TÜBİTAK

Turkish Journal of Chemistry

http://journals.tubitak.gov.tr/chem/

Research Article

Turk J Chem
(2017) 41: 48 – 60
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doi:10.3906/kim-1603-135

Investigation of the effects of purification and characterization of turkey (*Meleagris gallopavo*) liver mitochondrial thioredoxin reductase enzyme and some metal ions on enzyme activity

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Received: 31.03.2016 • **Accepted/Published Online:** 21.06.2016 • **Final Version:** 22.02.2017

Abstract: The thioredoxin system, found in all living creatures, consists of thioredoxin protein (Trx), thioredoxin reductase enzyme (TrxR), and NADPH. In this study, turkey liver mitochondrial TrxR enzyme with 3.07 EU × mg^{-1} specific activity was purified 990-fold in a yield of 2.05% using 2',5'-ADP Sepharose 4B affinity chromatography. The purity of the enzyme was measured and the molecular weight of its subunits was determined to be 45.5 kDa by SDS-PAGE. The molecular mass of the enzyme's natural state was found to be 88 kDa using Sephadex-G 150 gel filtration chromatography. In addition, characteristic and kinetic properties of the enzyme were determined. Then the inhibitory effects of some heavy metal ions (Ag^+, Fe^{3+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}, and Co^{2+}) on the activity of TrxR enzyme were examined under in vitro conditions. IC_{50} values were found with the heavy metal concentration with which 50% of the activity of the TrxR enzyme was inhibited. Finally, K_i values for these substances were calculated from the Lineweaver–Burk plots. It was determined that Ag^+, Fe^{3+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} ions inhibited TrxR enzyme, Pb^{2+} ion increased enzyme activity, and Ni^{2+} and Co^{2+} ions had no effect on enzyme activity.

Key words: Thioredoxin reductase, characterization, metal, inhibition

1. Introduction

The thioredoxin system, found in both prokaryotes and eukaryotes, consists of thioredoxin (Trx), thioredoxin reductase, and NADPH.[1] Thioredoxin reductases (E.C 1.6.4.5.; TrxR) are enzymes depending on the flavo-protein family, consisting of lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase. All members of this family are homodimeric proteins formed by an active region containing disulfide, an NADPH binding site, and an FAD prosthetic group.[2] TrxR activity is regulated by NADPH produced with glucose-6-phosphate dehydrogenase (G6PD), which is the allosteric enzyme of oxidative reactions of the pentose phosphate pathway.[3] Three types of thioredoxin reductase enzymes were determined in mammals: TrxR1 found in the cell cytoplasm,[4] TrxR2 found in the mitochondria,[5,6] and TrxR3 found in the testicles.[7] Mainly two TrxRs were cloned: an enzyme of 54 kDa found in the cytoplasm and an enzyme of 56 kDa found in the mitochondria.[8]

Thioredoxins (Trx) are the protein family of 10–12 kDa containing Trp-Cys-Gly-Pro-Cys-Lys catalytic section. Two cysteine groups of these proteins are exposed to oxidation/reduction reversibly. The reduced

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dithiol form [Trx-$(SH)_2$] of Trx reduces oxidized protein substrates containing a disulfide group, and the oxidized disulfide form [Trx-(S-S)] rejoins the cycle through an NADPH-dependent path regulated by TrxR. Trx is an important redox regulator. The formation of Trx protein is induced by oxidative stress, hypoxia lipopolysaccharides, viral infections, X-rays, and UV radiation. Trx affects the binding of transcription factors such as p53 and AP-1 to DNA through redox balance. Furthermore, Trx was demonstrated to increase the resistance of cancer cells to cytotoxic drugs.[8] Trx-1 was demonstrated to inhibit cell death when it was added to lymphoid cell culture or its gene was transferred to these cells.[9] It is not yet known by which mechanism Trx-2 provides protection against cell death. However, it is thought that the increase in cell reduction power reduces the proteins damaged by oxidative stress and thus prevents cell death.[10]

The thioredoxin reductase enzyme reduces the oxidized form of thioredoxin protein NADPH dependently; the reduced thioredoxin ensures the transformation of hydrogen peroxide into the water by providing an electron to thioredoxin peroxidase, performs the transformation of ribonucleotides to deoxyribonucleotides for DNA synthesis by providing an electron to ribonucleotide reductase, and affects the binding of transcription factors to DNA. It also leads to thioredoxin cell growth and inhibition in the apoptosis, which is programmed cell death.[11] The mammalian thioredoxin reductase, which is a homodimeric enzyme, consists of two subunits. One of the subunits contains N-terminal dithiol in the redox active center, and the other one contains a C-terminal selenathiol region.[12,13] In the mechanism proposed for the enzyme, electrons provided by NADPH reduce the disulfide bonds found in the N-terminal active region through FAD, which is the prosthetic group of the enzyme. Then these electrons are transferred from N-terminal disulfide to C-terminal selenium sulfide, and finally, the substrate is reduced by C-terminal selenathiol.[14,15]

Thioredoxin reductase was first discovered as a part of the ribonucleotide reductase system studies in *E. coli*.[16] Then the 'thioredoxin system' was purified from baker's yeast by Gonzalez et al. In their study, two different forms of thioredoxin protein were determined (thioredoxin-I and -II), and the molecular mass of its natural state was calculated as approximately 70 kDa.[17] Moreover, *Lactobacillus leichmannii*, Novikoff hepatoma, regenerated rat liver, and phage-infected *E. coli* were stated as the other sources in which the thioredoxin system was observed. Moreover, the enzyme was purified from rat tumors,[18] bovine liver and thymus,[19] rat liver,[20] human placentas,[21] rat liver mitochondria,[22] tomato,[23] and rainbow trout liver,[24] and it was characterized.

