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Purification and characterization of mitochondrial thioredoxin reductase enzyme from rainbow trout (*Oncorhynchus mykiss*) liver and investigation of the in vitro effects of some metal ions on the enzyme

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Abstract: Thioredoxin reductase (E.C 1.6.4.5.; TrxR) is an enzyme belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases. In this study, mitochondrial TrxR enzyme was purified from rainbow trout mitochondria. Thanks to the 2 consecutive procedures (preparation of homogenate and 2',5'-ADP Sepharose 4B affinity chromatography), the enzyme, having the specific activity of 11.9 EU mg protein-1, was purified with a yield of 2.38% and 672-fold. The purity of the enzyme was monitored and the molecular weight of its subunits was calculated as 70 kDa by SDS-PAGE. The native molecular mass of the enzyme was found to be approximately 151 kDa by gel filtration chromatography. Characteristic and kinetic properties of the enzyme were also determined. Furthermore, Se^{4+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, and Al^{3+} metal ions' in vitro effects on mitochondrial TrxR purified from rainbow trout was investigated. While Se^{4+} ion increased the enzyme activity, all of the other metal ions used in this study showed an inhibitory effect.

Key words: Thioredoxin reductase, characterization, purification, rainbow trout, metal ions

1. Introduction

The thioredoxin reductases (TrxRs) are enzymes belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases, which includes lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase.[1] TrxRs are homodimeric flavoproteins with two N- and C- terminally located interacting catalytic centers. The C-terminal redox center contains a selenocysteine (Sec) residue that is a part of a conserved Gly-Cys-Sec-Gly motif that is crucial for TrxR function.[2] There are two forms of the enzyme, mitochondrial and cytosolic.[1] However, mammalian TrxRs are expressed as three different isoforms. They are TrxR1, isolated from the cytosol/nucleus; TrxR2, isolated from the mitochondria; and TrxR3, purified from the testes.[3,4] The thioredoxin system, composed of TrxR, thioredoxin (Trx), and NADPH (nicotinamide adenine dinucleotide phosphate), plays a central role in regulating cellular redox homeostasis and signaling pathways. [5] Trx is a redox active protein that regulates several physiological and biochemical functions, such as growth, apoptosis, and cellular defense. The function of Trx is regulated by TrxR.[6] This redox system is ubiquitous in both prokaryote and eukaryote cells.[7]

The main function of the TrxR/Trx system in the metabolism is the production of deoxyribonucleotides,

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which are critical for DNA synthesis and cell reproduction. Trx provides the electrons required for the ribonucleotide reductase, an enzyme that catalyzes the transformation of ribonucleotide diphosphate into deoxyribonucleotides to reduce the ribose.[8] The thioredoxin system is a key antioxidant system in defense against oxidative stress with its disulfide reductase activity regulating protein dithiol/disulfide balance. The Trx system provides thiol-dependent peroxidases (peroxiredoxins) with the electrons to remove reactive oxygen and nitrogen species with a fast reaction rate.[9]

Almost all metals create a toxic effect when they are taken beyond a certain amount. This effect generally shows itself as enzyme inhibition. Metals may disturb the activity of important enzyme systems or affect protein structure when they bind with O, N, and S that exist in the structure of the enzymes.[10]

Nowadays the consumption of trout, which grows better in cold waters, is on the increase. In addition, as a result of industrial development, the wastes in the waters (especially metal ions) are increasing and they have negative effects on the metabolism of the creatures living there. This negative situation results in mass deaths of fish. Because of the high consumption of trout, TrxR enzyme was selected as the focus of this study.

Since no data have been encountered regarding the toxic effects of Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, and Al^{3+} on the activity of TrxR enzyme purified from rainbow trout liver in the literature, this study aimed to examine the purification and characterization of TrxR enzyme from rainbow trout mitochondria, the kinetic properties of this enzyme, and the effects of some metal ions (Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, and Al^{3+}) on enzyme activity.

