et al., 2001; Ben Achour et al., 2002). An atypical PDI, containing only a thioredoxin domain, was found in *Leishmania donovani* which helps the parasite evade the host immune system; hence, it is involved in parasite survival (Padilla et al., 2003). PDI from highly a virulent strain of *L. major* was expressed at higher levels; this suggests that PDI may have a role in the pathogenesis of *Leishmania* (Ben Achour et al., 2002).

In *Pseudomonas solanacearum* resistant tomatoes, PDI is present in significantly higher quantities than in susceptible tomatoes (Afroz et al., 2009). In wheat, PDI is a pathogen-induced, differentially expressed gene (Ray et al., 2003). Pathogens induce the production of reactive oxygen species (ROS) in wheat and PDI possesses the antioxidant activity to reduce ROS damage to host cells. Endothelial cells exposed to hypoxia show a very rapid upregulation of PDI (Graven et al., 2002). NADPH oxidase activity is functionally dependent on PDI, ROS production increases when PDI is over-expressed, and phagocytosis of the *L. chagasi* promastigote by macrophages requires PDI (Santos et al., 2009). During phagocytosis of parasites, the amount of expressed PDI remains unchanged and requires only PDI traffic to the membranes (Santos et al., 2009). Although PDI is not a direct receptor of *Chlamydia* (*C. Trachomatis* or *C. Psittaci* ), it is structurally required for *Chlamydia* attachment; the

*Abbreviations:* PDI, protein disulfide isomerase; ERp, endoplasmic reticulum protein; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; WSSV, white spot syndrome virus; TSV, taura syndrome virus; TGase, transglutaminase; PBS, phosphated buffered saline; HCV, hepatitis C virus.

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enzymatic activity of PDI is necessary for bacterial entry (Abromaitis and Stephens, 2009).

PDI was also reported in *Bombyx mori* (bPDI), which was not only regulated by hormones, but also plays an important role in bacterial infections (Goo et al., 2002). Ticks are blood-feeding arthropods and vectors of many mammalian pathogens (Jongejan and Uilenberg, 2004). Three PDIs (*HlPDI-1, HlPDI-2,* and *HlPDI-3*) from the tick *Haemaphysalis longicornis* contain two typical PDI active sites of CXXC and HlPDI-1 and HlPDI-3 is involved in blood feeding and *Babesia* infection (Liao et al., 2007).

Recently, shrimp PDIs have been reported to be involved in different biological processes including antiviral (Chongsatja et al., 2007; Bourchookarn et al., 2008) and anti-bacterial immune defense (Vargas-Albores et al., 2009) as well as stress response (de la Vega et al., 2007b) and they are also identified in sex-related genes in the vitellogenic ovaries of *Penaeus monodon* (Preechaphol et al., 2007). Significant increases in PDI could be observed at both mRNA and protein levels in the lymphoid organ of *P. monodon* upon yellow head virus (YHV) challenge (Bourchookarn et al., 2008). In *Litopenaeus vannamei*, the proteomics approach was used to analyze altered proteins in hemocytes upon Taura syndrome virus (TSV) infection and the results show that PDI was significantly upregulated among the altered proteins (Chongsatja et al., 2007). Transglutaminase (TGase; EC 2.3.2.13) from the human filarial parasite *Brugia malayi* shows significant homology to PDI; especially to ERp60, a PDI found in the endoplasmic reticulum (Devarajan et al., 2004). Human TGase also exhibits PDI activity (Hasegawa et al., 2003). PDIs from *Giardia lamblia* also show TGase activity (Knodler et al., 1999). In *L. vannamei*, TGase is downregulated by TSV infection (Chongsatja et al., 2007). A 56 kDa PDI from *L. vannamei* is strongly upregulated during the primary response to *Vibrio alginolyticus* in hemocytes (Vargas-Albores et al., 2009).

Past research focused on the expression patterns of shrimp PDIs in anti-YHV and anti-TSV immune defense. However, investigations regarding the function of PDI in anti-white spot syndrome virus (WSSV) or anti-bacterial immune defense have not been reported. This paper investigates the role of PDI in the innate immune defense of fleshy prawn *Fenneropenaeus chinensis* against WSSV and *Vibrio anguillarum*.

## 2. Material and methods

### 2.1. Preparation of viral inoculums

The WSSV inoculum was prepared and quantified based on a previously described method (Wang et al., 2009). WSSV was collected from the heavily infected gills of naturally infected shrimp *F. chinensis*. The gill tissues (1 g) were homogenized in 10 mL PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L $Na_2HPO_4$, and 1.8 mmol/L $KH_2PO_4$) and then centrifuged at 5000 g for 10 min at 4 °C. The supernatant was passed through a 450 nm membrane filter and used as the virus inoculum.

