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Full Length Research Paper

Molecular cloning and characteristic analysis of a thioredoxin from *Neobenedenia melleni*

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Thioredoxin (Trx) can regulate disulfide bond reduction of target proteins to maintain the reduced intracellular state in various organisms. Here, we cloned a cDNA sequence of thioredoxin from *Neobenedenia melleni*, which is a kind of platyhelminth parasite infecting many fishes of great economic value. The deduced *N. melleni* Trx (NmTrx) contained 170 amino acid residues with an active site consisting of four amino acid motif CPGC. Sequence comparison and phylogenetic tree analysis confirmed NmTrx as a distinct member of thioredoxin. Real-time quantitative polymerase chain reaction (PCR) revealed a significantly higher expression of NmTrx transcript in the adult stage compared with the egg and oncomiracidium stages. In the egg and adult stages, the NmTrx transcript level in the 32°C group was higher than those in the 18 and 25°C groups. NmTrx was expressed and purified from *Escherichia coli*, and antiserum against NmTrx was prepared. Western blot confirmed the higher NmTrx expression of the egg and adult stages in the 32°C group with respect to the other temperature groups. Recombinant NmTrx was able to reduce the disulfide bond in insulin, and its antioxidant capacity was determined to be 5.12 U/mg protein, similar to the classic thioredoxins. Trx activity was lower in the oncomiracidium stage and higher in the adult stage compared with the egg stage. These results indicate that NmTrx could function as an important antioxidant molecule under physiological conditions.

Key words: Thioredoxin, *Neobenedenia melleni*, redox regulation, mRNA expression, prokaryotic expression.

INTRODUCTION

The cellular damage due to the formation of reactive oxygen species (ROS) results in the development of antioxidant systems to maintain the reduced intracellular state by protecting molecule structure. In the cells, the mitochondrial respiratory chain is the major source of ROS (Chen et al., 2003). ROS might get attached to protein molecules and damage their functions (Roos and Messens, 2011). Thioredoxin (Trx), a hydrogen-carrying protein, plays an important role in maintaining the cellular redox state by control of reactive oxygen. It is first characterized as a hydrogen donor for ribonucleotide reductase in *Escherichia coli* (Holmgren, 1979). Trxs are characterized by a dithiol/disulfide active site (CGPC), which is conserved in bacteria, plants, and animals (Spyrou et al., 1997; Gelhaye et al., 2004). In human, there are three Trxs encoded by separate genes. Trx1, a cytosolic and nuclear form, is the most studied of the three forms of Trx (Nordberg and Arner, 2001). Trx2, a mitochondrial protein, contains a unique 60 amino acid N-terminal mitochondrial translocation signal (Spyrou et al., 1997). SpTrx, the third isoform of Trx, is highly expressed in spermatozoa (Miranda-Vizuete et al., 2001). Trx participates in a wide variety of physiological processes. Trx has shown anti-apoptotic functions by inhibiting apoptosis signal-regulating kinase-1 (Saitoh et al., 1998). Various oxidative stresses can induce Trx expression to perform an antioxidant effect (Nakamura et al., 2009). Trx shows redox regulatory functions in signal transduction

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Abbreviations: Trx, Thioredoxin; **GSH,** glutathione; **NmTrx,** a thioredoxin from *N. melleni*; **ANOVA,** one-way analysis of variance.

and regulates the DNA binding activity of transcription factors such as AP-1, NF-κB, p53 and hypoxia-inducible factor-1α (Watanabe et al., 2010). Trx can be secreted by antigen presenting cells to activate T lymphocytes (Angelini et al., 2002). In most organisms, antioxidant defenses include two major pathways: the glutathione (GSH) and the Trx systems (Toledano et al., 2007). The components of these pathways include GSH, Trx, and their enzymes glutathione reductase and thioredoxin reductase, both of which reduce the oxidized GSH and Trx. In parasites, the challenge for the control of ROS is greater because they must control not only their metabolic production but also the potential damage induced by the host immune attack. Some thioredoxin genes have been cloned and characterized in endoparasites, these includes, *Opisthorchis viverrini* (Suttiprapa et al., 2012), *Haemonchus contortus* (Sotirchos et al., 2008), *Haemonchus contortus* (Sotirchos et al., 2009) and *Schistosoma mansoni* (Alger et al., 2002). However, the Trx of body surface parasites is less known. Parasites possess unique antioxidant systems, which is different from conventional thioredoxin and glutathione systems (Otero et al., 2010). The platyhelminth *S. mansoni*, for example, lacks canonical thioredoxin and glutathione systems. Instead, it possesses a linked glutathione thioredoxin system that contains a selenoenzyme thioredoxin glutathione reductase for the provision of reducing equivalents to whole system (Bonilla et al., 2008). In trypanosomes, the two systems are joined to one system with a small peptide trypanothione [bis(glutathionyl)spermidine] which is oxidized by the enzyme tryparedoxin and is regenerated by the enzyme trypanothione reductase (Krauth-Siegel et al., 2005). *Neobenedenia melleni*, a kind of body surface platyhelminth parasite, can infect many important cage-cultured fishes, such as, the large yellow croaker (*Pseudosciaena crocea*), the miiuy croaker (*Miichthys miiuy*), the tilapia (*Oreochromis mossambicus*), the amberjack (*Seriola dumerili*), and *Epinephelus awoara* (Yang et al., 2001, 2007). Its outbreaks have caused fearful economic losses in fish mariculture. It would be beneficial to develop the potential method to control *N. melleni* infection by characterizing its antioxidant systems. However, no information is available for Trx in *N. melleni*. Here, we aimed to identify and characterize a thioredoxin from *N. melleni* (NmTrx). It is the first time that the thioredoxin of body surface platyhelminth parasite *N. melleni* was identified, and it is helpful to a better understanding of the antioxidant systems in this fish parasite.

