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ORIGINAL PAPER

Potential relationship among three antioxidant enzymes in eliminating hydrogen peroxide in penaeid shrimp

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Abstract Antioxidant enzymes, such as glutathione peroxidase (GPx), catalase (CAT), and peroxiredoxin (Prx), are essential components in cells to eliminate excessive reactive oxygen species such as hydrogen peroxide (H_2O_2). GPx, CAT, and Prx genes have been reported in penaeid shrimp, and they showed different expression profiles at transcription or protein level when shrimps were challenged by microbes. In order to learn the relationship among the above three genes in their function, *GPx*, *CAT*, and *Prx* transcripts were analyzed, and the variation of GPx and CAT enzyme activity was detected when shrimp was injected with H_2O_2 or one antioxidant enzyme gene was silenced in shrimp by double-strand RNA injection. The results indicated that there existed some relationships among three antioxidant enzyme genes, *CAT*, *GPx*, and *Prx* in shrimp at transcriptional level. The transcription of CAT and GPx could be directly induced by H_2O_2 injection, while the transcription of Prx cannot be induced by H_2O_2. Decreased transcription level of CAT or GPx could lead to increased transcription of the other two genes, which suggested that there existed some compensation among these three antioxidant enzyme genes. These data can help us to understand the roles of antioxidant enzymes in crustacean.

Keywords Antioxidant enzymes · RNA interference (RNAi) · Transcription expression · Enzyme activity · Penaeid shrimp

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Introduction

Invertebrates do not possess an adaptive immune system, which is based on highly specific antibodies and antigen receptors. They must rely on efficient innate immune defense to protect themselves against invaders (Iwanaga and Lee 2005). Great economical requirement of penaeid shrimp gives rise to researches on their innate immunity, but the aquaculture of shrimp is still limited by the occurrence of infectious diseases.

Phagocytosis is an important immune defense reaction when the organism is attacked by bacteria or viruses (Lee and Soderhall 2002; Smith et al. 2003). During phagocytosis in mammals, the host's NADPH oxidase gets activated, which in turn enhances the glycolytic reactions. They increase the consumption of oxygen and induce the production of a mass of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) (Bogdan et al. 2000; Roch 1999). Although ROS play an important role in host defense against microbial infection, their over expression and the residual ROS can cause cellular damage (Yu 1994). Most cells have acquired the relevant protective mechanisms to maintain the lowest possible level of ROS inside the cell. The protective mechanisms include both non-enzyme (ascorbic acid, β-carotene, glutathione, and α-tocopherol) and enzyme antioxidant systems, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and peroxiredoxin (Prx) (Aruoma 1998; Holmblad and Soderhall 1999; Schwarz 1996). SOD can scavenge the superoxide anions and detoxify them by converting to hydrogen peroxide and oxygen. Hydrogen peroxide is then transformed to water and oxygen by CAT, GPx, or Prx (Nordberg and Arnér 2001).

CAT is an important antioxidant enzyme and exists virtually in all oxygen-respiring organisms (Klotz et al. 1997).

The central role of CAT is eliminating excessive hydroperoxide and maintaining cellular redox balance. An extracellular immune-regulated catalase mediates a key host defense system that is necessary during host–microbe interaction in the gastrointestinal tract, highlighting the importance of these enzymes in the innate immune system of invertebrates (Ha et al. 2005a,b; Ryu et al. 2006). GPx plays an important role in detoxifying lipids and hydroperoxides, which protects biomembranes and other cellular components from oxidative damage by catalyzing the reduction of a variety of hydroperoxides, using glutathione as the reducing substrate during phagocytosis and/or physiological metabolism (Arthur 2000; Liu et al. 2004; Mills 1957; Speier et al. 1985). Prx, also named thioredoxin peroxidase (TPx), as an important peroxidase, was the latterly identified antioxidant protein, which can eliminate hydroperoxide with thioredoxin as an immediate hydrogen donor (Chae et al. 1994; Lim et al. 1993). Prx can protect the organisms against various oxidative stresses and regulate the intracellular signal transduction (Kang et al. 2005; Rhee et al. 2005). Expression of Prx gene can be induced when the organisms faced with oxidative stress caused by virus infection, temperature, or H_2O_2 stimulation (Kim et al. 2005; Lee et al. 2005). Due to the important roles of antioxidant enzymes, researchers are paying more attention to the antioxidant systems of shrimp in recent years, and made some progress already.

In penaeid shrimp, the complementary DNA (cDNA) sequence of CAT gene was identified in white shrimp, *Litopenaeus vannamei* (Tavaressanchez 2004) and Chinese shrimp, *Fenneropenaeus chinensis* (Zhang et al. 2008). The transcription level of CAT gene in hemocytes and hepatopancreas was upregulated when shrimp was infected by white spot syndrome virus (WSSV), which suggested that *CAT* might be involved in the shrimp immune response (Zhang et al. 2008). The full-length cDNA of *GPx* was cloned and characterized in *L. vannamei* (Liu et al. 2007), *Penaeus monodon* (Liu et al. 2010), and *F. chinensis* (Ren et al. 2009), and its transcript level and protein activity were significantly upregulated when shrimp were subjected to pathogen invasion, which suggested that *GPx* might play important role in shrimp immunity. Similarly, Prx genes have been isolated from several penaeid shrimp species including *F. chinensis* (Zhang et al. 2007) and *Marsupeneaus japonicus* (Bacano Maningas et al. 2008), and their expression at transcription level was changed when shrimp was challenged by bacteria or virus.

Although three antioxidant enzymes (CAT, GPx, and Prx), which might function in transforming H_2O_2 to harmless water and oxygen, were found in penaeid shrimp, no any study was reported on the relationship between these three genes. Shrimp has an open circulating system in which hemocytes distribute in most of the tissues. Besides hemocytes, hepatopancreas, and lymphoid organ, gills are also involved the immune function of shrimp. Since most of the organs are located in the cephalothorax of shrimp; therefore, the cephalothoraxes were usually used to represent the whole shrimp. In the present study, the relative expression of Prx, CAT, and GPx in the cephalothorax of Chinsese shrimp *F. chinensis* was studied at transcription and translation level after one of them was silenced by double-strand RNA (dsRNA) injection. The data can help us to understand the relationship of these genes in antioxidant reactions in shrimp.

