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Purification and partial characterization of thioredoxin reductase from the hepatopancreas of the mollusc *Mytilus galloprovincialis* Lam.

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ABSTRACT

Thioredoxin reductase (TrxR, EC 1.6.4.5) is a ubiquitous flavoenzyme that is present from Archaea to humans, and it is the only enzyme capable of catalyzing the reduction of thioredoxin (Trx) by nicotinamide adenine dinucleotide phosphate (NADPH). Although TrxR has been purified and characterized from different bacteria, plants, and mammalian organisms, a survey of the literature revealed no studies on the purification and characterization of TrxR from the mussel *Mytilus galloprovincialis* Lam. In this study, TrxR was purified to homogeneity from the hepatopancreatic tissue of *M. galloprovincialis* Lam. by extraction, ammonium sulfate precipitation, and DEAE-Sepharose CL-6B anion and 2',5'-ADP-agarose chromatographies, and some of its kinetic properties were examined. Molar mass determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed only a single protein band corresponding to a molecular weight of 35 kDa. Optimum pH and temperature were found to be 7.0 and 60°C, respectively. Km and Vmax values for NADPH were found to be 85 µmol and 4.82 µmol/min/mg, respectively. For 5,5' -dithiobis (2-nitrobenzoic) acid (DTNB), the Km and Vmax values were 193 µmol and 1.32 µmol/min/mg, respectively. Increasing the knowledge on the kinetic properties of TrxR will significantly increase the prospects of enzyme application as an oxidative stress biomarker in mussels and fishes for monitoring contamination in coastal environments.

Keywords: Enzyme purification, kinetic properties, *Mytilus galloprovincialis* Lam., thioredoxin reductase

INTRODUCTION

Thioredoxin (Trx), nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase (TrxR) comprise a thioredoxin system which exists in nearly all living cells (Arnér and Holmgren 2006). Trx, the physiological substrate for TrxR, occurs in either an oxidized or a reduced form. Reduced Trx prevents oxidation of various proteins by donating hydrogen atoms from two of the cysteine residues at its active site. Oxidized Trx is reduced by TrxR using NADPH as an electron donor (Seo and Lee 2010).

TrxR belongs to the pyridine nucleotide-disulfide oxidoreductase family and is a dimeric flavoenzyme (Lu et al. 2009). The preliminary purification and characterization studies were performed with TrxR from archaea, bacteria and anaerobic amino-acid-utilizing bacteria (Moore et al. 1964; Williams 1995; Harms et al. 1998; Horecká et al. 1998; Seo and Lee 2010; Yang and Ma 2010), fungi, some eucaryotes including plant (Reicheld et al. 2005) and intracellular parasites (Brown et al. 1996; Coombs et al. 2004; Maggioli et al. 2004; Arias et al. 2010; Kapoor and Banyal 2011). Extensive studies have been made also upon mammalian Trx system. Mammalian TrxR was purified to homogeneity from rat liver (Larsson 1973; Luthman and Holmgren 1982; Lu et al. 2009), bovine adrenal cortex (Watabe et al. 1999) and human placenta (Gromer et al. 1998).

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Bivalve molluscs like mussels, clams and oysters are highly nutritive commercially valuable seafood species on the worldwide basis. Populations of bivalves living in coastal areas are subject to many investigations for their possible use as enzyme resources. Many references can be found in the literature to the enzymes present in the digestive system of bivalves (Yalvaç and Kuşçu, 1993; Arısan-Ataç et al., 1994; Özsoy and Berkkan, 1997; Somar et al., 2000; Can et al. 2000; Dönmez et al. 2014). As TrxR is known to be involved in maintenance of redox homeostasis and antioxidant defense by reducing disulphide sites in oxidized proteins, it was used as a biomarker in the digestive gland of wild mussels (*M. galloprovincialis*) for biomonitoring the marine pollution (Sureda et al. 2011). However, no report has been found in the literature on the isolation and purification of TrxR from the mussel *M. galloprovincialis*. This work describes for the first time the purification and characterization of TrxR from the mussel *M. galloprovincialis*.

MATERIAL AND METHODS

The mussels belonging to the species *Mytilus galloprovincialis* Lam. were obtained from the Yenikapi seaboard on the day of experiment.