2. Results

Rainbow trout mitochondria TrxR was purified by 2',5'-ADP-Sepharose 4B gel affinity column. As shown in Table 1, specific activity was calculated for purified enzyme solution as 11.9 EU/mg protein, a yield of 2.38%, and a purification coefficient of 672-fold. Figure 1 exhibits the SDS-PAGE conducted for the purity and molecular weight of the enzyme. For the standard proteins and TrxR, Rf values were calculated, and an Rf-log MW graph (Figure 2a) was obtained according to Laemmli's procedure (19), showing a molecular weight of ∼70 kDa for GR. The molecular weight of the enzyme was also determined by gel filtration. A Kav-log MW graph was obtained (Figure 2b), which showed a molecular weight of ∼151 kDa for TrxR. The optimal pH was determined

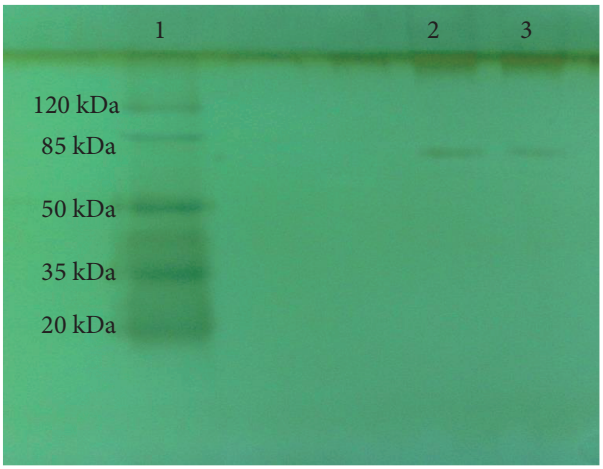

Figure 1. SDS-PAGE photograph: Lines 2 and 3; purified the enzyme from 2',5'-ADP Sepharose 4B matrix: Line 1; standard proteins (β-galactosidase: 120 kDa, BSA: 85 kDa, ovalbumin: 50 kDa, CA: 35 kDa, β-lactoglobulin: 25 kDa, lysozyme: 20 kDa).

as 7.5 using 100 mM K-phosphate (Figure 3a). The optimum ionic strength of the enzyme was estimated to be 500 mM in K-phosphate buffer (Figure 3b). The enzyme was seen to show the highest activity at 0 °C after being assayed between 0 °C and 80 °C (Figure 3c). The stable pH of the enzyme was 8.0 in Tris-HCl buffer (Figures 4a and 4b).

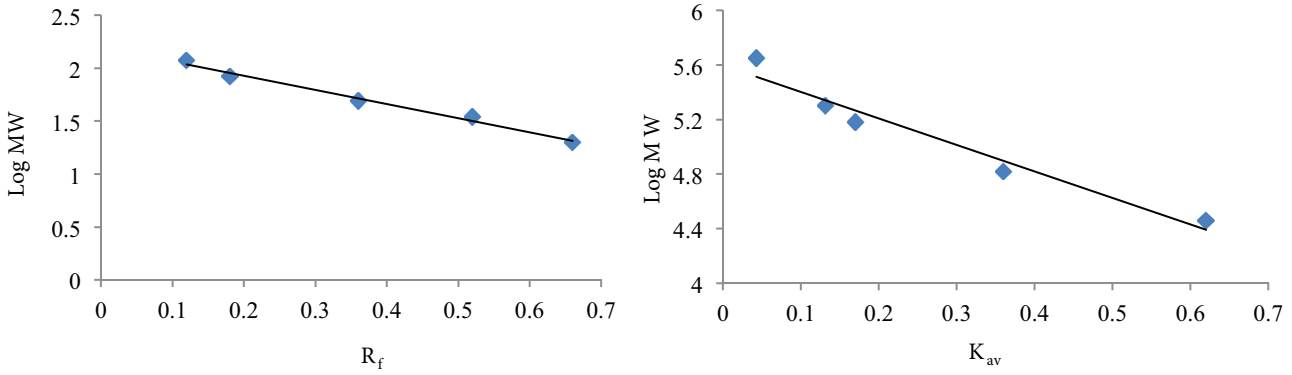

Figure 2. a. R_f values of rainbow trout liver mitochondrial TrxR were calculated by using the SDS-PAGE photograph. b. Standard K_{av}-log MW graph of rainbow trout liver mitochondrial TrxR using gel filtration. Standard proteins: Blue Dextran 2000 kDa, horse heart cytochrome-c 12.4 kDa, bovine erythrocyte carbonic anhydrase 29 kDa, bovine serum albumin 66 kDa, alcohol dehydrogenase 150 kDa, and β-amylase 200 kDa (MW-GF-200; Sigma).