The TrxR system is found in all living creatures from bacteria to animals, is vital for metabolism, and possesses a function in such events in the protection of the organism from the damaging effects of peroxide, the production of RNA from DNA, in apoptosis, in cell growth, and in gene transcription. Most of the studies indicated that the TrxR system was associated with various cancer types, AIDS, and immune diseases. For this reason, determination of the kinetics and characteristics of TrxR enzyme will guide the diagnosis and treatment of diseases associated with this enzyme. In the present study, we aimed to purify and characterize the TrxR enzyme from turkey liver mitochondrial tissue. Furthermore, the in vitro effects of some heavy metals on purified TrxR enzyme were examined as part of this study.

2. Results and discussion

Gradient centrifugation and 2',5'-ADP Sepharose 4B affinity chromatography were used to purify the mitochondrial thioredoxin reductase enzyme from turkey liver tissue. As a result of these purification steps, the enzyme was purified about 990-fold from the turkey liver mitochondria in a yield of 2.05% with 3.07 EU/mg protein specific activity. The purification results are shown in Table 1. The purity of the enzyme was determined by

looking at SDS-PAGE photos (Figures 1a and 1b). Thus, high purity enzyme was achieved in a very short time. Based on Laemmli's method,[25] a log MK-R_f plot was drawn by determining R_f values for standard proteins, and the molecular mass of the enzyme was calculated as about 45.5 kDa by means of the plot drawn (Figure 2a). For the determination of the molecular weight of the enzyme obtained by gel filtration chromatography, a log MW-K_{av} plot was drawn, and the molecular weight of the enzyme was found to be 88 kDa (Figure 2b).

Table 1. Purification results of turkey liver mitochondrial thioredoxin reductase enzyme with 2',5'-ADP Sepharose 4B affinity chromatography.

Sample type	Total volume (mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	% Yield	Purification coefficient
	Method 1					
Homogenate	60	1888.2	5.94	0.0031	100	1
2',5'-ADP Sepharose 4B affinity chromatography	8	0.0397	0.122	3.07	2.05	990

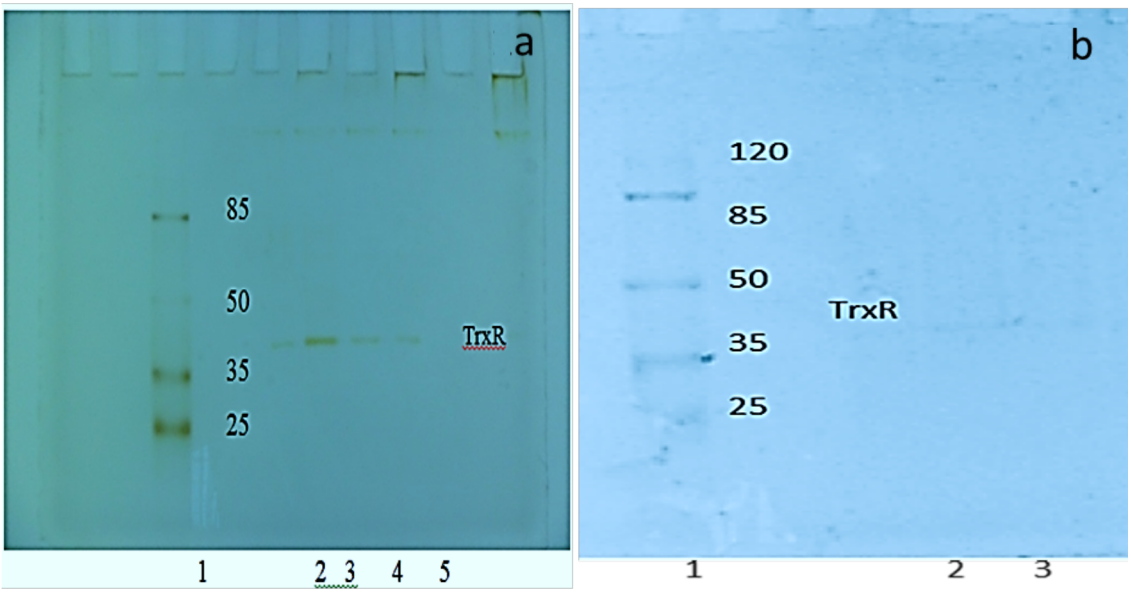

Figure 1a. Silver staining after the elution of turkey liver mitochondrial thioredoxin reductase enzyme from 2',5'-ADP Sepharose 4B affinity column. *(Well 1, Standard proteins: 85, 50, 35, 25 kDa. 2, 3, 4, 5; 2',5'-ADP Sepharose 4B) 1b. Coomassie Brilliant Blue dye Well 1; standard proteins (β-galactosidase: 120 kDa, BSA: 85 kDa, ovalbumin: 50 kDa, CA: 35 kDa, β-lactoglobulin: 25 kDa), 2, 3; after the elution of turkey liver mitochondrial thioredoxin reductase enzyme from 2',5'-ADP Sepharose 4B affinity column.

The optimum pH for the enzyme was determined as 600 mM potassium phosphate pH 7.5 buffer (Figure 3a). The optimum ionic strength for TrxR enzyme was determined as 600 mM potassium phosphate pH 7.5 buffer (Figure 3b). The activity measurements were performed at each 10 °C between 0 °C and 80 °C. As a result of these studies, the optimum temperature was determined as 50 °C (Figure 4). The stable pH for the enzyme was determined as 600 mM potassium phosphate pH 7.2 buffer (Figures 5a and 5b).

