



Shrimp thioredoxin is a potent antioxidant protein

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ARTICLE INFO

Article history:

Received 19 February 2008
Received in revised form 29 March 2008
Accepted 31 March 2008
Available online 7 April 2008

Keywords:

Thioredoxin
Shrimp
Litopenaeus vannamei
Antioxidant capacity
TEAC
cDNA

ABSTRACT

Thioredoxin (TRX) is a main component of the redox homeostasis machinery in the cell and it is required for ribonucleotide reductase function among others. In invertebrates, the redox balance is compromised during disease and changes in the physiological state and it is one of the components of the innate immune response. In this work, the shrimp (*Litopenaeus vannamei*) *LvTRX* cDNA was sequenced, cloned and over-expressed in bacteria to further characterize the function of the recombinant protein. *LvTRX* was able to reduce insulin disulfides and it was a better antioxidant compared to reduced glutathione and ascorbic acid, by means of the Trolox Equivalent Antioxidant Capacity (TEAC) assay. Interestingly, *LvTRX* contains aside of the canonical active site CXXC disulfide motif, one Cys (C73) residue in the interface of a putative dimer previously reported for human TRX. Using qRT-PCR, we found that shrimp *LvTRX* is mainly expressed in gills and pleopods; the variation of *LvTRX* mRNA upon hypoxia and re-oxygenation is not statistically significant. *LvTRX* stands as an important antioxidant that must be considered in future physiological and immune challenges studies.

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1. Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is one of the most cultivated and traded fishery resources worldwide, and these shrimps are commonly exposed to continuous environmental changes. Varying conditions of temperature, pressure, salinity, oxygen and nutrients activate mechanisms to maintain vital functions. Moreover, the exposure to solar radiation, pollution, microorganisms and pathogens also lead to activation of immune defense responses including the production of reactive oxygen species (ROS). Therefore, an oxidative stress occurs when the production and accumulation of these reactive species is beyond the organism capacity to remove or quench them, leading to cellular lipids, proteins and DNA damage (Nappi and Ottaviani, 2000; Lesser, 2006).

The immune response of invertebrates lacks memory effectors such as immunoglobulins and lymphoid cells. The arthropods defense system is based on the innate humoral and cellular components of the circulatory system to eliminate foreign invaders (Bachere, 2000; Nappi and Ottaviani, 2000). Therefore, in organisms without the vertebrate adaptive immune system, it is crucial to study the innate factors that play a role in cell protection against foreign and self-produced toxic substances, such as the redox systems. Grant (2001)

reviewed the importance of the sulphhydryl groups (–SH) in the response to oxidative stress and highlighted the role of the glutathione/glutaredoxin and thioredoxin systems in the maintenance of the cell redox homeostasis.

The thioredoxin system is formed by the small ubiquitous redox protein thioredoxin (TRX) and the enzyme thioredoxin reductase (TR) and it is conserved throughout all the species, from Archebacteria to humans (Holmgren, 1985). TR catalyzes the reduction of TRX with electrons donated by NADH. Hence, TRX is involved in multiple key biological processes including the selective redox regulation of some transcription factors, DNA synthesis via ribonucleotide reductase, extracellular growth factor activity, protein disulfide reduction and protection against oxidative stress (Powis and Montfort, 2001; Lillig and Holmgren, 2007). Most of the TRX biological functions depend on the dithiol group presence, formed by the two conserved cysteine residues located in its active site. Thus, disulfide exchange leads to a reduced target. Moreover, TRX is an efficient electron donor to human plasma glutathione peroxidase, and also acts as a cofactor to thioredoxin peroxidases, known as peroxiredoxins, leading to effective detoxification processes (Powis and Montfort, 2001; Watson et al., 2004). There are two TRX isoforms, the cytosolic TRX-1 and the mitochondrial TRX-2. The cytosolic TRX isoform is secreted by a leaderless pathway in mammals by an undescribed mechanism (Powis and Montfort, 2001).

Although invertebrate thioredoxins have been recently studied, their antioxidant properties have been tested as a protecting agent against metal-catalyzed oxidation of proteins (Kim et al., 2007) or DNA

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(De Zoysa et al., 2008). The recently reported crystal structure of *Drosophila melanogaster* TRX has the cognate (α/β)-fold associated with disulfide redox proteins (Wahl et al., 2005). Specifically in Crustacea, there are no reports on decapod TRX sequence or function. The most related work has focused on the peroxiredoxin protein (Zhang et al., 2007; Bacano Maningas et al., 2008). Therefore, the objective of the present research was to clone, over-express and characterize the *L. vannamei* thioredoxin (LvTRX) using molecular biology and biochemical approaches.