MATERIALS AND METHODS

Parasite source

Miiuy croaker (*Miichthys miiuy*) infected with *N. melleni* were maintained in 500 L tanks and fed with the basal diet, at Xiangshan Seaport Aquatic Seedling and fingerling Limited Company, Ningbo city, China. One hundred adult *N. melleni* worms were removed from the fish body surface using a scalpel blade. Adult parasites were placed in tissue culture dishes containing filtered seawater and were incubated at 25°C for a day to induce egg-laying (Lin et al., 2008). Deposited parasite eggs were collected in a 300 ml plastic beaker containing sand-filtered seawater and incubated at 25°C, with the water changed daily. After incubation for 5 to 9 days, the oncomiracidia were collected within 6 h of hatching. For the thermotolerance experiment, the *N. melleni* eggs and adults were incubated at 18, 25 and 32°C for 24 h, respectively. Collected eggs, oncomiracidia and adults samples were immediately frozen in liquid nitrogen before been stored at -70°C.

Cloning of the full-length cDNA of NmTrx

Express sequence tag (EST) sequence was employed to obtain important gene information in *N. melleni*. Total RNAs were extracted from the samples of eggs, oncomiracidia, and adults of *N. melleni* using RNAiso reagents (TaKaRa, Kyoto, Japan) and mRNA was purified using Oligotex-dT30<super> (TaKaRa), respectively. In order to generate EST resources with maximal efficiency of gene discovery, three individual mRNA samples were pooled together in equal amounts. The cDNA Library Construction Kit (TaKaRa) was used according to the manufacturer's protocols. A total of 30649 selected clones were partially auto-sequenced by an ABI 3730 automated sequencer (Invitrogen) and analyzed with BLASTX search (http://www.ncbi.nlm.nih.gov/).

Sequence analysis

The cleavage site of signal peptides was predicted by the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/). The incidence of conserved protein domains was assessed using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). Multiple sequence alignment, phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007).

Transcripts analysis of NmTrx in different developmental stages of *N. melleni*

Total RNA was isolated from eggs, oncomiracidia and adults of *N. melleni*, respectively using RNAiso reagent (TaKaRa, Kyoto, Japan), then treated with deoxyribonuclease I (TaKaRa) and reverse transcribed using Reverse Transcriptase M-MLV (RNase H^-) (TaKaRa). Primers NmTrx (+): 5'-AAATTAGAAGCCGTT-CTGGC-3' and NmTrx (-): 5'-GCTCTCTTTCTCCGCTTCTT-3' were used to amplify a 105 bp fragment of the NmTrx gene. As an internal PCR control, primers 28S rRNA(+): 5'-AAGCCACCATGCGTTTGTA-3' and 28S rRNA(-): 5'-TCATGCCAGAATACCAACC-3' were used to amplify a 149 bp fragment of the housekeeping 28S rRNA gene (accession number: FJ972005). One microliter of the resultant solution from each reverse transcription reaction served as the template in a 20 µl real-time polymerase chain reaction (PCR) reaction using SYBR premix Ex Taq (Perfect Real Time) (TaKaRa). The real-time PCR reaction was performed in triplicate on the RT-Cycler™ Realtime Fluorescence Quantitative PCR machine (CapitalBio, Beijing, China) using the following conditions (10 min at 95°C at the beginning, 40 cycles of 95°C for 30 s, 58°C for 30 s, and finally 72°C for 30 s). The mRNA expression of the NmTrx was normalized against 28S rRNA expression. All data were expressed as means ± SEM and statistified by one-way analysis of variance (ANOVA) with SPSS (version 13.0, Chicago, IL, USA). The tests were considered statistically significant at $p < 0.05$.

Prokaryotic expression and purification

Based on the previously determined NmTrx sequence, a primer pair was designed that would amplify the ORF and which included restriction sites for *Nde* I and *Bam*H I at the 5' and 3' ends of the upstream (NmTrx-F: 5'-CCATATGTACGATCATGTTACAACAGA-3') and downstream (NmTrx-R: 5'-CGGATCCTTAGTTTTTT-GGTGTTGTTATAT-3') primers respectively, in order to facilitate subsequent directional cloning into the *Nde*I/*Bam*HI-digested pET28a vector (QIAGEN, Shanghai, China). *Pfu* DNA Polymerase (Fermentas) was used for gene amplification according to the manufacturer's protocols. Prokaryotic over-expression of the protein was performed according to established protocols (Sambrook et al., 1989). The His-tagged recombinant proteins were purified by nickel-affinity chromatography according to the manufacturer's protocol (QIAGEN) and further analyzed by SDS-PAGE.