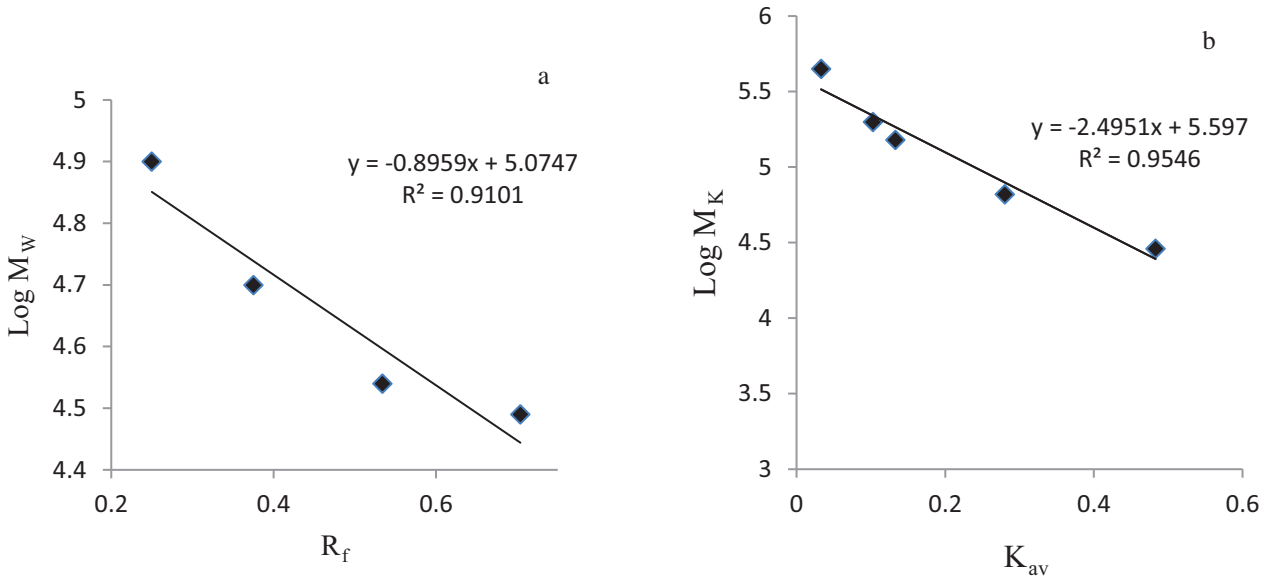

Figure 2. a. The standard plot used in the determination of the molecular mass of turkey liver mitochondrial TrxR enzyme by the SDS-polyacrylamide gel electrophoresis method. b. Log M_K, K_{av} plot used in the determination of the molecular mass of turkey liver mitochondrial TrxR enzyme.

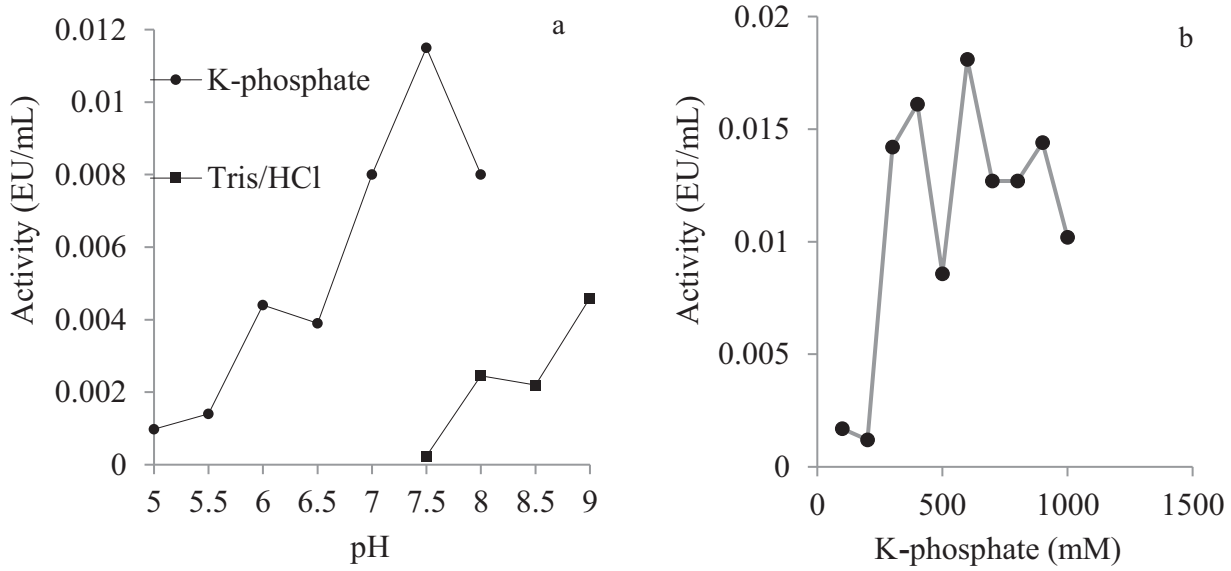

Figure 3. a. Results of the activity measurements performed for the optimum pH of turkey liver mitochondrial TrxR enzyme using 600 mM potassium phosphate and 600 mM Tris/HCl buffer solutions. b. Ionic strength activity plot drawn for turkey liver mitochondrial TrxR enzyme using potassium phosphate buffer solutions in different concentrations.

Lineweaver–Burk plots of K_M and V_{max} values were drawn for DTNB and NADPH substrates of the enzyme. K_M and V_{max} were calculated as 0.0318 mM and 0.0144 EU/mL, respectively, for DTNB, and K_M and V_{max} were calculated as 0.00137 mM and 0.0180 EU/mL, respectively, for NADPH (Figures 6a and 6b). kcat V_0 values were calculated as 87.8 s^{-1} and 2.7 $\times$ 10^6 M^{-1} s^{-1}, respectively, for DTNB substrate, and as 109.75 s^{-1} and 80 $\times$ 10^6 M^{-1} s^{-1}, respectively, for NADPH (Table 2).