Materials and methods

Shrimp source

Healthy juvenile shrimp with body weight of 6.80±2.29 g used in this study were reared in our lab from the nauplius stage. These shrimp were siblings from the same parents to ensure that they had the same genetic background. These shrimp would be used for H_2O_2 injection and dsRNA injection experiments.

Preparation of total RNA and cDNA synthesis by reverse transcription

Total RNA was extracted from cephalothorax of juvenile shrimp mentioned above with Unizol Reagent (Biostar Genechip Inc., Shanghai, China) as described in the manufacturer's protocol. RNA quality was assessed by electrophoresis on 1% agarose gel. The cDNA was synthesized from the total RNA using Moloney murine leukemia virus reverse transcriptase (Promega, USA) according to the manufacturer's instruction.

Construction of recombinant plasmids for dsRNA synthesis

In order to synthesize double-strand RNA, three plasmids, including *pGEM-T-FcGPx*, *pGEM-T-FcCAT*, and *pGEM-T-FcPrx*, were constructed based on the pGEM-T easy vector (Takara, Japan). The cDNA sequences used in dsRNA synthesis of *FcGPx*, *FcCAT*, and *FcPrx* are shown in Fig. 1. For the construction of plasmid *pGEM-T-FcGPx*, cDNA fragment of *GPx* with length of 646 bp was amplified from the cDNA synthesized above with forward primer GPx-F1 (5′-TCCTCTCCCCCCAATCTT-3′) and reverse primer GPx-R1 (5′-CGGTCTTCTTTGAATAACTT-3′). PCR program for amplification of *GPx* cDNA was 94°C for 4 min; followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; then extended at 72°C for 10 min. The cDNA fragment of *GPx* was ligated into the pGEM-T easy vector, and then the restriction enzymes, *Apa*I or *Sal*I, were used to make the

(a)

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TCCTCTCCCCCCAATCTTGTTTGAAAGAAGGAAAAAGACCTTTACTTCCAACCATGGCTTCCTCAGCTATCAAGTCCTTTTACGACCTGAGTGCCAAGGC 100
TCTCAGCGGTGAGGTGGTGTCGTTCAAGAAGTTCCAGGGGAAGGTGGTGCTGGTGCAGAACACGGCGTCGCTCTGAGGGACGACCACCAGGGACTTCCAC 200
CAGATGAACCAACTCAAGGAAGAGTTCGGGGACAAGCTGGAGGTCCTGGCCTTCCCCTGCAACCAGTTCGGGCACCAGGAGAACACCACCGAAGGAGAGT 300
TGCTGAGCTCCCTCCGCCACGTCCGCCCTGGCAACAACTTCGAGCCCAAGATGGTTATGTTTGGCAAAGTCGATGTCAACGGGTCAACAGCTGATCCCGT 400
CTTTAAGTACCTGAAGGAGCGACTGCCACTGCCAGCTGACGACAGCGTGAGCTTCATGTCAGACCCAAAGTGTATCATTTGGACACCTGTCTGCCGCTCT 500
GACATCGCCTGGAACTTCGAGAAATTCCTCATTGGCAAGGATGGACAGCCGTTTAAGAGGTTCAGCAAGAAGTATGAAACCATACTGCTTAAAAATGAAA 600
TCGCAAACTTGCTGTAAGCTTAAGAAAAGTTATTCAAAGAAGACCG 646
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(b)

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ACACTTCGACCGTGAGCGCATCCCAGAGAGAGTTGTGCATGCTAAAGGAGCAGGTGCCTTTGGTTACTTTGAGGTTACACATGATATAACCAAGTACTGT 100
AAGGCTGCTATGTTCAGTGAGATTGGGAAACAAACTCCCATTGCTGTTCGTTACTCCACTGTAGGAGGTGAGAGTGGATCAGCTGACACTGCCAGGGATC 200
CTCGAGGATTTGCTGTGAAGTTTTACACAGAAGAAGGAAATTGGGATCTCGTGGGGAACAACACCCCCATTTTCTTCATCAGGGATCCAATTCTGTTCCC 300
ATCCTTCATTCACACTCAGAAAAGAAACCCTGCAACTCATTTAAAGGATGCTGACATGTTTTGGGATTTCATCAGCCTGCGACCAGAAACAACACACCAA 400
GTGTCGTTCCTCTTCTCTGACAGAGGAACCCCTGATGGCTATCGTCACATGAATGGCTACGGTTCACATACTTTCAAGCTTGTCAATGCAAAGGGGGAAG 500
CTGTCTACTGCAAGTTCCATTACAAGACGGACCAAGGCATCAAGTGTCTGAGCTGCAAGAAGGCTGATGAACTGGCAGGCTCTGACCCGGATTATGCAAC 600
AAGAGACCTATACAATGCTATTTCAAGTGGCGATTACCCCTCATATACTATGTATATTCAGGTGATGACATTTGAAGAAGCAGAGAAGTGGAAGTTTAAT 700
CCATTTGACCTCACCAAAGTATGGCCACATGACGAGTTCCCACTCATCCCTGTTGGCCGTCTCACTTTTGACCGCAATCCAAAGAATTACTTTGCTGAGG 800
TGGAGCAGATTGCATTCTCACCTGCCAACATGGTGCCTGGTATTGAGGCTTCCCCTGACAAGATGTTGCAAGGCCGCCTCTTCTCCTACAACGACACTCA 900
CCGTCACCGTCTGGGCGCCAACTACACTCAGATCCCTGTGAACTGCCCATACCGTGCCCGTACAAAGAACTACCAGAGGGATGGCCCCATGTGTGT 996
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(c)