Purification of thioredoxin reductase

The purification of TrxR from hepatopancreas tissue of *M. galloprovincialis* involved four steps:

1. Isolation: 73.5 g hepatopancreas of freshly collected 60 mussels were homogenized in 200 mL of 10 mM Tris-HCl containing 1 mM EDTA, pH 7.5 buffer (TE buffer) by means of a homogenizer (Art-MICCRA D-1, Heitersheim, Deutschland). The homogenate obtained was centrifuged at 13,000 rpm for 20 min in a refrigerated centrifuge (Heraeus-Megafuge 1.OR, Thermo Fisher Scientific, Waltham, MA, USA) and the supernatant (the crude extract) was collected.

2. Ammonium sulphate precipitation: The crude extract was precipitated by ammonium sulphate at 80% saturation and left overnight in the refrigerator. The precipitate, separated by means of centrifugation at 13,000 rpm for 20 min was dissolved in TE buffer and dialyzed against the same buffer to remove the salt. The dialyzed solution was heated at 56°C in a water bath for 10 min, cooled, and after centrifugation at 13,000 rpm for 20 min the precipitate was discarded and the supernatant was used for further purification procedure.

3. DEAE-Sepharose Chromatography: The dialysate was applied to a column of DEAE-Sepharose CL-6B (1.5x25 cm) previously equilibrated with 10 mM TE buffer. The column was washed with approximately 150 mL of equilibration buffer until no protein could be detected in the effluent. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl in 10 mM TE buffer. The eluate was collected in 0.5 mL fractions and assayed for absorbance at 280 nm and for enzyme activity. The fractions showing TrxR activity were pooled and concentrated by ultrafiltration with a stirred cell (Millipore Corporation, Bedford, MA 01730 USA) equipped with a PM 10 membrane (Amicon, Inc., Beverly, Mass.) under nitrogen pressure of 20 lb/in^2. The concentrated enzyme fractions were subsequently dialyzed against TE buffer. All operations were performed at 4°C.

4. Affinity chromatography: The main activity peak was applied to a 2′,5′-ADP-agarose column (1x10 cm) equilibrated with 10 mM TE buffer. The column was eluted with a gradient of 0 to 0.5 M NaCl in TE buffer. The purified enzyme solution was concentrated by ultrafiltration and dialyzed against 10 mM TE buffer. The purified enzyme was stored at -80°C until used.

Determination of protein concentration

Protein concentration was determined either by the Bradford method (1976) using bovine serum albumin (BSA) as a standard or measurement of absorbance at 280 nm.

Determination of thioredoxin reductase activity

TrxR activity was measured by the reduction of DTNB with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm (Holmgren and Bjornstedt 1995) using Thioredoxine reductase Assay Kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. TrxR was determined also by the insulin-dependent reduction assay described by Arnér and Holmgren (2000). Enzyme activity was expressed as µmol/min/mL of formation of TNB by using extinction coefficient of 6.35 mM^{-1}. One unit is defined as NADPH-dependent production of 2 µmol of TNB per minute.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE*)* was carried out in 12.5% polyacrylamide gel slabs at pH 8.3 by using 1 M Tris-glycine buffer containing 0.1% (w/v) SDS according to Laemmli's method (1970). Subunit molar mass was analyzed under reduced conditions. The purified sample was prepared by heating a protein solution in a sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol, 5% 2-mercaptoethanol) at 100°C for 5 min. A low-molecular-weight calibration mixture (Amersham Biosciences, Buckinghamshire, UK) was used as standard.

Kinetic properties of thioredoxin reductase

The relationship between the pH variation and TrxR activity was investigated at a pH range of 6-10 by using 500 mM potassium phosphate buffer containing 1 mM EDTA. The effect of temperature on the activity of the enzyme was studied between 30°C and 85°C under assay conditions. The effect

Table 1. Purification of thioredoxin reductase of *Mytilus galloprovincialis* from 73.5 g of wet hepatopancreas tissue

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (U)*	Specific Activity (U/mg)**	Yield (%)	Purification Fold
1-Crude extract	200	3 642.4	112 298.0	30.8	100	-
2-80% ammonium sulphate fraction	12.5	371.3	1 336.3	3.6	10.2	0.1
3-DEAE-Sepharose CL-6B (10 mM)	10	10.6	775.4	73.2	0.3	2.4
4-2',5'-ADP-agarose (400 mM)	1	0.04	1 189.8	29 745.8	0.001	965.7

*µmol TNB/min

**µmol TNB/min/mg protein

of substrate concentration on the velocity of the enzyme reaction was investigated by using varying concentrations of NADPH (0.015-0.24 mM) and DTNB (0.19-3 mM) as substrates. Km and Vmax were calculated by means of the equation of Lineweaver-Burk plot.