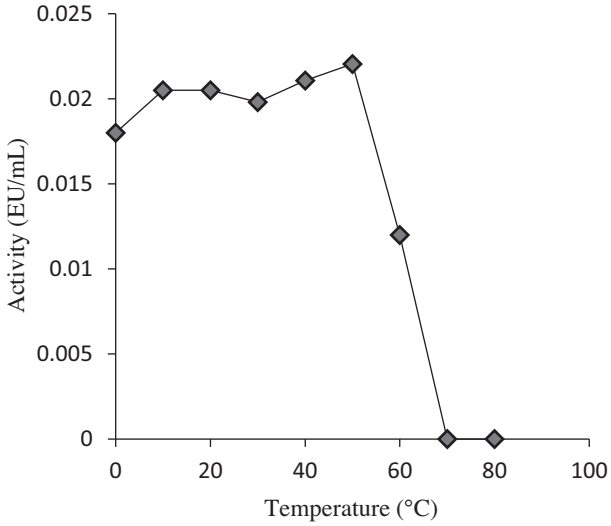

Figure 4. Temperature–activity plot drawn for measuring the optimum temperature of turkey liver mitochondrial TrxR enzyme.

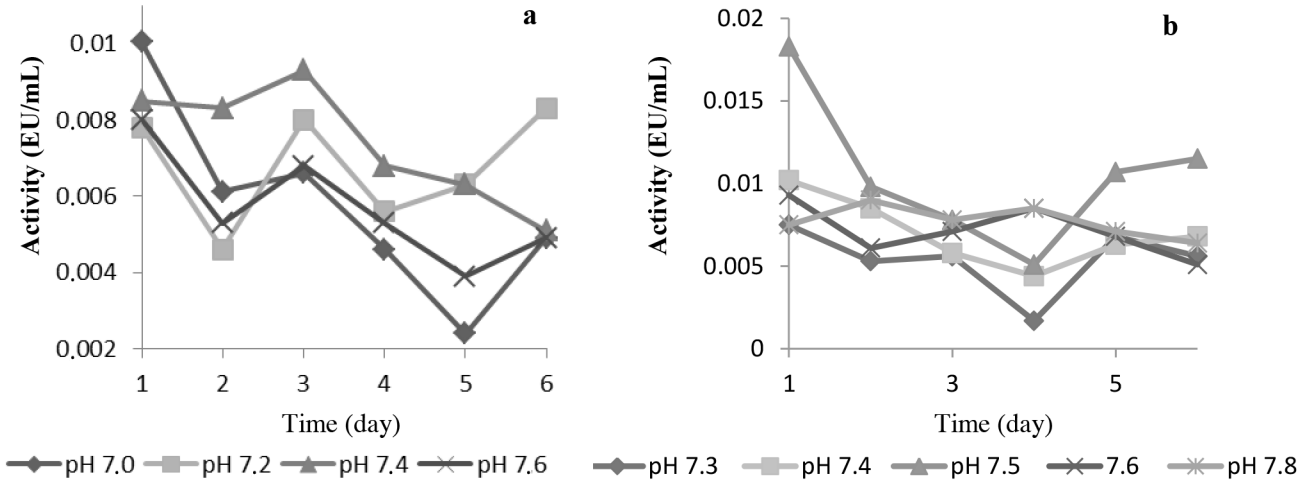

Figure 5. a. The plot of the activity measurement performed for the stable pH of turkey liver mitochondrial TrxR enzyme using 600 mM potassium phosphate buffer solutions. b. The plot of the activity measurement performed for the stable pH of turkey liver mitochondrial TrxR enzyme using 600 mM Tris/HCl buffer solutions.

Ag^{+}, Fe^{3+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}, and Co^{2+} inhibitory effects of some heavy metal ions on the activity of the turkey liver mitochondrial TrxR enzyme were examined under in vitro conditions. IC_{50} values with the heavy metal concentration with which 50% of the activity of TrxR enzyme was inhibited were found as 0.019, 0.529, 0.610, 1.460, and 3.400 mM for Ag^{+}, Cd^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} metal ions, respectively (Figure 7a; Table 3). It was determined that Ag^{+}, Fe^{3+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} ions inhibited TrxR enzyme, Pb^{2+} ion increased enzyme activity, and Ni^{2+} and Co^{2+} ions had no effect on enzyme activity (Figure 7b; Table 3). Finally, K_i constants for these metal ions were calculated from the Lineweaver–Burk plots as 0.013 $\pm$ 0.007, 0.435 $\pm$ 0.1920, 0.52 $\pm$ 0.093, 0.93 $\pm$ 0.130, and 3.15 $\pm$ 0.167 mM, respectively (Figures 8a and 8b; Table 2).