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ATGAGCAACACTGTCCCAGCTATTGGCAAGCCTGCCCCTGTCTTCAAGGGCACTGCCGTTGTTGATGGGCAGTTCAAGGAGATTTCCCTGGAGGATTACA 100
AGGGCAAATATGTCATTTTCTTCTTCTACCCTTTGGATTACACCTTTGTCTGCCCCACTGAAATTATCGCCTTCTCTGACCGTGTTGAAGAATTCAGGAA 200
AATTGGATGCGAAGTGGTTGCTTGCTCTACAGACTCCCACTTCTCCCACCTTGCTTGGATCAATACTCCTCGCAAGGAAGGTGGTCTTGGTACCATGAAG 300
ATTCCTCTTCTGGCTGACAAGTCAATGGAAGTTGCAAAGACTTACGGAGTTCTTAAGGAGGACGAAGGCATTGCTTTCAGAGGCCTCTTCGTTATTGACG 400
GCAAGCAGGATCTCCGTCAGGTAACCATCAATGACTTGCCAGTTGGGCGTGACGTAGATGAAACACTGCGATTAGTACAGGCCTTCCAGTTCACAGATGA 500
GCACGGTGAAGTATGCCCTGCTGGCTGGAAACCCGGAGCCAAGACAATGAAGGCTGATCCTGCTGGCAGCAAAGAATACTTCCAGAACGAAAATTAATAT 600
AATGACCTCTTTACTTGGATTTTTTTTTTTTTTTCTCCTTTTATAAAAGATTTTGGATCTGTCTTGTTCAAAAAGAGTTGGCATTCAGGATATGGCATTA 700
GGTTCATTCTGGTAATGATATTTCCTTTTGTTAAAACCGGTTTGAAAGCTCAAGTAATTACAGTACACAACATAGTTTGAATCAAAATAAGTTCAGGTTA 800
TTTTGAAGAAAAGCTGCGTTATTTCACAGTATCAG 835
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Fig. 1 The nucleotide sequences of cDNA fragment for *GPx*, *CAT*, and *Prx* double strand RNA synthesis. **a** The cDNA sequence for DsGPx synthesis. **b** The cDNA sequence for DsCAT synthesis. **c** The cDNA sequence for DsPrx synthesis

plasmid be linearized separately. For plasmid *pGEM-T-FcCAT*, cDNA fragment of *CAT* with length of 996 bp was amplified with forward primer CAT-OF1 (5′-ACACTTCGACCGTGAGCGCAT-3′) and reverse primer CAT-OR1 (5′-ACACACATGGGGCCATCCCT-3′). PCR program for amplification of *FcCAT* cDNA was 94°C for 4 min; followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; then extended at 72°C for 10 min. The cDNA fragment of *CAT* was ligated into the pGEM-T easy vector, and the restriction enzyme, *Nco*I or *Sal*I, was used to make the plasmid be linearized separately. For plasmid *pGEM-T-FcPrx*, the cDNA fragment of *Prx* with length of 835 bp, was amplified with forward primer Prx-TF (5′-ATGAGCAACACTGTCCCA-3′) and reverse primer Prx-

TR (5′- CTGATACTGTGAAATAACGC-3′). The PCR program for amplification of *Prx* cDNA was the same as that in *FcCAT* cDNA amplification. The amplified cDNA fragment of *Prx* was ligated into the pGEM-T easy vector, and the restriction enzymes *Nco*I or *Sal*I were used to make plasmid be linearized separately.

Preparation of dsRNA for *FcGPx*, *FcCAT*, and *FcPrx*

Procedures for the preparation of dsRNA, detection of silencing efficiency were the same as described previously (Li et al. 2009). Briefly, two single-stranded RNAs were transcribed in vitro from the linearized plasmid constructs (*pGEM-T-FcGPx*, *pGEM-T-FcCAT*, or *pGEM-T-FcPrx*) using T7 and SP6 RNA polymerases (Takara, Japan), and the DNA template was degraded by addition of DNase I (Promega, USA) at a ratio of 1 U/μg of template. The single-strand RNA transcripts obtained from T7 and SP6 RNA polymerase were then mixed and annealed by incubation at 95°C for 5 min, and then put at room temperature overnight to let them form dsRNA of *FcGPx*, *FcCAT*, and *FcPrx* (DsGPx, DsCAT, and DsPrx). The formation of DsGPx, DsCAT, or DsPrx was monitored by determining the size shift in agarose gel electrophoresis, and the concentration of dsRNA was measured spectrophotometrically after purification by phenol chloroform. The silencing efficiency was detected as described previously (Li et al. 2009).

H_2O_2 injection into shrimp

Different concentrations, including 0.1% and 0.01% of H_2O_2 diluted by phosphate-buffered saline (PBS), were used for juvenile shrimp injection. In the control group, each shrimp was injected with 20 μL PBS. In the experimental group, each shrimp was injected with 20 μL 0.01% or 0.1% H_2O_2. Cephalothoraxs of four shrimps in each group were dissected out and preserved in liquid nitrogen at 0 and 2 h post-injection (hpi). The sampled cephalothoraxs of shrimp were used for further activity assay and RNA isolation to analyze the transcriptional expression profiles.

DsGPx, DsCAT, or DsPrx injection into shrimp

Twenty micrograms of DsGPx, 30 μg DsCAT or DsPrx in volume of 20 μL PBS were injected into each juvenile shrimp separately in the RNA interference (RNAi) experiments (three experimental groups). At the same time, shrimp injected with 20 μL PBS were used as a control group. Before injection, cephalothoraxes of four shrimp were collected to be preserved in liquid nitrogen. Then cephalothoraxs of four shrimps in each RNAi group and control group were dissected out at 48 hpi and preserved in liquid nitrogen. These samples will be used for RNA isolation or enzyme activity analysis for *FcGPx*, *FcCAT*, or *FcPrx*.

H_2O_2 injection into shrimp after *FcGPx* was silenced for 48 h

Each shrimp injected with 20 μg DsGPx in the volume of 20 μL PBS were taken as RNAi groups and the shrimp injected with the same volume of PBS as the control group. Four shrimp in each group were taken out at 0 h and 48 hpi, and their cephalothoraxes were preserved in liquid nitrogen. After 48 h of post-DsGPx or PBS injection, the shrimp from each group were injected with 0.1%H_2O_2 or PBS, respectively. Then, their cephalothoraxes in each group were dissected out at 2 hpi and preserved in liquid nitrogen, respectively. These samples will be used for RNA isolation to analyze the transcriptional expression profiles or activity measurement.