### 2.2. Immune challenge of shrimp

Shrimp (about 10–20 g each), purchased from Qingdao in Shandong Province, China, were cultured in 500 L tanks filled with air-pumped seawater. In the experiment group, shrimp were challenged with *V. anguillarum* ($2\times10^7$cells, diluted in PBS) or WSSV ($3.2\times10^7$ per shrimp, diluted in PBS). About 4.5 mL hemolymph was collected from 5 shrimp from the ventral sinus using a 5 mL sterile syringe preloaded with 500 μL anticoagulant (10% sodium citrate, pH 7.0) at 0, 2, 6, 12, and 24 h. The hemolymph was then centrifuged at 800 *g* at 4 °C for 5 min to isolate the hemocytes. Other tissues such as heart, hepatopancreas, gills, stomach, intestines, and ovaries of unchallenged shrimps were also dissected for RNA extraction. RNA was extracted from the hepatopancreas, gills, and ovaries at 2, 6, 12, and 24 h post-injection.

### 2.3. Total RNA isolation and cDNA synthesis

Total RNAs were extracted from the collected tissues using the Unizol reagent (Biostar, China). The SMART PCR cDNA synthesis kit (BD Biosciences Clontech) was used for the first strand cDNA synthesis using the primer oligo-anchor R [5′-GACCACGCGTAT-CGATGTCGACT$_{16}$(A/C/G)-3′] and Smart F (5′-TACGGCTGCGAGAA-GACGACAGAAGGG-3′).

### 2.4. Gene cloning of FcPDI1 and FcPDI2 from shrimp

An expressed sequence tag (EST) with 5′ UTR was obtained from the hepatopancreas suppression subtractive hybridization (SSH) library in our lab and BLAST P analysis showed that it was similar to PDIs from other species. The 3′ fragment of FcPDI1 was cloned using a gene specific primer (FcPDI1F: 5′- GACTTGGCTTCTTTGGAGAGGTT-CATT-3′) and a 3′ anchor R primer. The full length of FcPDI1 was obtained by overlapping the two resulting fragments. The 5′ encoding region of FcPDI2 was cloned using a pair of primers (FCPDI2F1:5′-ATGAGGGTGGGAGCAACCGTC-3′, FCPDI2R:5′-CTGGTCATAGA-TAGGTGCCAAC-3′) based on the PDI from *L. vannamei*. The 3′ fragment of FcPDI2 was amplified using a gene specific primer (FCPDI2F2: 5′-GTTGGCACCTATCTATGACCAG-3′) and a 3′ anchor R. The full length of FcPDI2 was also obtained by overlapping the two fragments.

### 2.5. Sequence analysis

Similarity analysis was performed using BLASTX (http://www.ncbi.nlm.nih.gov/). The signal peptide and domain organization of FcPDI1 and FcPDI2 were predicted by SMART (http://smart.embl-heidelberg.de/). Translation of FcPDI1 and FcPDI2 was conducted with ExPASy (http://www.au.expasy.org/). MEGA 4.1 and Neighbor-Joining method were used for phylogenetic analysis (Kumar et al., 2008).

### 2.6. Tissue distribution using RT-PCR and expression pattern analysis of FcThem by Quantitative real-time PCR

The tissue distribution of FcPDI1 and FcPDI2 was analyzed in the hemocytes, heart, hepatopancreas, gills, stomach, intestines, ovaries, and testis via RT-PCR. Meanwhile, quantitative real-time polymerase chain reaction (qRT-PCR) was performed following manufacturer instructions included in the SYBR Premix Ex Taq kit (Takara, Japan) with a real-time thermal cycler (Bio-Rad, Hercules, CA, USA). The primers used for RT-PCR and qRT-PCR were, (QFcPDI1F:5′-AGCCATG-GAGACCTCAAGGCC-3′, QFcPDI1R:5′-GGAGCCATTCGCTTGCAGTGT-3′; QFCPDI2F: 5′-ACCATGGTGTGGACACTGC-3′, QFCPDI2R: 5′- GACATTC-CAGCCAATGTGCGT-3′). β-actin was amplified for internal standardization with the primers actin F (5′-CTGGAGAAGTCCTACGAGCTC-3′) and actin R (5′-GATGCCAGGGTACATGGTGGT-3′). The methods were from a previous paper (Ren et al., 2009). In detail, the comparative CT method was used to quantify the mRNA expression (Livak and Schmittgen, 2001). First, The CT of the β-actin gene subtracted from CT of *FcPDI1* or *FcPDI2* gene was equal to ΔCT. Then, to obtain ΔΔCT, the ΔCT of the control sample was subtracted from the ΔCT of *V. anguillarum* or WSSV treated sample. The normalized fold changes of *FcPDI1* or *FcPDI2* expression were expressed as $2^{-\Delta\Delta CT}$. Unpaired sample t-test was used in statistical analysis and $P<0.05$ indicates a significant difference.