Antiserum preparation and western blot

The purified NmTrx protein was used as an antigen to immunize mice to produce antiserum (Han et al., 2007; Han and Zhang, 2007). Antigen was mixed with an equal volume of Freund's complete adjuvant (Sigma, USA). The emulsion was injected intradermally into mice and two booster shots were administered at one week interval. One day after the last injection, blood was collected, clarified by overnight incubation at 4°C, and centrifuged at 1500 g for 15 min. The serum was stored at -70°C.

Collected egg and adult samples were lysed at 4 °C in a buffer composed of 20 mM Hepes, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM NaCl, 0.2 mM DTT, 0.5 mM sodium orthovanadate, and 0.4 mM PMSF (pH 7.4). The lysate was then centrifuged at 10,000 g for 30 min. The protein concentration of the supernatant was measured in each soluble fraction by using the Bradford method, and samples were subjected to SDS-PAGE (15% acrylamide gel) and transferred to PVDF (Pall, NY, USA). Membranes were blocked for 1 h in a 10% non-fat dry milk solution in TBS-Tween. After 1.5 h incubation with NmTrx antiserum, membranes were washed and incubated for 1 h with HRP-conjugated secondary antibodies. Proteins were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA), and semiquantitative analysis was performed by scanning densitometry.

Thioredoxin activity

The activity of NmTrx was measured by monitoring the reduction of insulin by the increase in turbidity (Holmgren, 1979). The reaction mixture contained 50 mM Tris (pH 7.5), 2 mM EDTA, 0.33 mM dithiothreitol (DTT), 0.13 mM insulin, and thioredoxin in concentrations 2 to 4 µM. The reaction was started by pipetting 3 ml DTT in all cuvettes. The measurements were performed at 650 nm using 2.0 min intervals for 60 min. The time for precipitation initiation was defined as an increase by 0.02 at A650 after a stable base-line recording. The antioxidant activity of NmTrx was detected by Fe^{3+} reducing power-based total antioxidant capacity Detection Kit (Jiancheng Bioengineering co., Ltd). An antioxidant capacity unit was defined as an increase by 0.01 at A520 min^{-1} per mg protein. Trx activities in the three development stages of *N. melleni* were measured with the insulin disulfide reduction assay as described elsewhere (Schulze et al., 2004). Total protein was extracted from egg, oncomiracidium, or adult with lysis buffer. Total protein extract was incubated with buffer (50 mmol/l HEPES pH 7.6, 1 mmol/l EDTA, 1 mg/ml BSA, 2 mmol/l DTT) at 37°C for 15 min before they were incubated with human Trx reductase (Sigma, St. Louis, MO) in the reaction buffer (0.3 mmol/l insulin, 200 µmol/l NADPH, 1 mmol/l EDTA, and 20 mmol/l HEPES pH 7.6) at 37°C for 20 min. The reaction was then terminated by adding 500 µl of stop mix (6 M guanidine HCl, 1 mM DTNB in 0.2 M Tris-HCl pH 8.0) and then absorption at 412 nm was measured.

RESULTS

NmTrx gene analysis

To obtain the cDNA sequence of important genes in *N. melleni*, a total of 30649 selected clones were single-pass sequenced, resulting in the characterization of 26548 ESTs that were longer than 100 bp after eliminating vector sequences. The average insert size was estimated to be 673 bp by PCR amplification of inserts from 50 randomly selected clones. The NmTrx was identified by the BLASTX search. The nucleotide sequence obtained was deposited into the DDBJ/GenBank/EMBL databases with the accession number GW920378 for the full-length cDNA sequence of NmTrx.

The full-length cDNA sequence of NmTrx was 735 nt long (Figure 1). The predicted translation product contains 170 amino acids (aa) with a calculated M_r of 19.0 kDa and a theoretical isoelectric point (*pI*) of 4.83. The amino acid sequence was aligned with the corresponding published sequences of other animals. The N-terminal half of the protein contained the thioredoxin active-site motif which consisted of four amino acid CPGC.

Amino acid sequence comparisons showed that NmTrx had a high degree of identity with the Trx of *Xenopus (Silurana) tropicalis* and *Drosophila willistoni* (47%) than that of other animals (36 to 46%). A phylogenetic tree was constructed using the reference amino acid sequences by the neighbor-joining method. Currently accepted relationships of the animal phyla were reflected in the phylogenetic tree. NmTrx is distantly related to other animal clusters (Figure 2). In Figure 2, the values at the branching points indicate the percentage of trees in which this grouping occurred after bootstrapping (1,000 replicates; shown only when > 60%). The scale bar shows the number of substitutions per base. Accession numbers of sequences used are *Homo sapiens*_Trx1, AAF86466; *Nomascus leucogenys*_Trx1, XP003260517; *Callithrix jacchus*_Trx1, AF353204; *Rattus norvegicus*_Trx1, AAH58454; *Mus musculus*_Trx1, AAH94415; *Anolis carolinensis*_Trx1, XP003227375; *Mesobuthus caucasicus*_Trx1, CAE54119; *Litopenaeus vannamei*_Trx1, EU499301; *Fenneropenaeus chinensis*_Trx1, ACX30746; *Neobenedenia melleni*_Trx, GW920378; *Lycosa singoriensis*_Trx2, ABX75495; *Bombyx mori*_Trx2, NP_001040283; *Xenopus laevis*_Trx2, NP_001080066; *Oreochromis mossambicus*_Trx2, ABO26636; *Homo sapiens*_Trx2, AAF86467 and *Mus musculus*_Trx2, CAM23426.