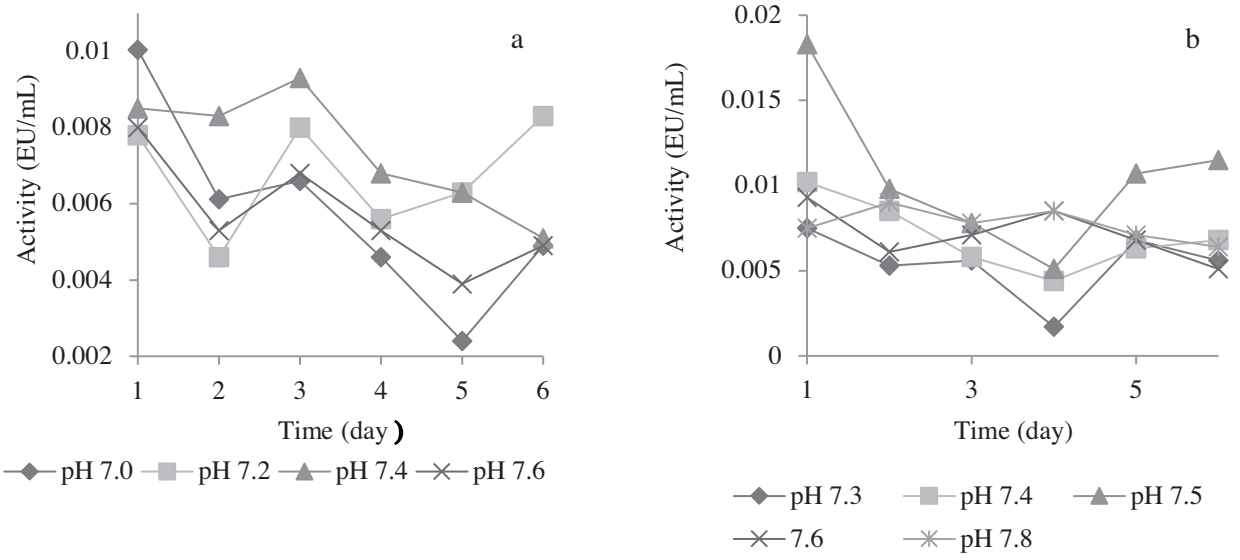

Figure 6. a. Lineweaver–Burk plot drawn for mitochondrial TrxR enzyme using 5 different NADPH concentrations b. Lineweaver–Burk plot drawn for mitochondrial TrxR enzyme using 5 different DTNB concentrations.

Table 2. Results of DTNB and NADPH substrates along with K_M, V_{max}, kcat, and Vo studies for turkey liver thioredoxin reductase enzyme.

Enzyme	Substrate	Km (mM)	Vmax (EU/mL)	k_{cat} (s^{-1})	V_0 (M^{-1} s^{-1})
Thioredoxin reductase	DTNB	0.0318	0.0144	87.8	2.7×10^6
	NADPH	0.00137	0.0180	109.75	80×10^6

Table 3. IC_{50} and K_i values and inhibition types found in different inhibitor concentrations for mitochondrial TrxR enzyme purified from turkey liver.

Inhibitor type	IC_{50} (mM)	K_i (mM)	Inhibition type
Ag^+	0.019	0.013 ± 0.007	Noncompetitive
Cd^{2+}	0.529	0.435 ± 0.1920	Noncompetitive
Fe^{3+}	0.610	0.520 ± 0.093	Competitive
Cu^{2+}	1.460	0.930 ± 0.130	Competitive
Zn^{2+}	3.400	3.150 ± 0.167	Noncompetitive

TrxR enzyme, which was first discovered as a part of ribonucleotide reductase system studies in *E. coli*, reversibly catalyzes the transfer of electrons to thioredoxin protein containing a redox-active disulfide bond with a molecular weight of 12 kDa through NADPH. The oxidation of NADPH ensures the formation of reduced thioredoxin (thioredoxin-S2) and provides electrons to the reduced thioredoxin ribonucleotide diphosphate reductase enzyme. Thioredoxin, thioredoxin reductase, and NADPH are called the thioredoxin system.[26] The thioredoxin protein, which is the physiologic substrate of TrxR enzyme, has various roles in different organisms in DNA synthesis, reduction of protein disulfide bonds, reduction of H_2O_2, reduction by methionine sulfoxide and protein repair, participation in the formation of phage fibrils, hydrogenation for 3'-phosphoadenylsulfate (PAPS) reductase, reduction of the photosynthetic enzymes in the chloroplast, redox regulations in transcription factors, and the regulation of apoptosis, the immune system, pregnancy, childbirth, and the central nervous system.[27]

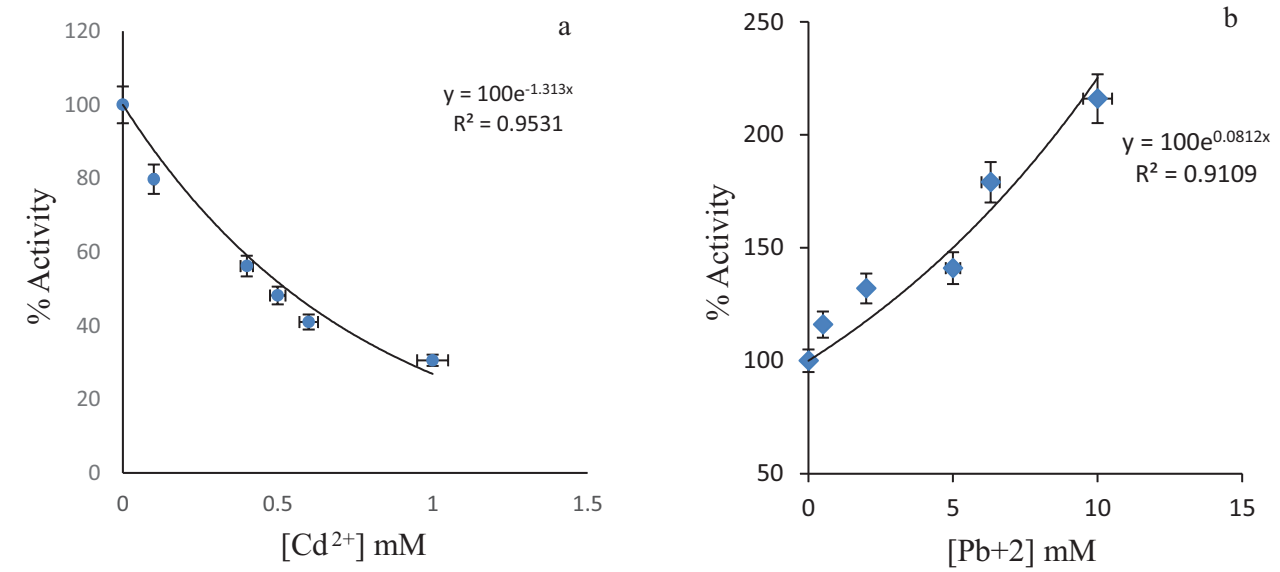

Figure 7. a. Effect of [Cd^{2+}] ion on turkey liver tissue mitochondrial TrxR enzyme b. Effect of [Pb^{2+}] ion on turkey liver tissue mitochondrial TrxR enzyme.