2. Materials and methods

2.1. LvTRX cDNA cloning and sequence analysis

The thioredoxin cDNA from *L. vannamei* was assembled from ESTs from a gill cDNA library previously described (Clavero-Salas et al., 2007). DNA sequencing was done at the Laboratory of Molecular and Systematic Evolution Facility at the University of Arizona (Tucson, AZ, USA) and the Laboratory of Parasitic Diseases of the National Institute of Allergy and Infectious Diseases (NIAID-NIH, Bethesda, MD, USA). The identity of the contig was assigned using the BLASTX algorithm and identities were obtained after alignment with CLUSTALW. The deduced amino acid sequence was modeled using the SwissModel (Schwede et al., 2003) and the *Drosophila melanogaster* TRX (PDB 1XWA) as the structural template, while the figures were made using PyMOL 1.0 (DeLano 2002).

2.2. Recombinant expression of LvTRX in *E. coli*

LvTRX was over-expressed in *E. coli* using the pET11a vector (Novagen). The coding region of the polypeptide was amplified from the cDNA clone using a high-fidelity DNA polymerase (Invitrogen) and primers TRX-F (5'-GGAATCCATATGGTTTACCAAGTTAAAG-3') and TRX-R (5'-CGGGATCCTTACTGTCTTCTC-3') containing the restriction sites *Nde*I and *Bam*HI, respectively. Restriction sites are in italics in each primer. The recombinant clone was sequenced on both strands and used to transform *E. coli* BL21(DE3)SI competent cells (Novagen).

A preparation of 1 L of bacterial culture was made. Fermentation was done in Fernbach flasks with 25 mL of an overnight culture as inoculum. Briefly, bacteria were grown in Luria-Bertani broth (without NaCl) containing ampicillin ($100 \mu\text{g mL}^{-1}$) until an optical density of 0.6 at 600 nm was reached, in an orbital shaker at 250 rpm. Expression was induced with addition of NaCl to a final concentration of 0.3 M. After 4 h of growth at 37 °C, the cells were harvested and washed in 0.9% NaCl (w/v). The bacterial pellet was sonicated (four pulses of 10 s at 0 °C) in lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM benzamidine, 5 mM dithiothreitol (DTT), 30 mM NaCl, 10 mM MgCl_2 and 0.5 mM phenylmethylsulfonyl fluoride), and the lysate was centrifuged at 35,000 g for 20 min at 4 °C for debris removal. LvTRX was soluble, as determined by SDS-PAGE analysis of soluble and insoluble fractions of the bacterial lysate.

2.3. LvTRX chromatographic purification

The recombinant thioredoxin was purified by ion-exchange chromatography. The debris-free supernatant was fractionated by two consecutive precipitation steps with 50 and 85% ammonium sulfate saturation. The precipitate was dissolved in 10 mM Tris-HCl, 63 mM NaPO_4 , 2 mM EDTA and 5 mM DTT, pH 7.5 (buffer A), heated to 70 °C for 15 min and clarified by centrifugation. The ammonium sulfate was further removed by dialysis against buffer A. The clarified solution was loaded on an 8 mL Fast Flow Q-Sepharose column (GE Healthcare) equilibrated with buffer A, in an ÄKTA Basic chromatographer (GE Healthcare). The bound proteins were eluted using a salt gradient from 0 to 1 M NaCl in buffer A and 2 mL fractions were collected and monitored by absorbance at 280 nm. Samples from

elution peaks were analyzed by SDS-PAGE and assayed for thioredoxin activity (see below). Those samples with thioredoxin activity and an 11-kDa band in the gel were pooled. Protein concentration was determined using the bicinchoninic acid assay (Smith et al., 1985) with bovine serum albumin (BSA) as the standard. Purity and homogeneity of the protein samples were determined by SDS-PAGE. Gels were run in a Mini-Protean 3 electrophoresis system (Bio-Rad), and stained with 1% Coomassie Brilliant Blue R250 or with silver stain.

2.4. Identification of the recombinant LvTRX

To confirm the identity of the recombinant LvTRX, the sample was digested with sequencing-grade trypsin and the peptides were separated using high-performance liquid chromatography coupled to a matrix-assisted laser desorption ionization/time of flight MALDI-TOF mass spectrometer (LC-MS/MS) at the Proteomics facility at the BIO5 Institute, University of Arizona (Tucson, AZ, USA) as previously described (Zakharyan et al., 2005).