The analysis of NmTrx mRNA expression

The transcript level of NmTrx was measured at the egg,

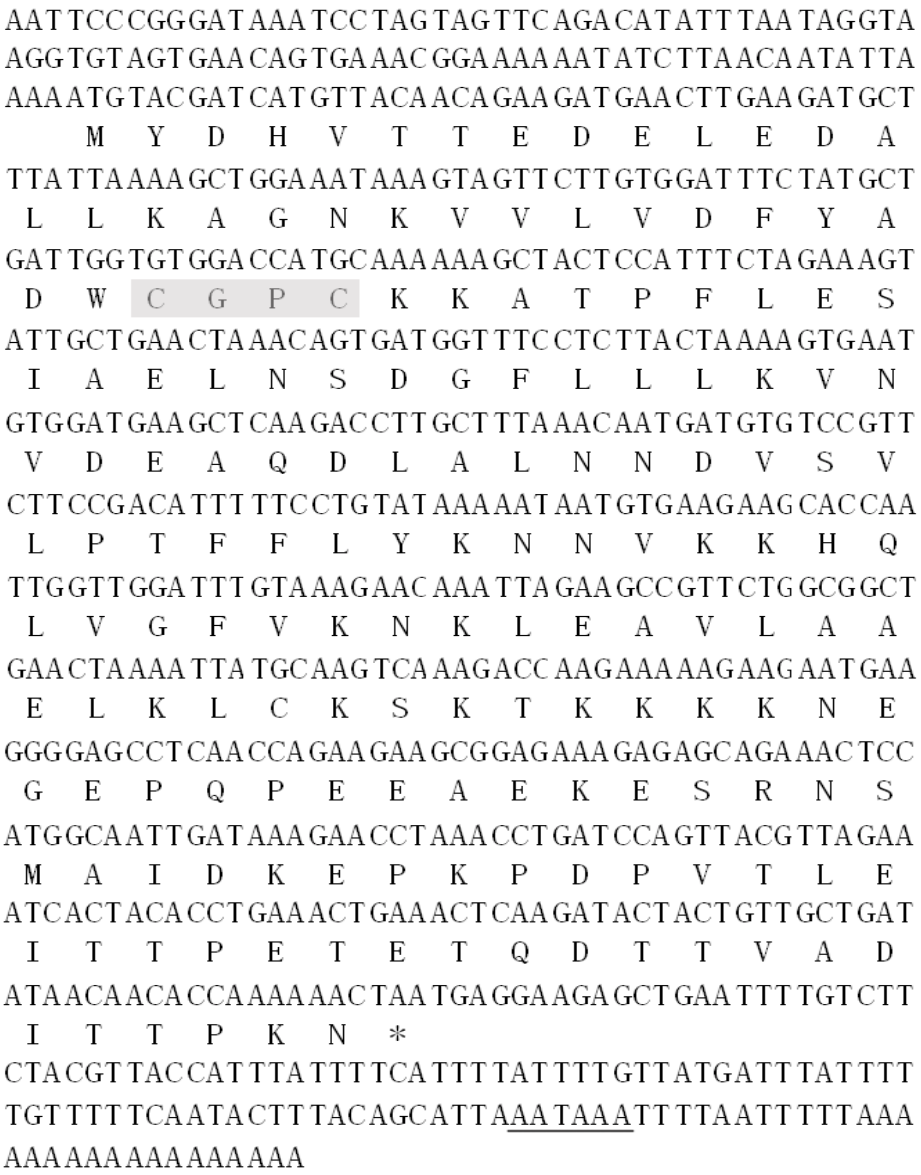

Figure 1. Nucleotide and predicted amino acid sequences of NmTrx. The catalytic center is shaded in dark. The polyadenylation signal is underlined. An asterisk indicates the stop codon.

the oncomiracidium, and the adult stages of *N. melleni* while 28S rRNA gene expression was used as an internal control. Real-time PCR data showed that NmTrx transcript was markedly lower at the oncomiracidium stage than that at the egg stage ($P < 0.01$), and higher at the adult stage compared with that at the egg stage ($P < 0.05$). Moreover, NmTrx transcript was higher at the adult stage compared with that at the oncomiracidium stage ($P < 0.001$, Figure 3A). The environment temperature induced change of NmTrx transcripts were measured at the egg and adult stages of *N. melleni*. There was no significant difference of NmTrx mRNA expression in the eggs between the 18 and 25°C groups. The NmTrx mRNA expression in the eggs in the 32°C group was higher than those in the 18 and 25°C groups ($P < 0.001$) (Figure 3B). In the adults, the NmTrx mRNA level in the 32°C groups was up-regulated compared with the 18 and 25°C groups ($P < 0.01$) (Figure 3C).