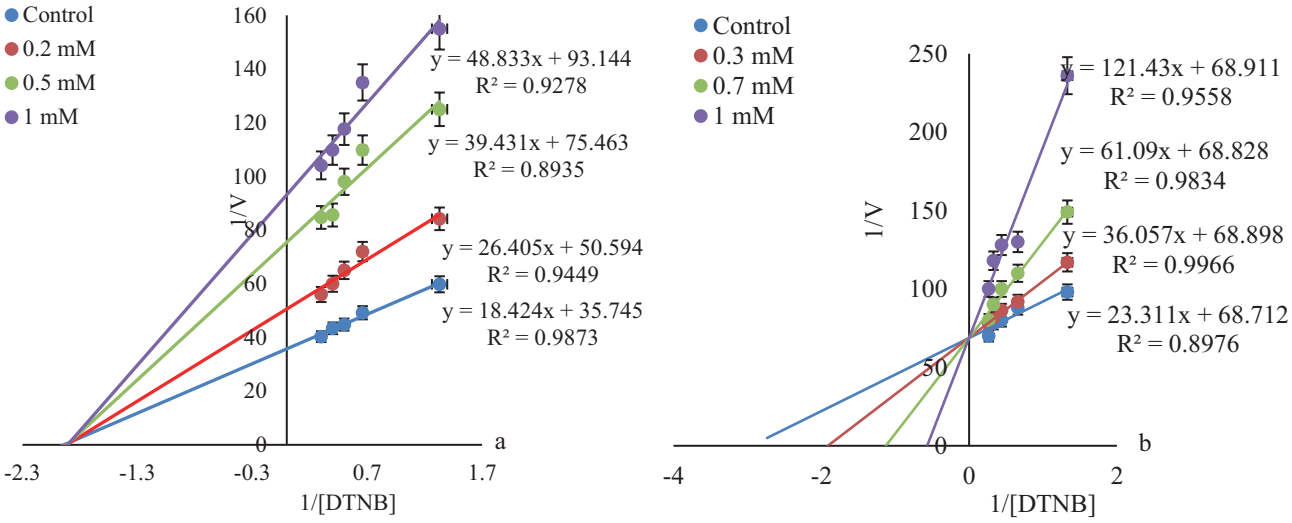

Figure 8. a. Effect of Cd^{2+} ion in different concentrations on turkey liver tissue mitochondrial TrxR enzyme b. Effect of Fe^{3+} ion in different concentrations on TrxR enzyme.

The thioredoxin system or thioredoxin reductase enzyme, as it is understood, is of great importance in living tissues. Many studies have been carried out to determine where this enzyme is found in the living creature metabolism and how it functions, and its kinetic properties have been investigated by being characterized from various creatures. In the present study, the effects of purification and characterization of the thioredoxin reductase enzyme from turkey liver tissues and some heavy metal ions on the enzyme activity were investigated.

In the purification studies carried out so far for TrxR enzyme, the enzyme was purified 1.083-fold from yeast[17] with 650 EU/mg specific activity using Sephadex G-50 chromatography and DEAE cellulose chromatography, and it was purified 4.375-fold from the rat liver[20] with 35 (EU/mg) specific activity using ammonium

sulfate precipitation, DEAE cellulose chromatography, Sephadex G-50 chromatography, CM-cellulose chromatography, 2',5'-ADP Sepharose chromatography, and ($\tilde{\omega}$-aminohexyl) agarose chromatography. Ammonium sulfate precipitation, DEAE cellulose chromatography, TEAE cellulose chromatography, and Sephadex G-200 chromatography were used for the enzyme purified from calf liver (with 82.760 EU/mg specific activity and a yield of 6%) and thymus (with 673 EU/mg specific activity and a yield of 27%).[19] In a different study carried out, it was purified 1.470-fold from the calf adrenal cortex mitochondria in a yield of 25.2% with 13.9 EU/mg specific activity using DEAE-Biogel A, Sephacryl S-300, Phenyl-Sepharose, and ADP-Agarose chromatography.[28] In the present study, turkey liver mitochondrial TrxR enzyme was purified 990-fold in one step in a yield of 2.05% with 3.07 EU/mg specific activity using 2',5'-ADP Sepharose 4B affinity chromatography. Thus we obtained the enzyme by an economical and effective method in high purity in a very short time compared to previous studies. A disadvantage of this method is low yield of purified protein but in a cold environment and a shorter time the purification fold and yield can be improved.

The purity of the TrxR enzyme obtained from turkey liver tissues was examined by SDS-PAGE. The enzyme was determined to be pure by the observation of a single band in the gel (Figures 1a and 1b). The molecular mass of the subunit of the enzyme was calculated to be about 45.5 kDa by this method. Sephadex G-150 gel filtration chromatography was performed to calculate the molecular mass of the active form of the enzyme. The molecular mass of the active enzyme was found to be 88 kDa by this method. The purified TrxR enzyme was determined to be in a homodimeric structure since the molecular mass of the enzyme calculated with Sephadex G-150 gel filtration chromatography was about twofold the size of the molecular mass found with SDS-PAGE. It is reported from various resources in the literature that the purified thioredoxin reductase enzyme is in a homodimeric structure and the subunits molecular masses are between 36.5 and 70 kDa.[17–24]

The optimum pH for the enzyme was determined to be 600 mM potassium phosphate pH 7.5 buffer (Figure 3a). In the literature, the optimum pH potassium phosphate buffer was found to be pH 7.0 for the enzymes purified from yeast,[17] rat liver,[20] calf liver, and adrenal cortex mitochondria,[21] and the optimum pH Tris/HCl pH 7.5 was found for the enzyme purified from tomato. It is seen that the optimum pH value found for TrxR enzyme purified from turkey liver is similar to the values in the literature. The optimum ionic strength for TrxR enzyme activity was determined to be 600 mM potassium phosphate pH 7.5 buffer (Figure 3b). As a result of the studies carried out, the stable pH for the enzyme was determined to be 600 mM potassium phosphate pH 7.2 (Figures 5a and 5b). The activities of the enzyme were measured spectrophotometrically with intervals of 10 °C between 0 °C and 80 °C. The optimum temperature was found to be 50 °C as a result of these measurements (Figure 4).