2.5. LvTRX disulfide reducing activity

The activity of the LvTRX was measured using a modified turbidimetric assay, based on the insulin disulfides reduction by thioredoxin (Holmgren 1979; Martinez-Galisteo et al., 1993). The turbidity produced by the insoluble reduced free-insulin polypeptides was followed by an increase in absorbance at 650 nm. The reaction was performed at 25 °C, and started with the addition of LvTRX. Final concentrations were 0.328 μM LvTRX, 5 mM Tris, 63 mM NaPO_4 , 1 mM DTT, 2 mM EDTA and 1 mg mL^{-1} insulin, at pH 7.0. Absorbance at 650 nm was continually read for 30 min against a blank solution by triplicate, which included all reagents except LvTRX. Slopes were obtained by a linear regression to calculate specific activity as $\Delta A_{650 \text{ nm}} \text{ mg LvTRX}^{-1} \text{ min}^{-1}$.

2.6. Trolox equivalent antioxidant capacity (TEAC) activity of LvTRX

TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich (Milwaukee, WI, USA). ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), reduced glutathione (GSH), ascorbic acid (vitamin C) and hen egg white lysozyme (HEWL) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade or better.

LvTRX-containing samples were dialyzed against 50 mM phosphate, 150 mM NaCl buffered saline (PBS), pH 7.4, and concentrated by ultrafiltration through a 1 kDa molecular weight cutoff filter (Amicon). All four samples (LvTRX, GSH, ascorbic acid and HEWL) were evaluated at a 335 μM concentration using a modified TEAC method (Re et al., 1999) to compare their antioxidant activity.

TEAC assay is based on the ability of the antioxidants to scavenge the blue-green ABTS^{•+} radical cation relative to the scavenging capacity of the water soluble vitamin E analogue TROLOX (Pellegrini et al., 1999; Re et al., 1999). The ABTS^{•+} radical cation was generated by mixing 5 mL of a 7 mM ABTS solution and 88 μL of a 140 mM $\text{K}_2\text{S}_2\text{O}_8$ solution. One mL of the ABTS^{•+}, was dissolved in 88 mL of PBS. The reaction contained 3900 μL of ABTS^{•+} plus 100 μL of each antioxidant sample or several dilutions of the TROLOX standard. The absorbance was monitored during 30 min after the initial mixing. Calculations were made correlating the scavenging capacity of TROLOX vs. each sample (Pellegrini et al., 1999; Re et al., 1999). The antioxidant capacity was expressed as g equivalent TROLOX per mol of each antioxidant tested.

2.7. Relative quantification of LvTRX gene expression in tissues and under hypoxia

The mRNA levels of LvTRX was investigated in four different shrimp tissues. Pleopods, gills, hepatopancreas and muscle were

individually dissected from three adult intermolt shrimp randomly collected from a laboratory maintained stock of experimental animals under controlled conditions. Each tissue was homogenized, and total RNA was isolated using TRIzol (Invitrogen) following manufacturer's specifications. Total RNA integrity was analyzed by 1% agarose-formaldehyde gel electrophoresis (Sambrook and Russell, 2001), and concentrations of total RNA samples were determined by absorbance at 280 nm using a ND-1000 spectrophotometer (NanoDrop). Two micrograms of total RNA samples were treated with DNase I (Sigma) (1 U/ μ g RNA) to eliminate any genomic DNA contamination. LvTRX and ribosomal protein L8 GenBank accession number DQ316258 (Gomez-Anduro et al., 2006) mRNA levels were analyzed by real time RT-PCR. Total RNA was reverse transcribed and amplified in a iQ5 thermocycler (Bio-Rad), each sample reaction was carried out in duplicates in a total volume reaction of 25 μ L using 12.5 μ L of 2X Brilliant II QRT-PCR one-step master mix (Stratagene), 1 μ L of RT/RNase block enzyme mix (Stratagene), 250 ng of total RNA, 1.25 μ L of primer mix including 18 μ M of each forward and reverse primers, and 5 μ M of a specific LvTRX or L8 Taqman probe using 5-carboxyfluorescein (FAM) as reporter dye and a non-fluorescent quencher (NFQ) (Applied Biosystems). After denaturing at 95 °C for 2.5 min, reverse transcription was done at 50 °C for 30 min followed by incubation at 95 °C for 15 min and then, PCR amplification using 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. A single fluorescence measurement was taken at 60 °C. Controls without template were included for each gene during the run. The following primers and probes were used: L8-SHRIMP-Fw 5'-ATTTGCAACCTTGAGGAGAAGACT-3'; L8-SHRIMP-Rv 5'-TGGGCAATGACCTGAGCATAATT-3'; L8-SHRIMP-Probe 5'-FAM ATCC-ACGGGCAATACG-NFQ-3'; TRX-SHRIMP-Fw 5'-GTGGATGTGGATGAA-TGTGAAGAC-3'; TRX-SHRIMP-Rv 5'-CAAGCTTCTGCCATCTTCATG-3'; TRX-SHRIMP-Probe 5'-FAM ATTGCCCAAGATAACC-NFQ-3'.