RESULTS AND DISCUSSION

The thioredoxin and glutathione systems are the two major thiol-dependent reductases that maintain a reducing intraenvironment in the presence of oxygen (Lu et al. 2009). In this study, approximately 40 µg of the purified enzyme was obtained from 73.5 g hepatopancreas of the mollusc *M. galloprovincialis*. The enhanced purification was primarily due to the use of 2,5-ADP as an affinity ligand for purification of NADPH-binding proteins, a procedure previously described by Brodelius et al. (1974). The use of the affinity step resulted in a 0.001% yield of enzyme with a specific activity of 29 745.8U/mg protein (Table 1). Earlier purification schemes for Trx and TrxR involved anion exchange and affinity column chromatography steps. Affinity column chromatography was used as the initial purification step by Pigiet and Conley (1977), who purified both TrxR and glutathione reductase (GR) 300-fold in one step. However, it was reported that the use of affinity chromatography after several initial purification steps resulted in greater column yield. Williams et al. (1967) reported that, GR and TrxR, each purified by a two-step chromatographic procedure including anion-exchange chromatography and affinity chromatography, bind to the affinity gel at the extent of 600 and 570 units/mL of gel respectively; while using a crude extract, only 116 and 26 units/mL gel of each enzyme bind to the affinity gel. This may be due to the presence of several NADPH-binding proteins competing with the desired proteins in the crude extract (Pigiet and Conley 1977). By a combination of anion exchange and affinity chromatography, TrxR was purified to homogeneity from *Streptomyces aureofaciens* 3239 (Horecká et al. 1998), a protozoan parasite *Giardia duodenalis* (Brown et al. 1996), and the worm *Fasciola hepatica* (Maggioli et al. 2004). TrxR from rat liver with specific activity of 625 U/mg was obtained by chromatography on Sephadex and on DEAE-cellulose (Larsson 1973). The method described here for the purification of TrxR from the hepatopancreas tissue of *M. galloprovincialis* has resulted in a preparation with higher specific activity.

The procedure used for the purification of the TrxR from *M. galloprovincialis*, was similar to that used for the purification of this enzyme from anaerobic amino-acid utilizing bacteria (*Eubacterium acidaminophillum*, *Clostridum litorale*, *C. sticklandii*, *C. sporogenes*, *C. cylindrosporum* and *Tissierella creatinophilla*) as described by Harms et al. (1998). The proteins isolated in this study did not bind to DEAE-Sepharose and were eluted with the washing buffer (Figure 1). However, they were bound tightly to the affinity gel material and eluted with 0.4 M NaCl (Figure 2) as reported by Harms et al. (1998). The affinity step permitted the rapid and high yield purification of large quantities of enzyme for subsequent use in structural studies.

All described purification schemes for the TrxR involve a heat denaturation step; 65°C, 5 min (Moore et al. 1964); 70°C, 8 min (Williams et al. 1967); 60°C (Maggioli et al. 2004); 56°C, 10 min (Lu et al. 2009). In this study, heat treatment was also an important step for the enrichment of the enzyme, in which otherwise difficult-to-remove contaminating proteins were eliminated. The heat treatment was effective in simplifying the purification procedure and increasing the yield.

Optimum pH for TrxR have been reported to exist generally at pH 7.5 (Watabe et al. 1999), 7.4 (Kapoor and Banyal 2011), 7.7 (Williams 1995) and 6.5 (Yang and Ma 2010). Optimum pH values for TrxR determined in the present study was 7.0, which is within the mentioned range (Figure 3).

The optimum temperature of TrxR was found to be 60°C (Figure 4). However, the enzyme activity was reduced at 70°C. Similarly, the activity of TrxR from *D. radiophilus* was drastically reduced at 80°C and completely inactivated at 90°C (Seo and Lee 2010). TrxR from the hyperthermophilic bacterium *Thermotoga maritima* was reported to display a relatively high thermostability (up to 95°C) (Yang and Ma 2010). Thermostable enzymes have considerable potential in biotechnical applications because of their resistance to heat denaturation and consequently lower replacement costs when enzymes are integrated into high

temperature processes. TrxR enzymes with optimum temperature as low as -4°C and 0°C has been reported by Kapoor and Banyal (2011) and Özgençli and Çiftçi (2016), respectively. It might be the result of the necessity for these organisms to adapt itself to environmental conditions.