Quantification of gene transcript by real-time PCR

Based on the cDNA sequences of *GPx* (Ren et al. 2009), *CAT* (EU102287.1), and *Prx* (DQ205423) in GenBank, primers used for real-time PCR analysis were designed. The primer sequences and the expected size of the amplified

Table 1 Primer sequence and corresponding annealing temperature of genes

Gene	Primer name	Direction	Primer sequence(5′–3′)	Expected size (bp)	annealing temperature (°C)
GPx	GPx-RTF1	Forward	TGCAACCAGTTCGGGCACCA	173	69
	GPx-RTR1	Reverse	AGTGGCAGTCGCTCCTTCAGGT		
CAT	CAT-RT-F	Forward	ACTCCCATTGCTGTTCGT	114	58.5
	CAT-RT-R	Reverse	ATCCCAATTTCCTTCTTCTG		
Prx	Prx-RT-F	Forward	GCTTGCTCTACAGACTCCC	295	58.5
	Prx-RT-R	Reverse	ATACTTCACCGTGCTCATC		
18S rRNA	18S2F	Forward	TATACGCTAGTGGAGCTGGAA	147	55
	18S2R	Reverse	GGGGAGGTAGTGACGAAAAAT		

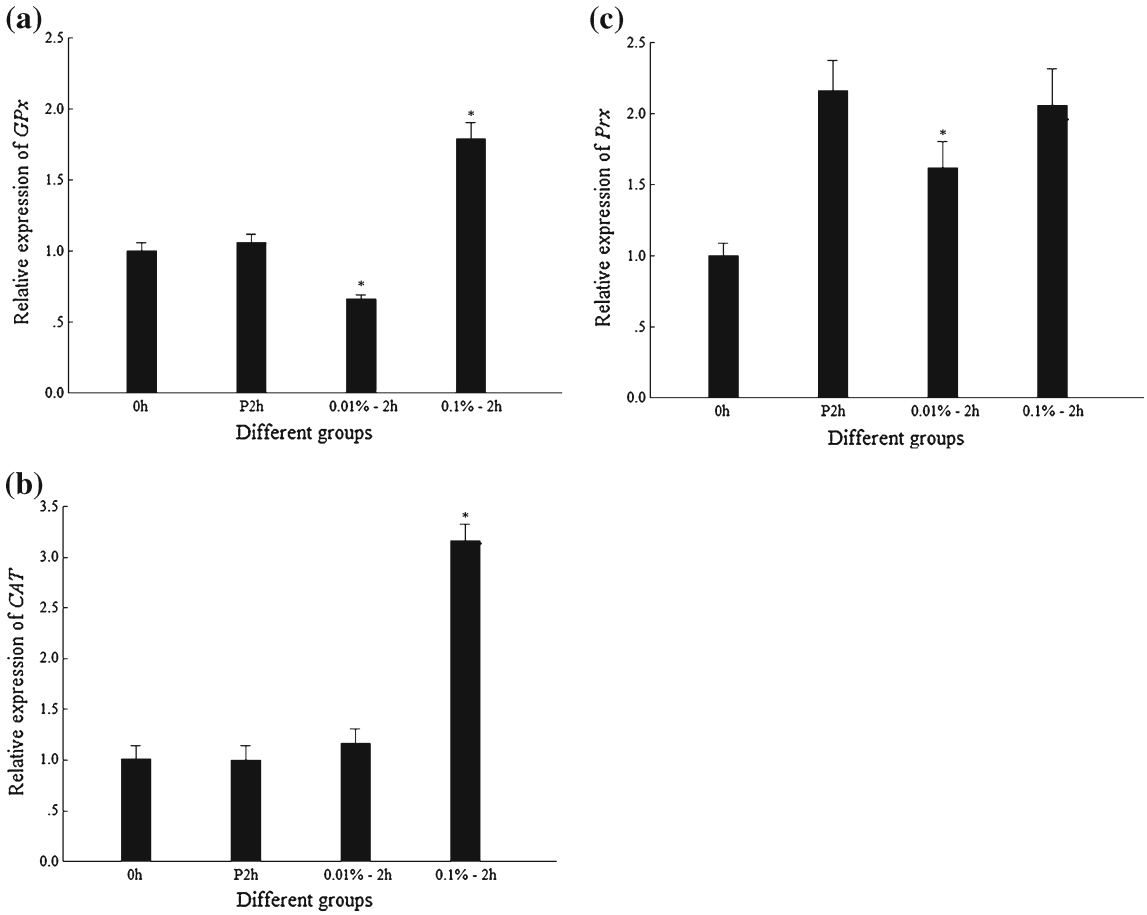

Fig. 2 Transcriptional expression profiles of *GPx*, *CAT*, and *Prx* in shrimp after H_2O_2 injections. *0h* blank group without any injection, *P2h* 2 hpi of PBS, *0.01%–2 h* 2 hpi of 0.01% H_2O_2; *0.1%–2 h* 2 hpi of 0.1% H_2O_2. *Asterisk* means that the difference between 0.01%–2 h or 0.1%–2 h and P2h group is statistically significant. **a** Expression profile of *GPx*. **b** Expression profile of *CAT*. **c** Expression profile of *Prx*

fragments with the annealing temperature are shown in Table 1. The synthesized cDNAs from RNA isolated from samples preserved above were used as template for real-time PCR analysis. *18S rRNA* was quantified as a stably expressed reference gene. The amplification conditions were as follows: 1 cycle of 94°C for 2 min; 40 cycles of 94°C for 15 s, annealing for 20 s, and 72°C for 20 s. In order to confirm whether only one specific PCR product was

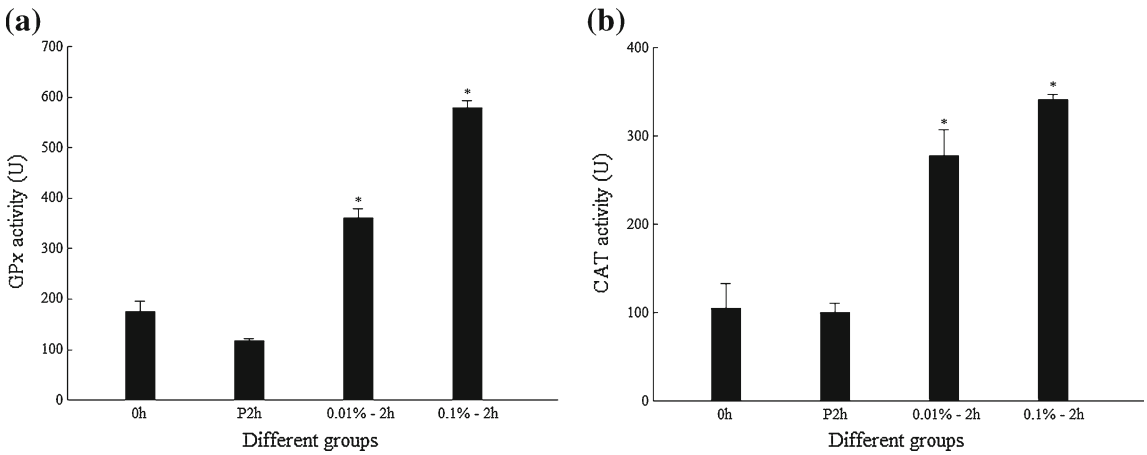

Fig. 3 Enzyme activities of GPx and CAT in shrimp after H_2O_2 injections. *Asterisk* means that the difference between 0.01%–2 h or 0.1%–2 h and P2h group is statistically significant. **a** GPx activity. **b** CAT activity