Prokaryotic expression and purification of NmTrx

The open reading frame (ORF) encoding NmTrx was amplified and cloned into the pET28a vector. For expression, plasmid was transformed into an *E. coli* strain BL21. The expression of recombinant proteins was

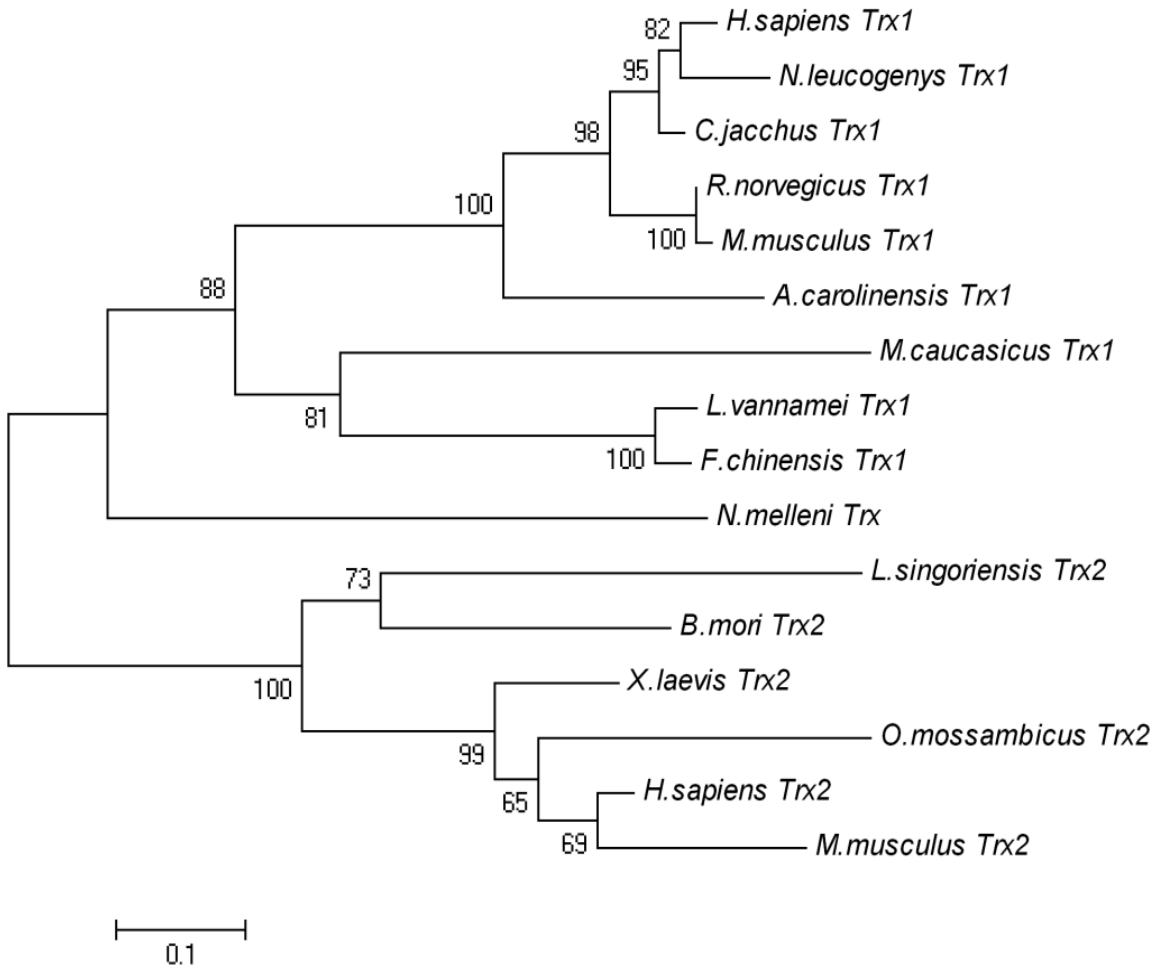

Figure 2. Phylogenetic (neighbor-joining) analysis of complete amino acid sequence of different thioredoxin molecules using the MEGA 4.0 program.

induced with 1 mM IPTG (Figure 4). The observed MW from SDS-PAGE of recombinant NmTrx was about 27.4 kDa. The resulting protein has a higher molecular weight than our predicted one because of the His-tag insertion. The target protein was further purified by nickel-nitrilotriacetic acid chromatography. The purity of recombinant NmTrx preparation was checked by SDS-PAGE, which showed a single band moving at the position expected from its molecular size (Figure 4). An NmTrx antiserum was prepared in mice immunized with the purified recombinant protein.

The temperature dependent NmTrx protein expression

To investigate the temperature dependent NmTrx protein expression, whole eggs and adults were homogenized for protein preparation after 24 h induction under the 18°C, 25°C, or 32°C. Different incubation temperatures were found to be able to effectively change the protein expression of NmTrx in the eggs and adults by Western blot method. In the eggs, the protein expression level of NmTrx showed a 2.35 fold increase at 32°C compared with 18 and 25°C (Figure 5A). There was no significant change of NmTrx protein expression in the eggs between the 18 and 25°C groups. In the adults, NmTrx protein expression was also 1.8-fold up-regulated in the 32°C group than the 18 and 25°C ones (Figure 5B).