Km and Vmax values for NADPH were 85 µmol and 4.8 µmol/min/mg, respectively (Figure 5). Also, Km and Vmax values for DTNB were found to be 193 µmol and 1.32 µmol/min/mg, respectively (Figure 6). Km and Vmax values for DTNB were lower or comparable to that reported for *Plasmodium berghei* (Km=125 µmol; Vmax=100 µmol/min) (Kapoor and Banyal 2011). *Deinococcus radiophilus* (Km=463 µmol; Vmax=756 µmol/min) (Seo and Lee 2010) and rat liver (Km=660 µmol) (Luthman and Holmgren 1982) and higher than that reported for rainbow trout (Km=0.828

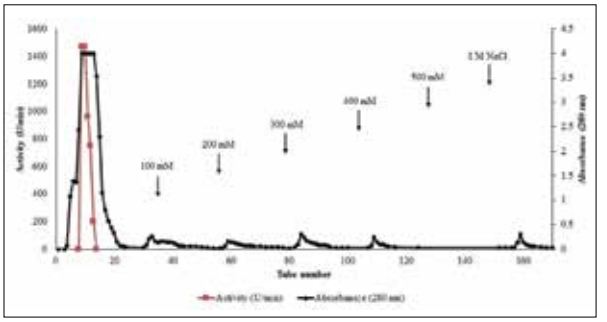

Figure 1. DEAE-Sepharose CL-6B ion exchange chromatography. Elution profile of the 80% ammonium sulphate fraction of Mytilus galloprovincialis hepatopancreas tissue crude extract.

Column: 25x1.5 cm, sample volume: 12.5 mL (371.3 mg protein), flow rate: 48 mL/hour, the enzyme was eluted with a linear gradient of 0-1 M NaCl in 10 mM TE (pH 7.5) buffer.

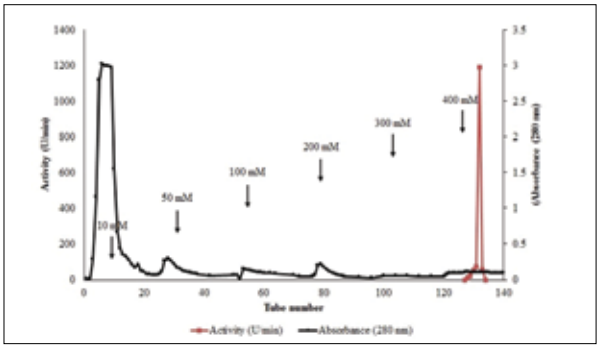

Figure 2. 2',5'-ADP-agarose chromatography of thioredoxin reductase after DEAE-Sepharose CL-6B purification.

Column: 1x10 cm, sample volume: 1 mL (10.6 mg protein), flow rate: 1 mL/min, the enzyme was eluted with a linear gradient of 0-0.5 M NaCl in 10 mM TE (pH 7.5) buffer.

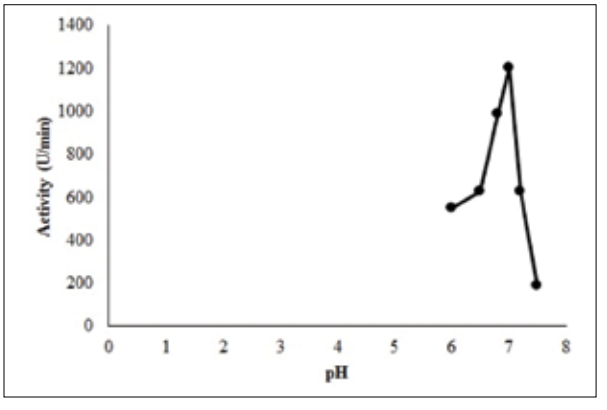

Figure 3. Effect of pH on *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity.