Ribosomal protein L8 was used to normalize LvTRX mRNA expression levels and the relative expression was calculated using the Equation $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

The effect of hypoxia was evaluated in gills. Fifteen shrimp of 30.58 \pm 2.5 g were randomly distributed in three tanks containing five animals and 300 L of sea water each. The shrimp were maintained for acclimatization under controlled laboratory conditions for a period of 24 h (water temperature 28 °C, salinity 34 ppt, and dissolved oxygen concentration of 6 mg L⁻¹). After acclimatization, one tank was

maintained under normoxic conditions as a control (6 mg L⁻¹). The air stones were retired from the other two tanks and oxygen concentration in water was continuously monitored using a submersible oxymeter. The lowest concentration of dissolved oxygen persistently registered in water in each tank was 2 mg L⁻¹ with the same shrimp density and water content. Shrimp from the second and third tanks were maintained in hypoxia (2 mg L⁻¹) for 3 h. The animals were collected from the control (6 mg L⁻¹) and second (2 mg L⁻¹) tanks. The third tank was re-oxygenated using the air stones and after 2 h, the oxygen concentration of the water reached 7 mg L⁻¹ and the shrimp were sampled 3 h later.

Once each experimental group was exposed up to 3 h to each condition (6, 2 and 7 mg L⁻¹), the gills of three shrimp from each group were individually dissected, homogenized in TRIzol reagent (Invitrogen) and total RNA obtained. Each RNA sample was treated with DNase I and mRNA levels determined as mentioned before.

2.8. Statistical analysis

mRNA expression was analyzed using one-way ANOVA followed by Tukey's Multiple Comparison test. All the statistical analyses were carried out using the Statistica software V 6.0.

3. Results

3.1. Shrimp TRX cDNA cloning

The deduced amino acid sequence of shrimp LvTRX was obtained from a gill cDNA library prepared for a massive sequencing project to generate shrimp ESTs (Clavero-Salas et al., 2007). The cDNA and deduced amino acid sequence were deposited in GenBank under accession number EU499301 (Fig. 1). The identity of LvTRX with insect TRXs is around 60–65%, and it is 53% identical to human TRX (Fig. 2). After molecular modeling of the LvTRX, it was found that the amino acid sequence is consistent with the canonical α/β thioredoxin fold present in disulfide redox proteins such as glutaredoxins, peroxiredoxins and all of the thioredoxin family members (Fig. 3, panel A).

The presence of a C73 residue in LvTRX suggests that this protein is able to form covalent dimers. Many conserved residues in mammalian TRXs are conserved in the LvTRX dimer interface, including D60

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1  ACTCGAGCGTAGACTGCCGCCGGGACAACAGTGACAGAAAATTTCCGTCCTCCTCTTCCC
1          M V Y Q V K D Q E D S T K Q L N
61  ACATTCGCCAAGATGGTTTACCAAGTGAAAGACCAGGAAGATTCCACTAAGCAGCTTAAC
17  E A G N K L V V I D F Y A T W C G P C K
121 GAGGCTGGAAACAAGCTGGTTGTTCATCGACTTCTACGCCACCTGGTGTGGGCCGTGCAAA
37  M I A P K L E E L S Q S M S D V V F L K
181 ATGATCGCACCTAAGCTGGAGGAGCTAAGTCAGTCTATGAGCGATGTCGTTTTCTCTGAAG
57  V D V D E C E D I A Q D N Q I A C M P T
241 GTGGATGTGGATGAATGTGAAGACATTGCCCAAGATAACCAGATTGCATGCATGCCTACT
77  F L F M K N G Q K L D S L S G A N Y D K
301 TTTCTATTTCATGAAGAATGGCCAGAAGCTTGACAGCTTGTCTGGTGCCTACTATGATAAG
97  L L E L V E K N K -
361 CTCTCGAACTCGTTGAGAAGAACAAGTAAACCATTCCACTGCTCTCTCTGGCACCAGA
421 GCATGAAAGATGGACCATCTTTGCAAATTAGATCTGCTAATAGTATTTGTTTTAGATTC
481 ATTGTGTGTGATTAAGACAAAATGGAGCTGTTTTGTTATTTATTTGTTCATAGATGTT
541 CACTTTTTCCAAATTTCTCGGTAAAAGATGATATAGCACCTTTTATGAAAATTTATC
601 ATTGTTTTGATAGAAGGTAGTTTAGGCGGCTAATTAATTCAGGTGAATATCCAAGATCTT
661 TTCATTATTGCATTATATTTAGTACCAGTGACTGTTTGTGCCTTCTATGTAATGTTGAA
721 CTTGGATTTATAAGATTTCAATAAAGTCTGAATATCTACCGAAA

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Fig. 1. cDNA nucleotide and deduced amino acid sequence of *L. vannamei* LvTRX. Peptides of the recombinant protein identified by mass spectrometry are underlined.