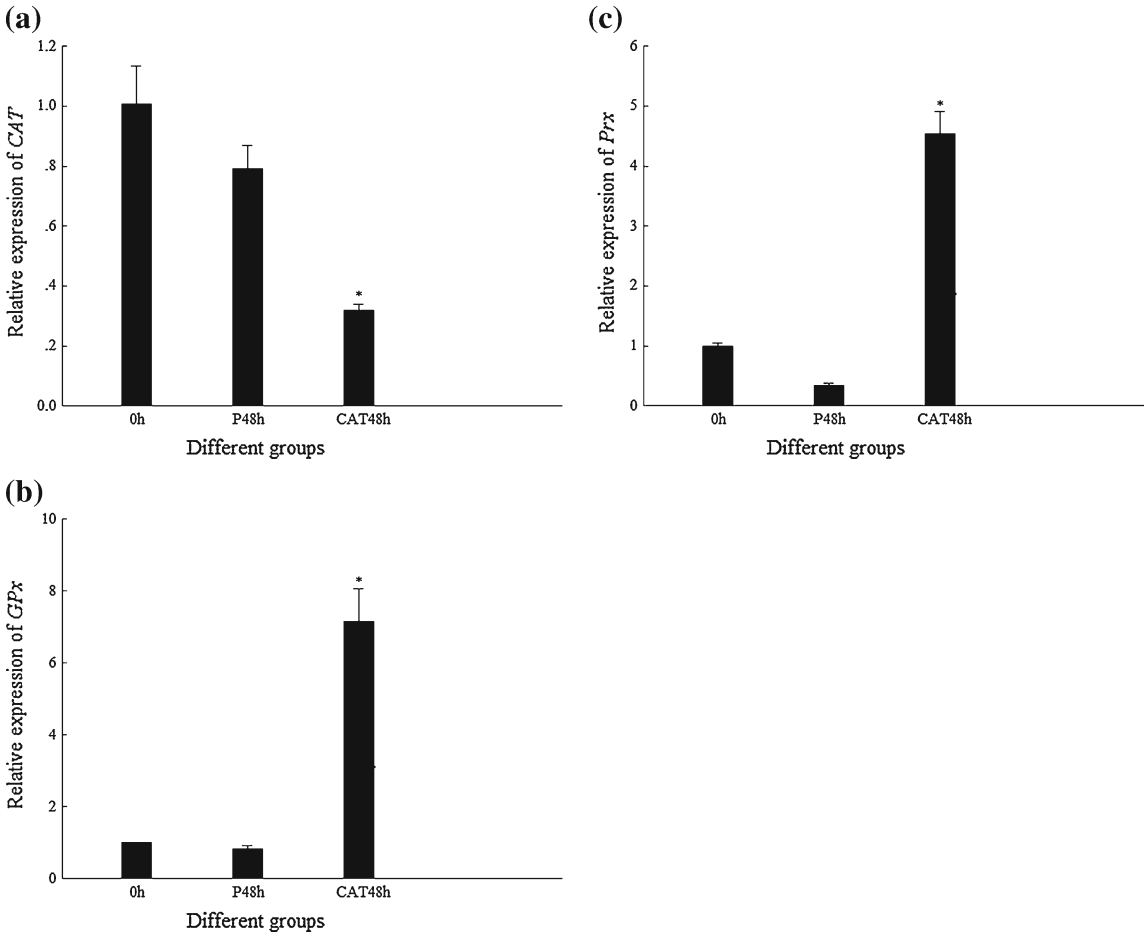

Fig. 4 Transcriptional expression profiles of *CAT*, *GPx*, and *Prx* in shrimp when *CAT* were silenced by DsCAT. *P48h* 48 hpi of PBS, *CAT48h* 48 hpi of DsCAT. *Asterisk* means that the difference between CAT48h and P48h group is statistically significant. **a** Expression profile of *CAT*. **b** Expression profile of *GPx*. **c** Expression profile of *Prx*

amplified, a melt cycle, in which PCR product was denatured from 68°C to 100°C, was added to each thermal profile to produce the melting curves. Expression of other target genes was normalized to *18S rRNA*, which served as an internal control for calibrating the quantity of RNA isolated from each sample. Experiments were performed in quadruplicate, and at least three shrimps were analyzed for each sample. The relative expression of each gene was

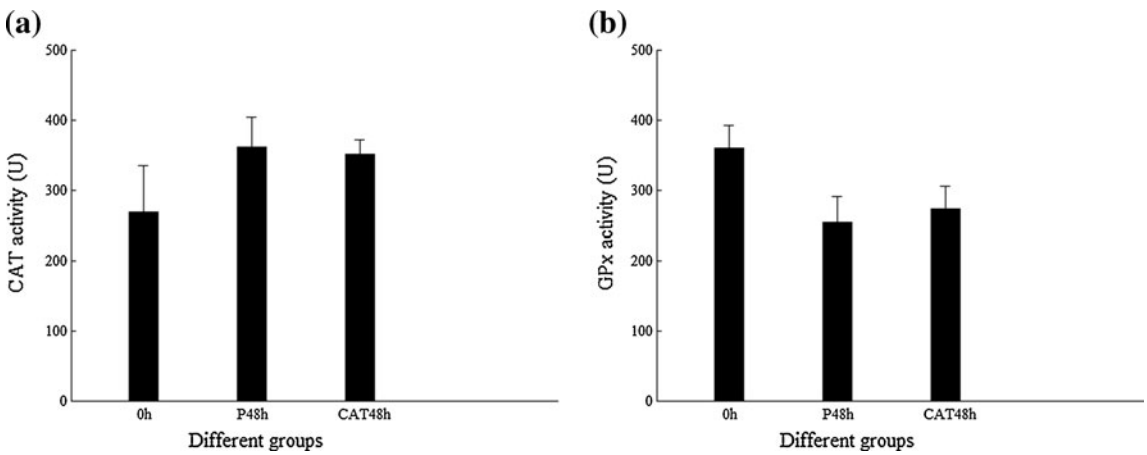

Fig. 5 Enzyme activities of CAT and GPx in shrimp when *CAT* were silenced by DsCAT. **a** CAT activity. **b** GPx activity