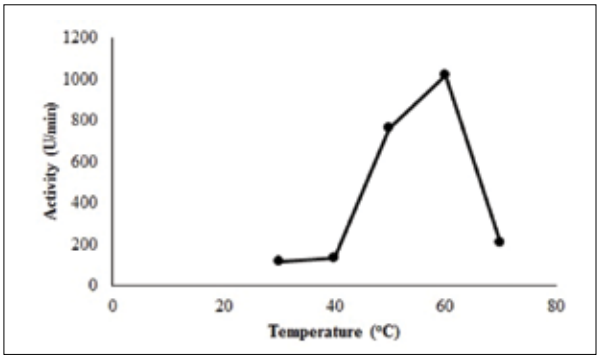

Figure 4. Effect of temperature on *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity.

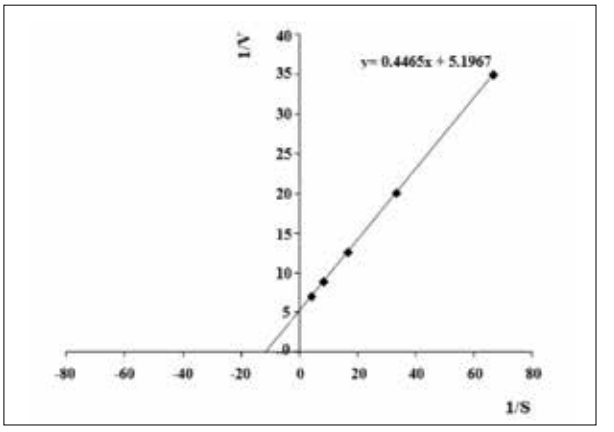

Figure 5. Effect of different concentrations of NADPH on the activity of *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity (-1/Km = -11.76).

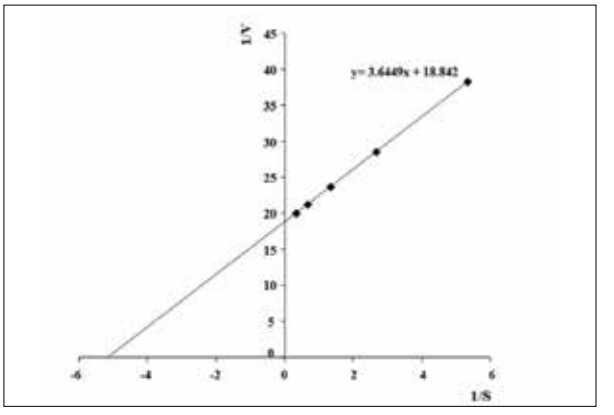

Figure 6. Effect of different concentrations of DTNB on the activity of *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity (-1/Km = -5.18).

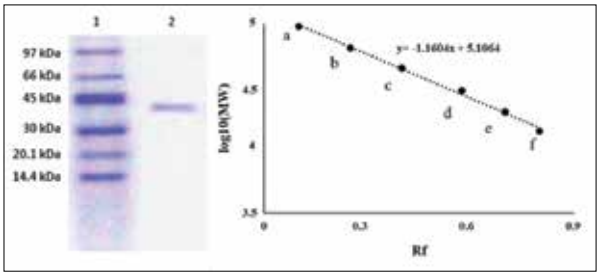

Figure 7. SDS-PAGE electrophoresis of the purified thioredoxin reductase after affinity chromatography.

Line 1: Low molecular weight proteins (a: phosphorylase 97 kDa, b: albumin 66 kDa, c: ovalbumin 45 kDa, d: carbonic anhydrase 30 kDa, e: trypsin inhibitor 20 kDa and f: α-lactalbumin 14,4 kDa; Line 2: Purified enzyme after affinity chromatography.

µmol) (Özgençli and Çiftçi (2016). Km and Vmax values for NADPH were higher than that reported for *Deinococcus radiophilus* (Km=12.5 µmol; Vmax= 25 µmol/min) (Seo and Lee 2010), rat liver (Km=6 µmol) (Luthman and Holmgren 1982) and rainbow trout (Km=12.65 µmol) (Özgençli and Çiftçi (2016).

The purity of the enzyme was confirmed by SDS-PAGE showing a single band with a molecular mass of about 35 kDa (Figure 7). This value is the same with that reported for TrxR in prokaryotes, archaea and lower eukaryotes, but different from a protein in higher eukaryotes that was found have a MW of 55 kDa (Williams et al. 2000).

CONCLUSION

The TrxR enzyme from mussel *M. galloprovincialis* was purified to homogeneity, and its properties were investigated. The results may contribute to a great number of studies applying oxidative biomarkers in mussels and fishes for monitoring environmental pollution.

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