<i>D. melanogaster</i>	---MVYQVKDKADLDGQLTKASGKLVVLDFFAT <u>W</u> <u>C</u> <u>G</u> <u>P</u> <u>C</u> <u>K</u> <u>M</u> <u>I</u> <u>S</u> <u>P</u> <u>K</u> <u>L</u> <u>V</u> <u>E</u> <u>L</u> <u>S</u> <u>T</u> <u>Q</u> <u>F</u> <u>A</u> <u>D</u> <u>N</u> <u>V</u> <u>V</u> <u>L</u> <u>K</u> 57
<i>L. vannamei</i>	---MVYQVKDQEDSTKQLNEAGNKLVVIDFYAT <u>W</u> <u>C</u> <u>G</u> <u>P</u> <u>C</u> <u>K</u> <u>M</u> <u>I</u> <u>A</u> <u>P</u> <u>K</u> <u>L</u> <u>E</u> <u>E</u> <u>L</u> <u>S</u> <u>Q</u> <u>S</u> <u>M</u> <u>S</u> <u>D</u> -VVFLK 56
<i>H. sapiens</i>	---MVKQIESKTAFQEALDAAGDKLVVVDFSAT <u>W</u> <u>C</u> <u>G</u> <u>P</u> <u>C</u> <u>K</u> <u>M</u> <u>I</u> <u>K</u> <u>P</u> <u>F</u> <u>F</u> <u>H</u> <u>S</u> <u>L</u> <u>S</u> <u>E</u> <u>K</u> <u>Y</u> <u>S</u> <u>N</u> -VI FLE 56
<i>E. coli</i>	MSDKI IHLTDDSPD TDVLKADG--AILVDFWAE <u>W</u> <u>C</u> <u>G</u> <u>P</u> <u>C</u> <u>K</u> <u>M</u> <u>I</u> <u>A</u> <u>P</u> <u>I</u> <u>L</u> <u>D</u> <u>E</u> <u>I</u> <u>A</u> <u>D</u> <u>E</u> <u>Y</u> <u>Q</u> <u>G</u> <u>K</u> <u>L</u> <u>T</u> <u>V</u> <u>A</u> <u>K</u> 58
<i>D. melanogaster</i>	VDVDECEDIAMEYNIS <u>S</u> <u>M</u> <u>P</u> <u>T</u> <u>F</u> <u>V</u> <u>L</u> <u>K</u> <u>N</u> <u>G</u> <u>V</u> <u>K</u> <u>V</u> <u>E</u> <u>F</u> <u>A</u> <u>G</u> <u>A</u> <u>N</u> <u>A</u> <u>K</u> -RLEDVIKANI- 106
<i>L. vannamei</i>	VDVDECEDIAQDNQIA <u>C</u> <u>M</u> <u>P</u> <u>T</u> <u>F</u> <u>L</u> <u>F</u> <u>M</u> <u>K</u> <u>N</u> <u>G</u> <u>Q</u> <u>K</u> <u>L</u> <u>I</u> <u>S</u> <u>L</u> <u>S</u> <u>G</u> <u>A</u> <u>N</u> <u>Y</u> <u>D</u> -KLELLEVEKNK- 105
<i>H. sapiens</i>	VDVDDCQDVASECEVK <u>C</u> <u>M</u> <u>P</u> <u>T</u> <u>F</u> <u>Q</u> <u>F</u> <u>F</u> <u>K</u> <u>Q</u> <u>K</u> <u>V</u> <u>G</u> <u>E</u> <u>F</u> <u>S</u> <u>G</u> <u>A</u> <u>N</u> <u>K</u> <u>E</u> -KLEATINELV- 105
<i>E. coli</i>	LNIDQNPGTAPKYGR <u>G</u> <u>I</u> <u>P</u> <u>T</u> <u>L</u> <u>L</u> <u>L</u> <u>F</u> <u>K</u> <u>N</u> <u>G</u> <u>E</u> <u>V</u> <u>A</u> <u>A</u> <u>T</u> <u>K</u> <u>V</u> <u>G</u> <u>A</u> <u>L</u> <u>S</u> <u>K</u> <u>G</u> <u>Q</u> <u>L</u> <u>K</u> <u>E</u> <u>F</u> <u>L</u> <u>D</u> <u>A</u> <u>N</u> <u>L</u> <u>A</u> 109

Fig. 2. Sequence alignment of the shrimp LvTRX. Invariant active site catalytic cysteines are underlined, conserved residues of the hydrophobic dimerization interface are in italics.