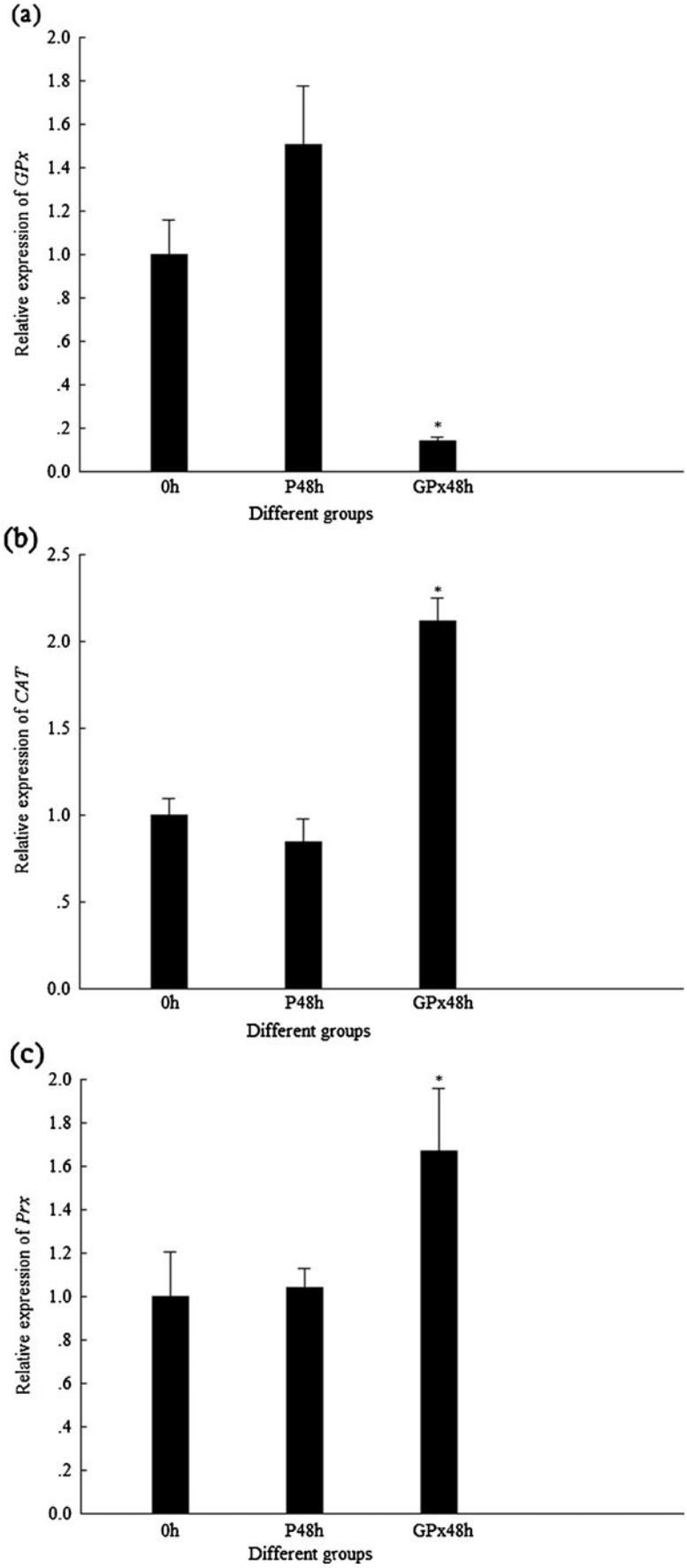

Fig. 6 Transcriptional expression profiles of *GPx*, *CAT*, and *Prx* in shrimp when *GPx* were silenced by DsGPx. *P48h* 48 hpi of PBS, *GPx48h* 48 hpi of DsGPx. *Asterisk* means that the difference between GPx48h and P48h group is statistically significant. **a** Expression profile of *GPx*. **b** Expression profile of *CAT*. **c** Expression profile of *Prx*

calculated using the comparative Ct method with the formula $2^{-\Delta\Delta Ct}$ (where $\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$). Data obtained from real-time PCR analysis were subjected to one-way analysis of variance using SPSS v 16.0, and $P<0.05$ were considered statistically significant.

The activity assay of GPx and CAT

GPx and CAT activity assays were performed respectively using the GPx and CAT activity assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instruction provided by the producer. U was expressed as the activity unit of the enzyme. One unit of GPx activity means the amount of enzyme in 1 mg tissue proteins that catalyze the reaction to make the concentration of reduced glutathione to decrease for 1 μmol/L in a minute without regard to non-enzymatic effects. One unit of CAT activity means the amount of enzyme in 1 mg tissue proteins that can reduce 1 μmol H_2O_2 in a second.

Results

Expression of *FcGPx*, *FcCAT*, and *FcPrx* in shrimp after H_2O_2 injection

The transcription level of the genes encoding different enzymes in juvenile shrimp after injection of different concentration of H_2O_2 is shown in Fig. 2. The data showed that injection of 0.1% H_2O_2 could significantly induce the upregulation of *FcGPx* and *FcCAT* on the transcription level at 2 hpi compared to those with 0.01% H_2O_2 or PBS injection (Fig. 2a, b), while the transcription level of *FcPrx* showed no apparent upregulation when shrimp was injected with 0.1% H_2O_2 (Fig. 2c). The activity assay of FcGPx and FcCAT showed that the activities of both FcGPx and FcCAT were upregulated when shrimp were injected by 0.01% or 0.1% H_2O_2 compared with their PBS controls (Fig. 3).