In kinetic studies carried out for the enzyme, K_M values for DTNB and NADPH, which are substrates of the enzyme, were calculated as 0.0318 and 0.00137 mM, respectively; V_{max} values for DTNB and NADPH were calculated as 0.0144 and 0.0180 mM, respectively. When these values were compared, the enzyme's relation with NADPH substrate was observed to be more than its relation with DTNB substrate. Although K_M values found show similarity with rat liver and calf liver TrxR enzymes, they are different from the values found for *E. coli* TrxR enzyme. Furthermore, kcat values were calculated as 87.8 s^{-1} for DTNB and as 109.75 s^{-1} for NADPH, and V_O values were calculated as 2.7×10^6 M^{-1} s^1 for DTNB and as 80×10^6 M^{-1} s^{-1} for NADPH (Table 2). All kinetic studies were carried out at 25 °C and at optimal pH (600 mM potassium phosphate pH 7.5).

Proteins gave a reaction via functional R groups of amino acids. For example, the –SH group in cysteine shows weak acid properties and it is very likely to react. Cysteine is a critical component of the active catalytic site of many enzymes. If cysteine contain enzymes treated with heavy metal ions such as Ag^+ and Hg^{2+}, –SH

mercaptans occur and it loses its enzyme activity.[29] The inhibitory effects of various metal ions on purified TrxR enzymes from different sources were examined. It was reported that Mn^{2+} and Zn^{2+} ions inhibit TrxR enzymes from purified rat liver.[22] In another study, the effect of Ca^{2+} ions on three different isoforms of TrxR was investigated. According to result of that study, cytosolic and mitochondrial forms were inhibited; on the other hand, the *E. coli* form of TrxR enzyme was not sensitive.[30] While it was reported in a different study that Ag^{+}, Cu^{2+}, and Hg^{2+} ions had an inhibitory effect on glutathione S-transferase enzyme purified from turkey liver,[31] it was reported in another study that Ag^{+}, Cd^{2+}, Cr^{2+}, and Mg^{2+} ions had a noncompetitive inhibitory effect on rainbow trout glutathione S-transferase enzyme.[32] Considering previous studies, it is thought that previously mentioned types of heavy metal ions can also inhibit enzymes.

Ag^{+}, Fe^{3+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+}, and Co^{2+} were chosen as metal ions in our study. From these metal ions selected, it was determined that Ag^{+}, Fe^{3+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} ions inhibited TrxR enzyme, Pb^{2+} ion increased enzyme activity, and Ni^{2+} and Co^{2+} ions had no effect on enzyme activity (Table 3; Figure 7b). From % Activity - [I] and Lineweaver–Burk plots drawn for metal ions showing the inhibitory effects on TrxR enzyme, the sequence of IC_{50} and K_i values from the smallest to the biggest between each other is $Ag^{+} < Cd^{2+} < Fe^{3+} < Cu^{2+} < Zn^{2+}$. From these results, it is seen that Ag^{+} and Cd^{2+} metal ions caused maximal inhibition on the enzyme. As a result, the methodology provided an effective and inexpensive way in addition to high purity in a very short time and the above-mentioned metal ions were determined to have a toxic effect on the enzyme.

2.1. Experimental

2.2. Chemicals

NADPH, DTNB, standard serum albumin, and electrophoresis chemicals were obtained from Sigma Chem. Co, 2',5'-ADP Sepharose 4B was obtained from Pharmacia, and the other chemicals used were obtained from Fluka and E. Merck AG.

2.3. Preparation of homogenate

The turkey liver (20 g) was cut into small pieces with the help of a knife to prepare homogenate. Then 60 mL of 0.05 M Tris HCl and 0.1 M DTT pH 7.5 buffer were added to the cut liver, and it was suspended with the homogenizer. First, the suspension was centrifuged at 6000 $\times g$ for 30 min at +4 °C for the mitochondrial enzyme. The precipitate was added, and the supernatant was centrifuged at 22,000 $\times g$. The obtained precipitate was homogenized in 0.05 M Tris HCl pH 7.5 buffer, and a freezing and thawing procedure (enzyme activity decreases due to the heating of the sonicator and so we preferred the freezing and thawing procedure) at –20 °C was applied. This suspension was centrifuged at 24,000 $\times g$ for 30 min at +4 °C, and the supernatant was prepared to be used in analyses.

2.4. Preparation of 2',5'-ADP Sepharose 4B affinity column and purification of turkey liver mitochondrial thioredoxin reductase enzyme

For 10-mL bed volume, by weighing 2 g of dry 2',5'-ADP Sepharose 4B gel it was eluted several times with 400 mL of distilled water for the removal of solid materials. The gel was inflated during eluting. The inflated gel was deflated with a vacuum using a water trompe, and then the gel was suspended by adding equilibration buffer (10 mM Tris/HCl, 1 mM EDTA pH 7.4) (buffer A). The suspended gel was packed into a cooled column consisting of a 1 × 10 cm closed system. After the gel had been settled, the column was equilibrated by eluting

with buffer A with the help of a peristaltic pump. The fact that the column was equilibrated was understood from the equalization of absorbance and pH at 280 nm in the eluate and buffer solution. Thus, the affinity column was prepared. The obtained homogenate was applied to the column equilibrated with buffer A. Then the column was eluted with buffer A. The elution process was maintained until the absorbance value at 280 nm became 0.05. Then the gradient elution procedure was carried out with buffer A solutions containing 2 mL of 2, 4, 6, 8, and 10 mM $NADP^+$ for each, and pure thioredoxin reductase enzyme was achieved.