Activity analysis of NmTrx

To estimate the activity of recombinant NmTrx, the ability to reduce the interchain disulfide of insulin was analyzed. In the control cuvette of no NmTrx, there was no precipitation throughout the whole test period. The addition of 2 and 4 μM rEsTrx1 resulted in rapid precipitation appearing after 12 and 8 min, respectively (Figure 6A). The Trx activities of eggs, oncomiracidia, and adults were further measured in whole homogenates.

Compared with the eggs, the Trx activity was down-

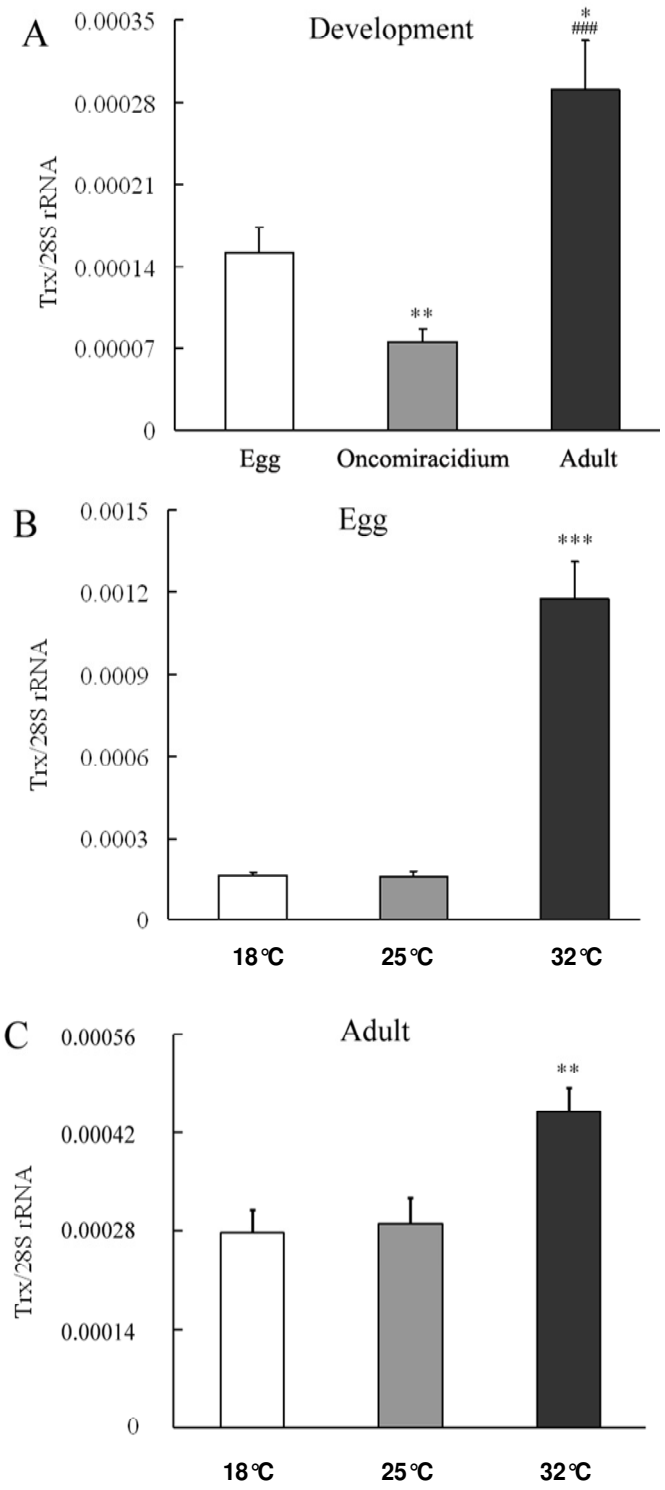

Figure 3. RT-qPCR analysis of NmTrx mRNA expression. (A), NmTrx transcript levels were different in the three development stages. $^{**}P < 0.01$ vs. Egg; $^{*}P < 0.05$ vs. Egg; ### $P < 0.001$ vs. Oncomiracidium. (B), the environmental temperature induced the change of NmTrx mRNA expression in the egg stage. (C), the change of NmTrx mRNA expression in the adult stage was induced by the environmental temperature. *** $P < 0.001$ vs. 18℃; ** $P < 0.01$ vs. 18℃. NmTrx transcript levels were normalized dividing by the 28S rRNA content. Each bar represents the mean ± SEM of the results from four samples.

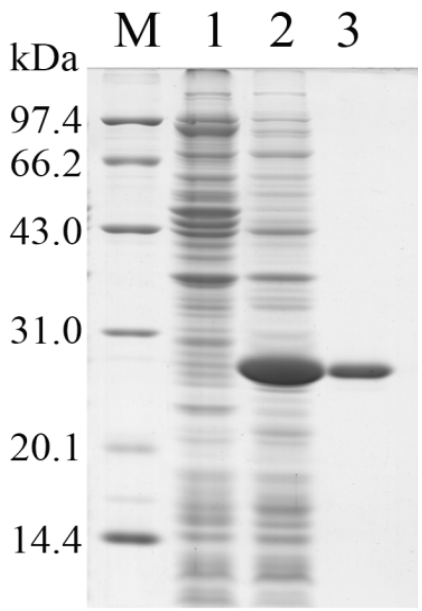

Figure 4. Prokaryotic expression and purification of recombinant NmTrx. The proteins were resolved using 15% SDS-PAGE. Lane M: protein marker; lane 1: before IPTG induction; lane 2: after IPTG induction; lane 3: purified recombinant protein.