Detection on the expression of *FcCAT*, *FcGPx*, and *FcPrx* in DsCAT, DsGPx, or DsPrx injected shrimp

The transcription level of genes encoding different antioxidant enzymes, *FcCAT*, *FcGPx*, and *FcPrx* in shrimp, is shown in Fig. 4 when *FcCAT* were silenced by DsCAT injection for 48 h. The transcription level of *FcCAT* in DsCAT-silenced shrimp was much lower than that of its control. In this situation, the transcription levels of *FcGPx* and *FcPrx* were much higher than those of their controls. However, the enzyme activities of FcGPx and FcCAT in DsCAT-silenced shrimp did not show any difference from their PBS controls (Fig. 5).

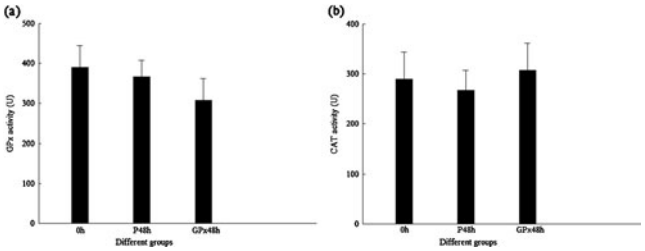

Fig. 7 Enzyme activity of GPx and CAT in shrimp when *GPx* were silenced by DsGPx. **a** GPx activity. **b** CAT activity

In DsGPx-silenced shrimp, the transcription level of *FcGPx* was much lower than that in their control group, while the transcription levels of *FcCAT* and *FcPrx* were significantly higher than those in their control groups (Fig. 6). Similar with the results of DsCAT-silenced shrimp, the enzyme activities of FcGPx and FcCAT in DsGPx-silenced shrimp did not show any difference from their PBS controls (Fig. 7).

In DsPrx-injected shrimp, the transcription level of *FcPrx* did not show any suppression compared with their control group; in reverse, it showed upregulation (Fig. 8). Therefore, the injection of DsPrx in shrimp could not silence the expression of *FcPrx*. In this case, the expression of FcGPx and FcCAT was not detected further.

Expression analysis on *FcGPx*, *FcCAT*, and *FcPrx* when DsGPx-silenced shrimp were injected with H_2O_2

In order to know whether there existed some compensation among the expression of these three genes, the transcriptions of *FcCAT* and *FcPrx* were analyzed when DsGPx-silenced shrimp were injected with H_2O_2. The transcription levels of *FcGPx*, *FcCAT*, and *FcPrx* in DsGPx-silenced shrimp injected with 0.1% H_2O_2 for 2 h are shown in Fig. 9.

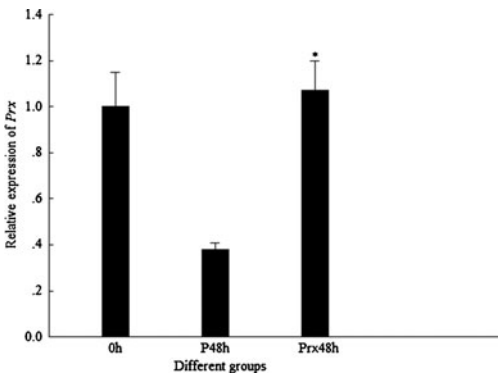

Fig. 8 Transcriptional expression profile of *Prx* in shrimp after injected by DsPrx. *P48h* 48 hpi of PBS, *Prx48h* 48 hpi of DsPrx. *Asterisk* means that the difference between Prx48h and P48h group is statistically significant

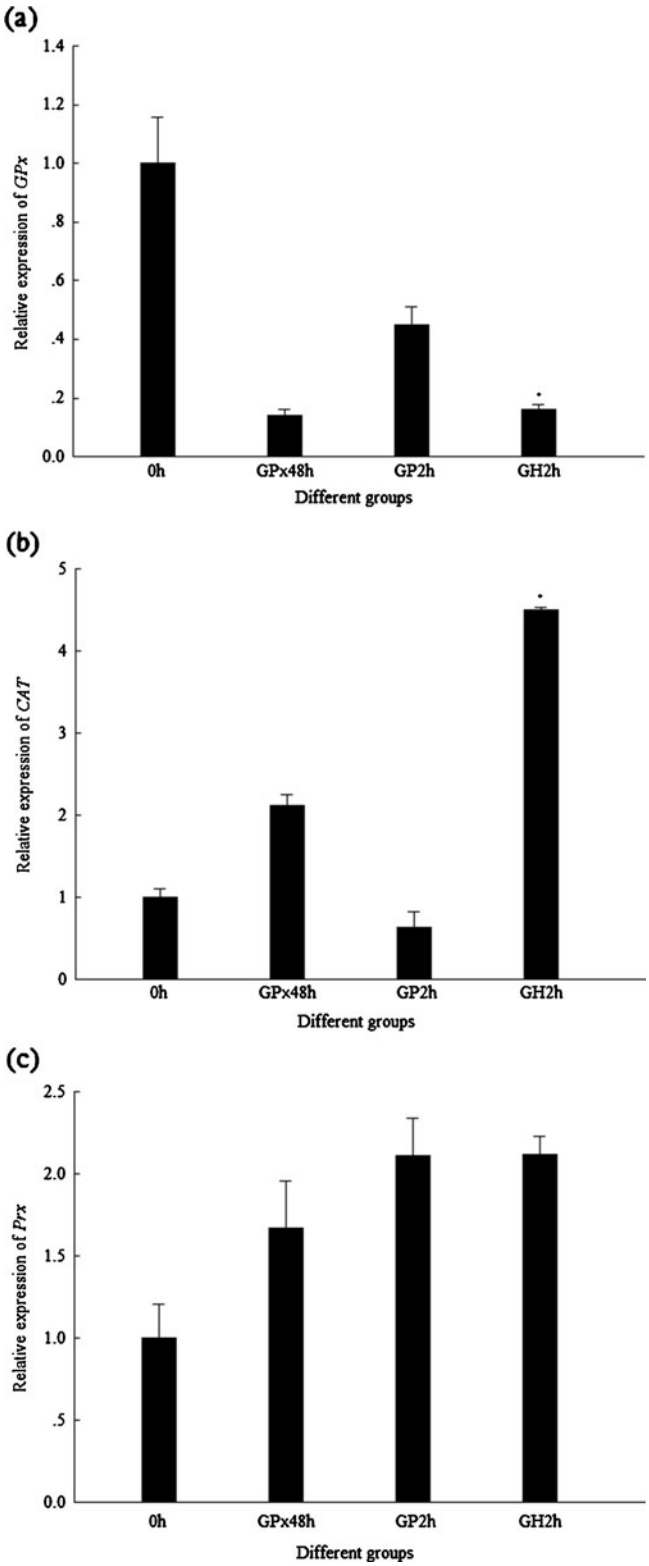

Fig. 9 Transcriptional expression profiles of *GPx*, *CAT*, and *Prx* in DsGPx-silenced shrimp injected with 0.1% H_2O_2. *GPx48h* 48 hpi of DsGPx, *GP2h* 2 hpi of PBS after DsGPx silenced, *GH2h* 2 hpi of H_2O_2 after DsGPx silenced. *Asterisk* means that the difference between GH2h and GP2h is statistically significant. **a** Expression profile of *GPx*. **b** Expression profile of *CAT*. **c** Expression profile of *Prx*

