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Cloning, structural features, and expression analysis of the gene encoding thioredoxin reductase 1 from *Apis cerana cerana*

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ABSTRACT

Previous studies have demonstrated that mammalian thioredoxin reductases 1 (TrxRs; EC 1.6.4.5) play roles in protection against oxidant injury, cell growth and transformation, and the recycling of ascorbate from its oxidized form. However, little is known about the function of TrxRs in insects, especially in *Apis cerana cerana*. To gain a better understanding of its role in insects, we cloned *TrxR1* from *A. cerana cerana* (*AccTrxR1*) and investigated its structural features and expression. The full-length cDNA is 1998 bp long and contains an open reading frame (ORF) of 1485 bp. The deduced AccTrxR1 protein has 494 aa, a calculated molecular mass (MW) of 54.24 kDa, and an isoelectric point of pH 7.35. The full-length genomic DNA of *AccTrxR1* is 3500 bp and contains eight exons and seven introns. In addition, the sequence and putative transcription factor-binding sites of the 5′-flanking region were examined. We also looked for conserved domains/motifs, generated homologous alignments, conducted phylogenetic analysis, and made secondary and tertiary structure predictions using web software. RT-PCR revealed that the expression of *AccTrxR1* could be induced by UV and heat (37 °C). These results indicate that AccTrxR1 may play a key role in protection against oxidant stress.

1. Introduction

The thioredoxin reductases (TrxRs) are selenoproteins belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases (Tamura and Stadtman, 1996; Williams, 1995). Members of this family are homodimeric proteins in which each monomer contains an FAD prosthetic group, an NADPH-binding site and an active site that contains a redox-active disulfide (Mustacich and Powis, 2000). The primary function of TrxRs is to catalyze the NADPH-dependent reduction of thioredoxin (Trx), a ubiquitous 12-kDa protein that is the major protein disulfide reductase in cells (Matsui et al., 1996). Reduced Trx can powerfully catalyze either the transport of electrons to ribonucleotide reductase and other reductive enzymes or the redox regulation of enzymes and transcription factors.

Three mammalian TrxRs have been identified, named TrxR1, TrxR2 and TrxR3 (Gladyshev et al., 1996; Gasdaska et al., 1999; Sun et al., 2001). The first mammalian TrxR1 was cloned from human placenta and found to have only 31% sequence identity with prokaryotic TrxRs, but to have 44% identity with eukaryotic and prokaryotic glutathione reductases (Gasdaska et al., 1995). TrxR1 and TrxR2 are expressed universally in cells and tissues, whereas the expression of TrxR3 is mainly confined to the testes. The studies demonstrated that TrxRs play a role in protection against oxidant injury, cell growth and transformation, and the recycling of ascorbate from its oxidized form (Mustacich and Powis, 2000). Additionally, cytoplasmic TrxR has been shown to be essential for embryogenesis but dispensable for cardiac development (Jakupoglu et al., 2005). Furthermore, the neuron-specific inactivation of TrxR1 did not result in cerebellar hypoplasia, suggesting a vital role for TrxR1 in Bergmann glia or neuronal precursor cells (Soerensen et al., 2008). Finally, Trx and TrxR influence estrogen receptor {alpha}-mediated gene expression in human breast cancer cells (Rao et al., 2009).

Dipteran insects such as *Drosophila melanogaster* have no glutathione reductase, so a Trx system is particularly important (Kanzok et al., 2001). In additional to its normal functions, it also reduces GSSG. Recently, function of His-464 and Glu-469 in the acid–base catalysis of TrxR from *D. melanogaster* was discussed (Huang et al., 2008a,b). Moreover, TrxR from *Anopheles gambiae* (AgTrxR) in which glutathione reductase was absent was investigated. *AgTrxR* represents three splice variants coding for two cytosolic and one mitochondrial variant and it is not a selenoenzyme but instead contains a highly unusual redox-active Cys-Cys sequence (Bauer et al., 2003).

The Chinese honeybee, *Apis cerana cerana*, is one of the main subspecies of *Apis cerana* with higher vitality and resistance to adversity (Yang, 2005). To determine the possible implication of antioxidant enzymes in the higher vitality and resistance to adversity of Chinese honeybee, we cloned *TrxR1* from *A. cerana cerana* and structural features, and expression levels under UV and heat stresses

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were also been analyzed. In present study, we cloned the cDNA and genomic DNA of *TrxR1* from *A. cerana cerana*. In addition, we firstly sequenced the 5′-flanking region and identified putative transcription factor-binding sites. RT-PCR analysis revealed that whereas the expression of *AccTrxR1* could be induced by UV and heat (37 °C), there were no obvious changes in its expression level between different tissues or developmental stages. These results revealed that AccTrxR1 might play an important role in protection against oxidant stress.

2. Materials and methods

2.1. Animals and treatments

The Chinese honeybees, *A. cerana cerana*, maintained at the Shandong agricultural university of China, were used in the experiments using different treatments. Worker bees were captured as they emerged from the combs of outdoor hives. They were divided into groups of 40 individuals, kept at constant temperature and were treated using UV (30 mj/cm^2) and heat (37 °C). The larvae, pupae and adult bees were grouped according to their age. All bees were flash-frozen in liquid nitrogen at the indicated time points and stored at −80 °C.

2.2. RNA extraction, cDNA synthesis and DNA isolation

Total RNA was extracted using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. Total RNA was digested with RNase-free DNase-I to remove the genomic DNA. The first-strand cDNA was then synthesized from 2 μg of total RNA by MMLV (moloney-murine-leukaemia virus) reverse transcripase (TaKaRa) with an adaptor primer oligo d(T)$_{18}$ (TaKaRa) at 42 °C for 60 min. Genomic DNA was isolated using the EasyPure Genomic DNA Extraction Kit according to the manufacturer's instructions (TransGen biotechnology, Beijing).