(Fig. 3, panel B), a residue that controls the pH-dependent dimerization (Andersen et al., 1997). Due to sequence similarity, LvTRX belongs to the cytosolic TRX-1 isoform.

3.2. Shrimp LvTRX recombinant expression

Shrimp LvTRX was successfully over-expressed in a bacterial system, since it does not require any posttranslational modification (Fig. 4). From 1 L of cultured media, 12.98 mg of pure LvTRX was obtained as shown by silver stained SDS-PAGE. Further analysis with mass spectrometry confirmed the identity of the polypeptide. Four tryptic peptides were identified by MALDI-TOF: ¹³KQLNEAGNKL²², ⁴¹KLEELSQMSD⁵⁷VVFLK⁵⁷, ⁸⁵KLDSLSGANYDKL⁹⁷ and ⁹⁶KLELLEVEKN¹⁰⁵ (Fig. 1). A peptide corresponding to the N-terminal sequence ¹MVYQVKD⁷ was also found but with a low score.

Moreover, the activity of the recombinant LvTRX was tested using the insulin disulfide reduction assay. The specific activity for shrimp LvTRX was 10.44 $\Delta A_{650 \text{ nm}} \text{ mg TRX}^{-1} \text{ min}^{-1}$. This value is comparable to the specific activity of TRX from *E. coli* (4.93), calf thymus (6.50) and calf liver (5.09) (Holmgren, 1979) and much larger than the mitochondrial TRX-2 from abalone (1.83) (De Zoysa et al., 2008).

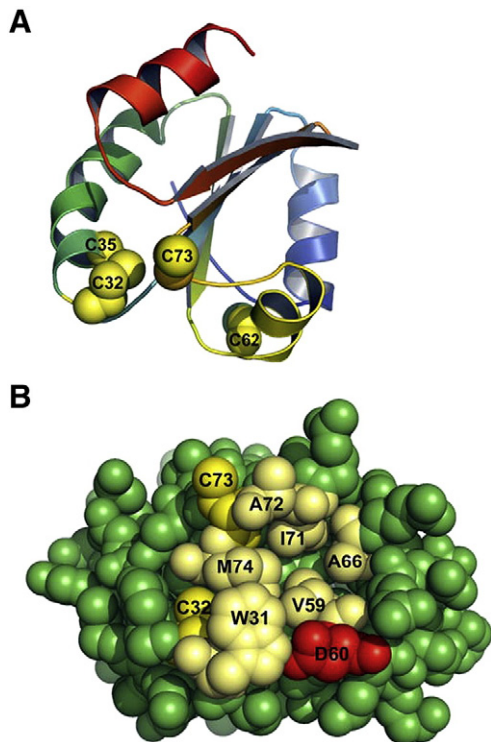


Fig. 3. Molecular modeling of the shrimp LvTRX. Panel A) Ribbon diagram of the thioredoxin fold showing the presence of all four cysteine residues. Panel B) Space-filling representation of the putative dimerization interface of shrimp LvTRX. Cysteines are represented in yellow, hydrophobic and non-polar residues in beige and negatively charged residues in red.

3.3. Shrimp LvTRX antioxidant capacity

Antioxidant capacity of proteins and enzymes has been less addressed compared to that of bioactive compounds such as vitamins C and E, carotenes and phytochemicals such as phenolics and flavonoids. However, proteins that contain cysteines or the chemical nature of their cofactors may naturally work as antioxidants. Therefore in this work the evaluation of the antioxidant capacity was addressed for the shrimp LvTRX using TEAC (Pellegrini et al., 1999; Re et al., 1999). It was found that LvTRX has a higher antioxidant capacity in a molar basis compared to GSH, ascorbic acid or a reference protein such as lysozyme (HEWL) (Fig. 5). TRX and GSH are important intracellular reductant agents although they appear to have distinct functions in the cell (Watson et al., 2004).

3.4. Shrimp LvTRX mRNA levels in tissues and under hypoxia stress

The levels of LvTRX mRNA were different in the evaluated tissues ($p < 0.01$). Gills have higher steady state levels of LvTRX mRNA compared to pleopods, hepatopancreas and muscle (Fig. 6, panel A). These differences may be related to tissue functions. The original LvTRX clone was obtained from a White Spot Syndrome Virus infected gills cDNA library, but changes in expression were not evaluated (Clavero-Salas et al., 2007). Nevertheless, since gills appear to be the tissue where TRX transcripts are more abundant, this may suggest an immunity-related function of LvTRX. Furthermore gills are highly active metabolic tissues and play a key role in oxygen transport.