2.5. Activity measurement of thioredoxin reductase

Holmgren's 1977 method was used while measuring the activity of the thioredoxin reductase enzyme; in this method, the fact that the thioredoxin reductase enzyme catalyzed the reduction of disulfide bonds in DTNB NADPH dependently was taken as the basis.[19] Total activity was expressed as enzyme units. The following formula was used to calculate enzyme unit:

$$\frac{EU}{ml} = \frac{(\Delta A_{412nm}/\min Num - \Delta A_{412nm}/\min Blind)\,(1)(df)}{(13,6 \times 2)(0.05)}$$

EU = the amount of enzyme that converts 1 micromole of substrate into product in one minute at 25 °C and optimal conditions,

1 = total volume (mL)
df = dilution factor
13.6 = extrusion coefficient
0.05 = amount of enzyme (mL)

2.6. Protein determination

The quantitative protein was determined spectrophotometrically at 595 nm with bovine serum albumin used as a standard by the Bradford method.[33]

2.7. Testing of the enzyme purity with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The enzyme purity was tested by doing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by Laemmli's[25] method (Figure 1). In this method, separation gel containing 0.1% SDS and 3% acrylamide and stacking gel containing 8% acrylamide were used. The molecular masses of the subunits of the enzyme were found from SDS-PAGE and the log MW-Rf plot obtained as a result of gel filtration chromatography (Figure 2a). The Rf value is a function of migration speed in the gel and was calculated from the formula

R_f = Xe/X dye
Xe = Distance travelled by protein, Xdye = Distance travelled by dye

2.8. Determination of the molecular mass of the enzyme with Sephadex G-150 gel filtration chromatography

Andrew's method was used in the determination of the molecular mass of the enzyme.[34] After the void volume had been calculated with Blue Dextran 2000 kDa, standard proteins (β-amylase; 200 kDa, yeast alcohol dehydrogenase; 150 kDa, albumin (bovine serum); 66 kDa, bovine erythrocyte carbonic anhydrase; 29 kDa

(MW-GF-200; Sigma)) and the enzyme solution were passed through a gel filtration column. The absorbance measurement at 280 nm was performed in the eluates obtained, and the absorbance measurement at 280 nm and activity measurement at 412 nm were performed for TrxR enzyme. The natural molecular mass of the enzyme was determined by using the log MW-Kav plot formed by standard proteins (Figure 2b). Kav defines solute behavior independently of the bed dimensions and packing. Kav value is calculated from the formula

Kav = (Ve – Vo)/(Vt – Vo)

Ve = elution volume of peak, Vo = void volume of column, Vt = packed bed volume

2.9. Determination of optimum pH

The potassium phosphate buffers with 7.5–9.0 pH 0.6 M Tris/HCl and 5.0–7.5 were used to determine the optimum pH of the enzyme. The separate enzyme activity was determined in each buffer with appropriate substrate solutions (Figure 3a).

2.10. Determination of optimum ionic strength

Potassium phosphate pH 7.5 solutions with concentrations ranging from 100 to 1000 mm were used to determine the optimum ionic strength of turkey liver mitochondrial thioredoxin reductase enzyme (Figure 3b).

2.11. Determination of optimum temperature

A heated–cooled water bath was used to determine the optimum temperature value of the enzyme (Poly Science bath; model 9105). The mixture containing the buffer and substrates required for the activity measurement was kept in the water bath set to the corresponding temperature in the bathtub, and the activity measurement was performed at intervals of 10 °C between 0 °C and 80 °C (Figure 4).

2.12. Determination of stable pH

Phosphate buffers ranging between Tris/HCl and 7.0–7.6 with pHs ranging between 7.3 and 7.8 were used to determine the stable pH of the enzyme; 2 mL of buffer solutions in pHs specified were mixed with 1 mL of enzyme solution and stored at +4 °C. The stable pH of the enzyme was determined by the activity measurements performed at intervals of 24 h for 7 days (Figures 5a and 5b).

2.13. Kinetic studies

The activity measurements were performed with 5 different concentrations of NADPH in fixed DTNB concentration (5 mM) to determine K_M and V_{max} values for DTNB and NADPH substrates of the enzyme. A Lineweaver–Burk plot was drawn with the values obtained.[35] K_M and V_{max} values were determined for NADPH using this plot (Figure 6a). Likewise, a Lineweaver–Burk plot was created by performing activity measurements with 5 different concentrations of DTNB in a fixed concentration of NADPH (0.2 mM), and K_M and V_{max} values were calculated for DTNB (Figure 6b). Activity measurements were performed at 25 °C and at optimal pH (600 mM potassium phosphate pH 7.5). Then the k_{cat} value showing the number of turnover of the enzyme for DTNB substrate was calculated using K_M and V_{max} values found, and from here the specificity constant of the enzyme V_0 was determined.

Inhibition studies were performed using DTNB substrate activity of the enzyme. For chemical substances such as $AgNO_3$, $CuCl_2.2H_2O$, $CdCl_2.H_2O$, $FeCl_3$, and $Zn(NO_3)_2.6H_2O$ that inhibit the enzyme, IC_{50} values

(inhibitor concentration that reduces the total enzyme activity by half) were determined by using five different inhibitor concentrations in fixed substrate concentration (5 mM DTNB) by drawing Activity % - [I] plots (Figure 7a). The activation curve was drawn for a Pb^{2+} ion that increased TrxR enzyme activity (Figure 7b). Then 1/V – 1/[S] values were determined in five different substrates and three different constant inhibitor concentrations for mitochondrial thioredoxin reductase enzyme purified from turkey liver tissues, and K_i constants and inhibition types were determined by drawing Lineweaver–Burk plots separately for each inhibitor (Figures 8a and 8b; Table 3). The kinetic studies and inhibitory studies were done in triplicate. K_i constants were calculated from these formulae

$V^I_{max} = V_{max}/(1 + [I]/K_i)$ for noncompetitive inhibitors.

$K^I_M = K_M\ (1 + [I]/K_i)$ for competitive inhibitors.

Acknowledgment

The authors thank the Research Fund of Atatürk University for funding the project (BAP 2012/150).

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