When shrimps were injected with PBS for 2 h, no any upregulation at transcription level for *FcGPx*, *FcCAT*, and *FcPrx* was detected; however, when shrimps were injected with H_2O_2, although the transcription level of *FcGPx* and *FcPrx* was not upregulated, the transcription level of *FcCAT* was greatly upregulated. However, there was no big difference for the enzyme activity of FcGPx and FcCAT (Fig. 10).

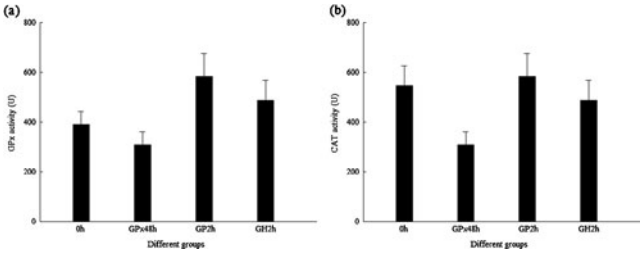

Fig. 10 Enzyme activities of GPx and CAT in DsGPx-silenced shrimp injected with 0.1% H_2O_2. **a** GPx activity. **b** CAT activity

Discussion

In high animals, the produced superoxide radicals after cell stress will be converted to hydrogen peroxide (H_2O_2) and oxygen (O_2) by SOD, and the residual H_2O_2 will be catalyzed into harmless water (H_2O) and oxygen (O_2) by CAT, GPx, and Prx (Aruoma 1998; Holmblad and Soderhall 1999; Schwarz 1996). The role of the antioxidant defense system in invertebrates could be of even greater importance than that in vertebrates, since phagocytosis—a highly ROS and reactive oxygen intermediate (ROI) producer process—is a major line of defense against invading microorganisms due to their lack of antibodies and acquired immunity (Johansson et al. 1999). The respiratory burst increased significantly in giant freshwater prawn, *Macrobrachium rosenbergii*, to eliminate the invading pathogen; therefore, the up-regulation of antioxidant enzyme activity and messenger RNA transcription were upregulated in order to convert the excess ROS caused by pathogen infection (Yeh et al. 2009). In black tiger shrimp *P. monodon*, respiratory bursts of *Photobacterium damsela* and WSSV-injected shrimp significantly increased (Liu et al. 2010). Therefore, the antioxidant enzymes might play key roles in the innate immunity of shrimp.

In the present study, when shrimp were injected by two doses of H_2O_2 (0.01% and 0.1%), a low dose (0.01%) did not induce upregulation of GPx and CAT at transcription level, but a high dose (0.1%) led to significant upregulation of GPx and CAT. In contrast to the variation at transcription level, the activity of GPx and CAT increased after both doses of H_2O_2 injection. These data suggested that both GPx and CAT were important for shrimp to eliminate H_2O_2. However, two doses of H_2O_2 injection did not induce any upregulation of Prx at the transcription levels; this might be related to the existence of abundant Prx protein in the cell. It was reported that Prxs are fairly abundant proteins and present in an approximate range from 1 to 10 μg/mg of soluble protein (Chae et al. 1999). On the other hand, it might be possible that the dose of 0.1% H_2O_2 is not enough to induce the variation of Prx at transcription or protein level. When CAT was silenced by DsRNA injection, the transcription of GPx and Prx was significantly upregulated. When GPx was silenced by DsRNA injection, the transcription level of CAT and Prx was apparently upregulated. Therefore, we inferred that the expression of CAT, GPx, and Prx could compensate each other to sustain the homeostasis of the cell in shrimp. When CAT or GPx was silenced for 48 h, we did not detect any variations of their activity. The reason might be that the decreased activity of CAT or GPx would be shown later than 48 h. DsRNA injection for 48 h could only cause the depressing at the transcription level of CAT or GPx, but their activity variation might be at a later time. In the present study, we did not succeed in silencing the expression of Prx by DsRNA injection in shrimp, this might also be caused by high amount of Prx that existed in the cell. In order to confirm the roles of CAT, GPx, and Prx in eliminating H_2O_2 in shrimp, we injected H_2O_2 in DsGPx-silenced shrimp. In GPx-silenced shrimp, the transcription level of GPx at 2 h post-0.1% H_2O_2 injection was not upregulated, which was different from the data that the transcription level of GPx was elevated in normal shrimp at 2 h post-0.1% H_2O_2 injection. This might indicate that the injected double-strand RNA of GPx was still effective in shrimp to silence GPx. Therefore, even if more transcripts of GPx were synthesized, they would be degraded due to the existence of double-strand RNA of GPx. Although injection of H_2O_2 could not cause the upregulation of Prx at transcription level, silencing of GPx or CAT can lead to upregulation of Prx at transcription level, which suggest that potential relationships among CAT, GPx, and Prx might exist in penaeid shrimp just like the situation in higher animals. When we detect the enzyme activities of FcGPx and FcCAT in DsGPx or DsCAT-silenced shrimp for 48 h, we did not detect any variation on GPx or CAT activity. We guess that the expression at translation level might compensate each other in later time because the regulation at translation level is always later than that at transcription level.

In summary, close relationships among three antioxidant enzymes, CAT, GPx, and Prx, existed in shrimp. The transcription of CAT and GPx could be directly induced by H_2O_2 injection; however, the transcription of Prx cannot be induced by H_2O_2, but decreased expression of CAT and GPx at transcription level could lead to increased transcription of Prx. Above data suggested that there exist some compensation among these three antioxidant enzyme genes.

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