2.3. Primers

The primers used in this study are listed in Table 1.

2.4. Cloning of the internal conservative sequence of AccTrxR1

To obtain the internal conservative fragment, we designed and synthesized the primers AMP1 and AMP2 based on amino acid and nucleotide sequences that are conserved among *Nasonia vitripennis*, *Tribolium castaneum* and *Apis mellifera* (Shanghai Sangon Biotechnological Company, China). The PCR reaction used 35 cycles as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C and extension for 2 min at 72 °C. The PCR product was purified, cloned into the pMD18-T vector (TaKaRa) and sequenced.

2.5. 3′ and 5′cDNA end amplification of AccTrxR1

Base on the internal sequence of *AccTrxR1*, we obtained the 3′ and 5′ ends using the rapid amplification of cDNA ends (RACE) approach and using gene-specific and adapter primers. For 5′ RACE, the cDNA was purified and polyadenylated as described by Wang et al. (2008), and the gene-specific primers 5AP1 and 5AP2 were designed based on the sequence of the cloned internal fragment. The first-round PCR primers were 5AP1 and the Abridged Anchor Primer (AAP), and the PCR product was diluted 100-fold for nested PCR with a second round of amplification using the Abridged Universal Amplification Primer (AUAP) and 5AP2. The two steps were carried out under the following conditions: pre-denaturation at 94 °C for 5 min; followed by 35 cycles of amplification (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s).

Based on the sequence of the cloned internal fragment, we designed the two gene-specific primers (3AP1 and 3AP2) for 3′ RACE. The primary PCR primers were 3AP1 and B26. The secondary PCR primers were 3AP2 and B25. The first PCR used 28 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. The second PCR reaction was programmed as follows: pre-denaturation at 94 °C for 5 min, followed by 35 cycles of amplification (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s). The PCR product was purified, cloned into the pMD18-T vector and sequenced.

Table 1
The primers used.

Abbreviation	Primer sequence(5′–3′)	Description
AMP1	GGGTGGATCAGGTGGTTTAG	cDNA genomic sequence primer, forward
AMP2	CCATACAGCGCAATGTGTTCTAC	cDNA genomic sequence primer, reverse
5AP1	GTACCACCTAGACCCCAAGTAC	Reverse primer for 5′ RACE, outer
5AP2	GCACCAAAATTCACTGCTTC	Reverse primer for 5′ RACE and genomic sequence
AAP	GGCCACGCGTCGACTAGTAC(G)$_{14}$	Abridged Anchor Primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged Universal Amplification Primer
3AP1	TTGCATTGACCGTTCGCTCTAG	Forward primer for 3′ RACE, outer
3AP2	GTAGAACACATTGCGCTGTATGG	Forward primer for 3′ RACE and genomic sequence
B26	GACTCTAGACGACATCGA(T)$_{18}$	3′ RACE universal adaptor primer
B25	GACTCTAGACGACATCGA	3′ RACE universal primer
AQ1	GTAGTTCGCTTTTGTCTTTTAGTTG	Full-length cDNA and genomic sequence primer, forward
AQ2	CATACAGCGCAATGTGTTCTAC	Full-length cDNA and genomic sequence primer, reverse
JYZ1	TTGTCACACCCTCTCCACG	Genomic sequence primer, forward
JYZ2	TCTTCTGTGAGTGGTTTACGAC	Genomic sequence primer, reverse
JYZ3	GTCGTAAACCACTCACAGAA	Genomic sequence RT-PCR primer, forward
JYZ4	TCTTCTTGCCAATAATCGACC	RT-PCR primer, reverse
AS1	GGCTTGCATGTGCTGTGAG	Inverse PCR primer, forward
AS2	TATCTATTTGTATATTGACAACCGG	Inverse PCR primer, forward
AX1	CTCTGTAAGGAATGTTCTCTG	Inverse PCR primer, reverse
AX2	GCACTTAATCGAACGACTTCTTAC	Inverse PCR primer, reverse
R	TCCTGCTATGTATGTCGC	RT-PCR primer, forward
F	AGTTGCCATTTCCTGTTC	RT-PCR primer, reverse

2.6. Amplification of the full-length cDNA and genomic sequence of AccTrxR1

By aligning the sequences of the 3′ RACE, 5′ RACE and middle region products, we deduced the full-length cDNA sequence of *AccTrxR1*. The sequence was obtained using one-step RT-PCR amplification, which we performed using the primers AQ1 and AQ2 as follows: pre-denaturation at 94 °C for 5 min and followed by 35 cycles of amplification (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min).

To obtain the genomic sequence of *AccTrxR1*, we designed and synthesized three pairs of primers based on the cDNA sequence. The first pair was AQ1 and 5AP2, the second pair was JYZ1 and JYZ2, and the third pair was JYZ3 andAQ2. All of the above PCR reactions were performed as follows: 94 °C for 10 min, followed by 35 cycles of amplification (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min 30 s). The PCR product was purified, ligated into the pMD18-T vector and sequenced.

2.7. Cloning of the 5′-flanking region of AccTrxR1

The 5′-flanking region of *AccTrxR1* was obtained using inverse PCR as described by Wang et al. (2008). The restriction endonuclease used was AluI. Two pairs of primers were designed and synthesized based on the genomic DNA sequence. The first PCR was performed using the primers AS1 and AX1 under the following conditions: pre-denaturation at 94 °C for 10 min, followed by 35 cycles of amplification (94 °C for 30 s,