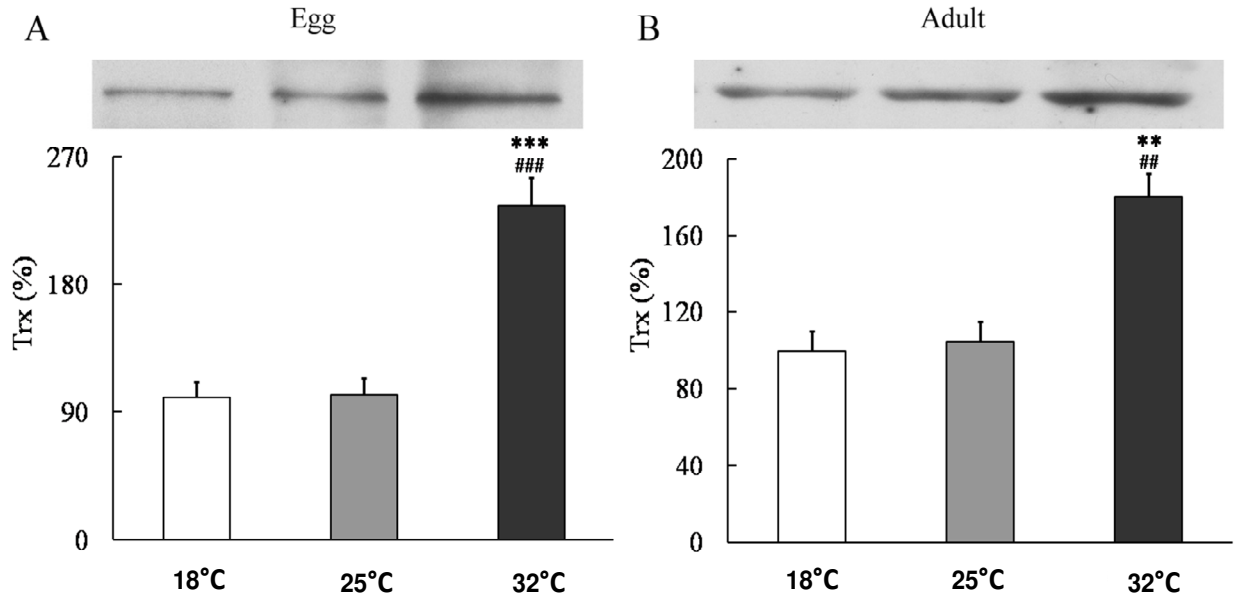

Figure 5. Analysis of NmTrx in the eggs (A) and adults (B) at different temperatures by Western blot. The eggs and adults of *N. melleni* were incubated at 18, 25, and 32°C for 24 h, respectively. The proteins were isolated from whole homogenates of eggs or adults and analyzed by Western blot (N = 3). $^{**}P < 0.01$ vs. 18°C; $^{***}P < 0.001$ vs. 18°C; ## $P < 0.01$ vs. 25°C; ### $P < 0.001$ vs. 25°C.

regulated in the oncomiracidia, but up-regulated in the adults (Figure 6B). Fe^{3+} reducing power assay was employed to evaluate the antioxidant capacity of NmTrx. The antioxidant capacity of NmTrx was determined to be