Levels of the LvTRX mRNA from normoxic, hypoxic and re-oxygenated experimental groups were not statistically different (Fig. 6, panel B). However, a trend was observed where hypoxia induces a 2.9-fold increase in the LvTRX mRNA levels compared to normoxic conditions, and a subsequent decrease of approximately 53% of the LvTRX mRNA levels (1.36-fold compared to normoxia), when shrimp were re-oxygenated.

Gills have been reported as the major site of bacterial accumulation under normoxic conditions in decapod crustaceans (Alday-Sanz et al., 2002). It has also been suggested that gills actively take up bacteria

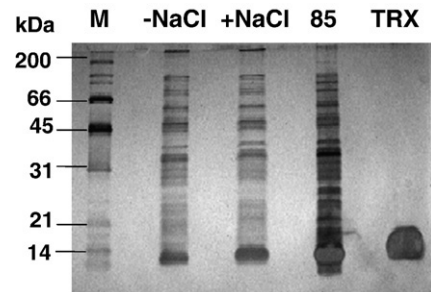


Fig. 4. SDS-PAGE analysis of the recombinant shrimp LvTRX purification. Lanes show uninduced (-NaCl), induced (+NaCl), protein precipitated at 85% saturation with ammonium sulfate (85) and purified LvTRX (TRX).

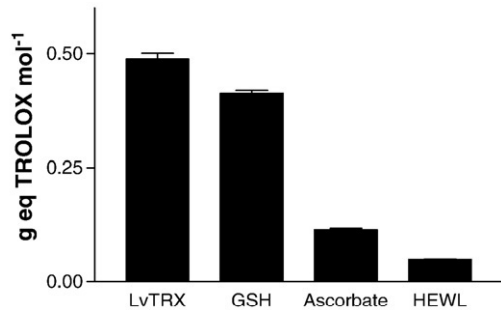


Fig. 5. Antioxidant Capacity of shrimp LvTRX. Bars represent g equivalent TROLOX per mol of each antioxidant tested. Assay done by triplicate.

from the hemolymph, and that hypoxia does not affect the mechanism responsible for this in the white shrimp (Burgents et al., 2005). However, there are no reports about TRX specific changes in response to hypoxia, re-oxygenation or immune challenges in marine crustaceans. Consequently, the high levels of LvTRX transcripts found in shrimp gills may be due to their high metabolic activity and are a target tissue for bacterial infections. The effect of hypoxia and re-oxygenation upon LvTRX mRNA steady-state levels deserves further investigation, considering its relevance upon circadian changes in dissolved oxygen in aquaculture ponds.

4. Discussion

Gills express high amounts of antioxidant proteins such as cytosolic Mn superoxide dismutase (Gomez-Anduro et al., 2006), catalase (Tavares-Sanchez et al., 2004) and detoxifying enzymes such as glutathione-S-transferase (GST) (Contreras-Vergara et al., 2004). The cytosolic LvTRX-1 isoform and GSH maintain the reducing status of the cell, although they appear to have different targets. Intracellular concentrations of GSH are ~1 mM while TRX is less than 1 μ M (Watson et al., 2004). GSH is one of the main reductants that protect the cell against ROS and electrophiles aside of its role in xenobiotic detoxification (Watson et al., 2004). GSH also can reduce sulfur adducts such as sulphenic acids and some disulfides as TRX does. The results presented in this investigation point out that the antioxidant capacity for LvTRX is slightly larger than GSH.

Another issue to consider particularly in shrimp, is the effect of metals on LvTRX. Metals are able to produce ROS by Fenton reactions, and shrimp hemolymph contains copper, which is a cofactor of hemocyanin (Figueroa-Soto et al., 1997). One recent study addressed the inhibition of LvTRX by metals, finding that copper, iron and nickel do not greatly affect TRX but they promote GSH oxidation. In addition, arsenic, cadmium and mercury do promote TRX oxidation but do not affect GSH (Hansen et al., 2006).

Other roles of TRX are to change gene expression of transcription factors. TRX is known to promote the expression and activity of hypoxia-inducible factor (HIF 1- α) and likewise to promote expression of epidermal growth factors (Maulik and Das, in press). As for hypoxia, in humans the process of ischemia and reperfusion injury may be used as a model for hypoxia-re-oxygenation. In the former case, ROS are involved in reperfusion damage for the increased production of ROS during re-oxygenation, and TRX or the antioxidant resveratrol are claimed to have beneficial effects on infarcted rat myocardium (Maulik and Das, in press). Whether LvTRX is involved in such processes in shrimp remains to be investigated.