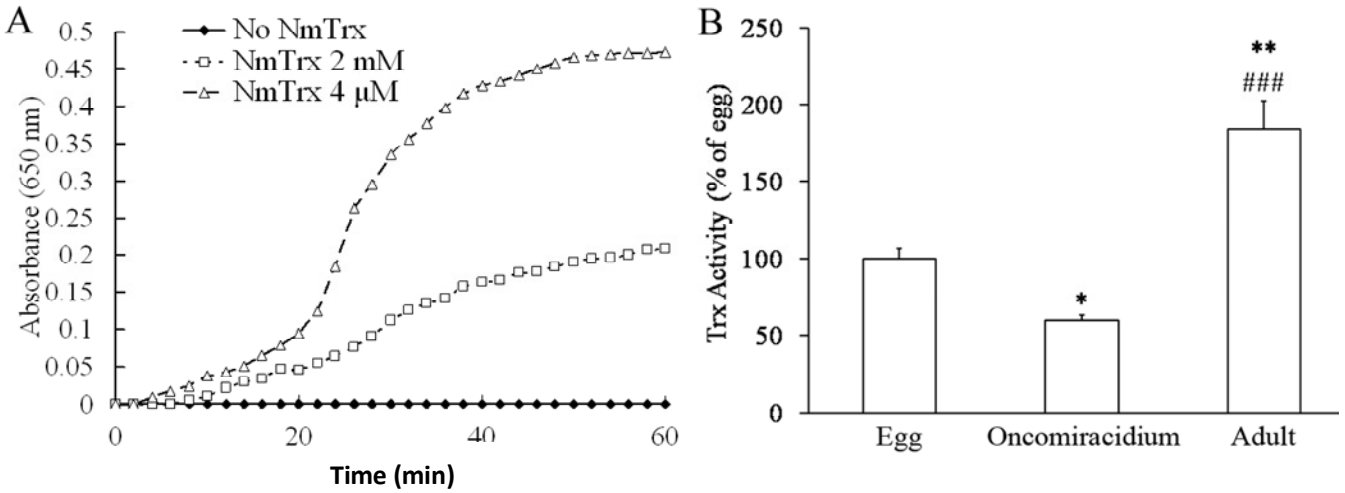

Figure 6. NmTrx-catalyzed reduction of insulin. (A) Activity analysis of recombinant NmTrx by by insulin turbidity assay. The absorbance at 650 nm was plotted against time. The cuvettes devoid of NmTrx at the 0 min served as blank and control. (B) Relative Trx activities in the lysates of the three developmental stages of *N. melleni* were assessed, normalized, and expressed as the percentage of the egg stage. Data represent as means ± SEM (N = 3). **P* < 0.05 vs. Egg; ***P* < 0.01 vs. Egg; ### *P* < 0.001 vs. Oncomiracidium.

5.12 U/mg protein.

DISCUSSION

Here, we identified a thioredoxin from *N. melleni*, which is known for having the broadest host-specificity of any monogenean species. The characteristic active site sequence CGPC of thioredoxins, structurally important amino acid residues, is highly conserved in NmTrx. This motif can control protein function via the redox state of structural or catalytic thiol groups (Nkabyo et al., 2002). The NmTrx has a molecular weight of 19.0 kDa, which is higher than the molecular weight of classic thioredoxin in other animals. In a phylogenetic tree, the observed relationships reflected the taxonomic positions of the species. However, the NmTrx is distantly related to the thioredoxin of other animal clusters. The uniqueness of the NmTrx sequence may reflect the specific life environment of *N. melleni* as a body surface parasite. The study also supports the idea of considering the NmTrx as a drug target for the treatment of the parasite *N. melleni*.

The expression of NmTrx at the transcriptional level revealed that NmTrx transcripts were all present in the three development stages examined, with the highest mRNA and protein expression level in the adult stage. The ubiquitous expression of NmTrx expression in the different developmental stages of *N. melleni* may suggest that the NmTrx is involved in important physiological functions. Thioredoxin, the crucial component of redox control systems, has versatile functions in DNA synthesis, defense against oxidative stress and apoptosis or redox signaling with reference to many diseases (Holmgren and Lu, 2010). In addition, the high level of NmTrx expression at the adult stage suggests that the *N. melleni* possibly suffer from stronger oxidized stress in the adult stage. The life cycle of *N. melleni* involves a fish host and they mostly spread by way of eggs and free-swimming infective larvae (oncomiracidia) in the seawater (Bondad-Reantaso et al., 1995). Therefore, *N. melleni* in the egg and oncomiracidium stages are only required to control free radicals produced by their metabolism. They are required to control potential damage from metabolism and host immune attack together in the adult stage (Henkle-Dührsen and Kampkötter, 2001). We conclude that more NmTrx protein and activity in the adults is needed to control potential damage. It has been found that thioredoxin pathways differ in parasitic and free-living flatworms (Otero et al., 2010). This result may also suggest that NmTrx plays more important roles in the adult stage than in the egg and oncomiracidium stages.

Thioredoxin functions are to regulate redox homeostasis in response to stresses, such as thermal stress, hypoxia, and osmotic stress (Kouwen et al., 2009; Muniyappa et al., 2009; Ying et al., 2010). In this study, the mRNA expression of NmTrx is higher in the egg stage at 32°C compared with 18 and 25°C. 25°C was the most suitable environment temperature to hatch *N. melleni*. The development of the *N. melleni* eggs will almost stop when the environment temperature is higher than 32°C (Lin et al., 2008). It has been found that heat exposure will influence the redox state (Hadzi-Petrushev et al., 2011). Our work supports the conception that thioredoxin participates in the thermal stress induced redox change.

Thioredoxin possesses a general intracellular anti-

oxidant activity and protects against oxidative stress (Holmgren et al., 2005). In this study, recombinant NmTrx is able to reduce insulin at 2 and 4 μM. Furthermore, the dithiol-reducing activity (5.12 U/mg) of NmTrx was comparable to the specific activity of Trx1 from *E. coli* (4.93 U/mg), calf thymus (6.50 U/mg) and calf liver (5.09 U/mg) (Holmgren, 1979). This result suggests that this thioredoxin from *N. melleni* is functionally similar to classic thioredoxins. In another platyhelminth parasite *S. mansoni*, two thioredoxins have been found by analyzing genome sequence (Berriman et al., 2009). It is still unknown whether other thioredoxins exist in *N. melleni* besides NmTrx. The Trx activity of *N. melleni* was lower in the oncomiracidia and higher in the adults than that in the eggs. The Trx activities in the three development stages are consistent with NmTrx mRNA. These results might suggest that NmTrx is the major Trx protein in *N. melleni.*

In summary, we cloned and characterized the first thioredoxin from the fish parasite *N. melleni* and examined the mRNA expression of NmTrx in different developmental stages at different environmental temperatures. Moreover, the recombinant protein of NmTrx showed antioxidant activity. The NmTrx may play important roles in the physiological oxidation-reduction of disulfide bonds to maintain redox homeostasis.

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