The presence of C73 in the LvTRX sequence opens the question of physiological homodimerization *in vivo*. In mammals, TRX is secreted by a leaderless pathway, and the extracellular presence of the dimer is proposed to function as a redox signal (Powis and Montfort, 2001). Non-classical leaderless secretion can be predicted by computational

methods (Bendtsen et al., 2004). Analysis of the human and shrimp LvTRX sequences using the SecretomeP algorithm gave a non-optimal score for leaderless secretion prediction, but nonetheless the scores are similar between both species. Structural analysis of the human (Weichsel et al., 1996) and *D. melanogaster* TRX (Wahl et al., 2005) suggested that dimerization may occur in these proteins. In humans, the TRX dimer was identified by crystallographic symmetry with an intermolecular disulfide bond between subunits. Further work demonstrated that dimerization is *bona fide*, which means that human TRX dimer exists under physiological conditions and functions as a pH-dependent redox sensor (Andersen et al., 1997). The fruit-fly TRX, which does not have a Cys residue in position 73, can only dimerize under non-physiological conditions (Wahl et al., 2005). We propose that the shrimp LvTRX will dimerize as in the human protein, since many residues around C73 are invariant (Fig. 3, panel B).

This work opens several hypotheses about the identification of LvTRX in the crustacean hemolymph, and to whether homodimers do exist physiologically in the hemolymph, and if they are increased under physiological or pathological challenges. Those questions may open the view of redox regulation further ahead of mRNA steady state levels of genes and lead to new vistas where protein levels, homodimerization, nitrosylation (Weichsel et al., 2007) or other posttranslational modifications may be also part of the complex regulation of redox homeostasis.

In conclusion, we have demonstrated that the LvTRX belongs to the cytosolic isoform, with an antioxidant capacity comparable to reduced glutathione (GSH) and that is highly expressed in gills. This biochemical evidence strongly supports a physiological role for LvTRX as a key component of the antioxidant response of marine crustaceans.

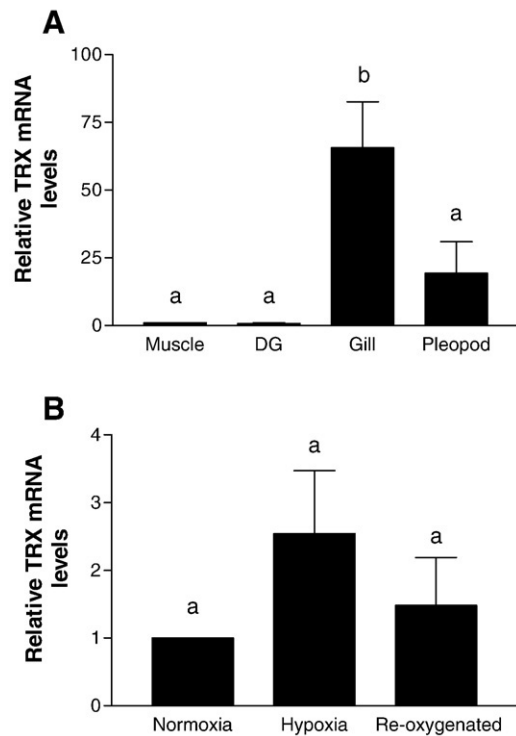


Fig. 6. Relative LvTRX mRNA levels. Panel A) Normalized LvTRX levels relative to muscle. Panel B) Normalized LvTRX levels relative to normal oxygen conditions (normoxia). Bars represent the means \pm SE. Differences were evaluated with one-way ANOVA and Tukey test ($p < 0.05$).

Acknowledgements

This research was sponsored by a CONACYT grant 45964 to G. Yepiz-Plascencia. E. Aispuro-Hernandez, L. del-Toro-Sanchez and R. M. Robles-Sanchez received scholarships from CONACYT (National Research and Technology Council, Mexico). We thank Monica Resendiz (CIAD) for technical assistance with the qRT-PCR experiments, and Dr. Maria Islas-Osuna for review and critical comments to the manuscript. E.A-H designed and performed research, analyzed data and wrote the paper, K.D.G-O designed experiments, analyzed data and wrote the paper, A.M-A designed and performed research, analyzed data and wrote the paper, L.d-T-S and R.M. R-S designed and performed research, J.H designed experiments, analyzed data and contributed new reagents, G.G-A designed experiments, contributed reagents and analytical tools and wrote the paper, G.Y-P designed experiments, contributed reagents and wrote the paper, R.R.S-M designed experiments, analyzed data and wrote the paper.

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