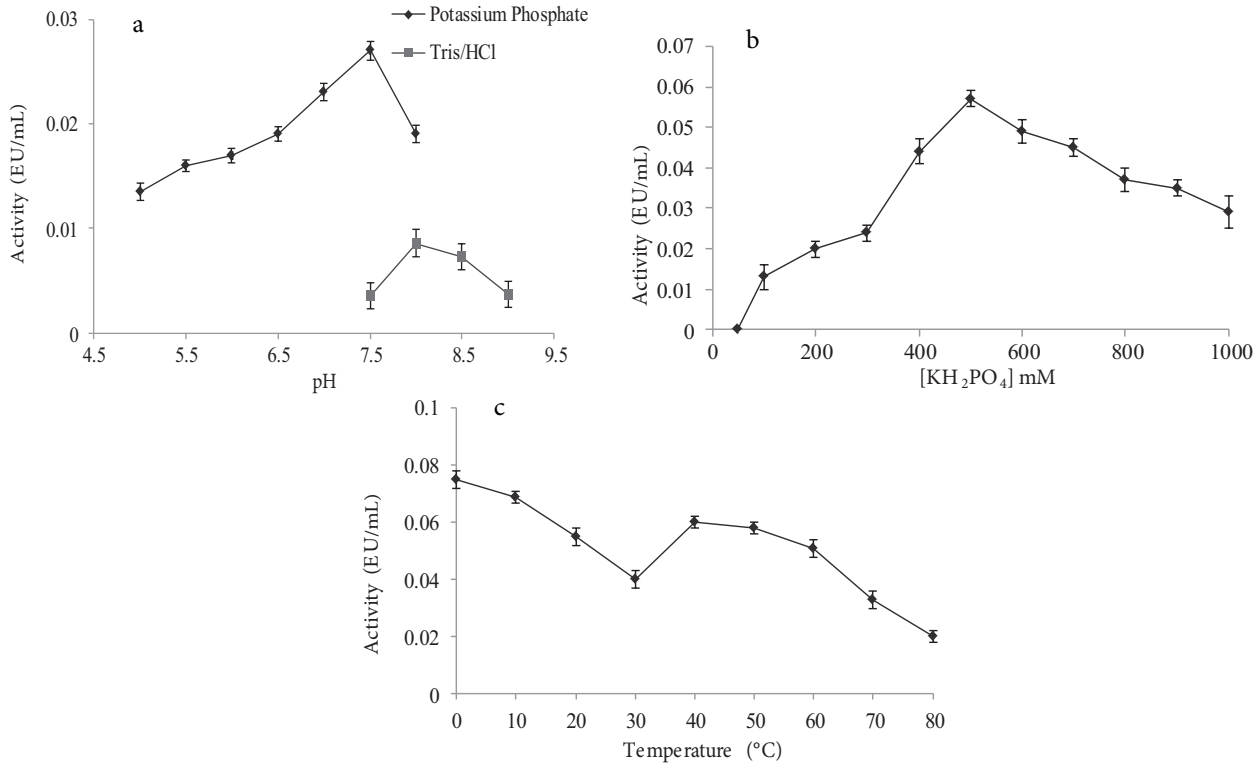

Figure 3. a. Effect of pH on activity of rainbow trout mitochondrial TrxR. The buffers used were 0.1 M Tris-HCl buffer (pH 7.5–9.0) and 0.1 M K-phosphate buffer (pH 5.0–8.0). b. Effect of ionic strength on activity of rainbow trout mitochondrial TrxR. The activity was assayed in different concentrations between 0.05 and 1 M of K-phosphate buffer at room temperature. c. Effect of temperature on activity of rainbow trout liver mitochondrial TrxR. The activity was assayed in 0.5 M K-phosphate buffer (pH 7.5).

Table 1. Purification scheme of mitochondrial thioredoxin reductase enzyme from rainbow trout liver tissue.

Fractions	Total volume (mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield %	Purification fold
Homogenate	60	744.8	13.2	0.0177	100	1
2',5'-ADP Sepharose 4B affinity chromatography	8	0.0264	0.314	11.9	2.38	672

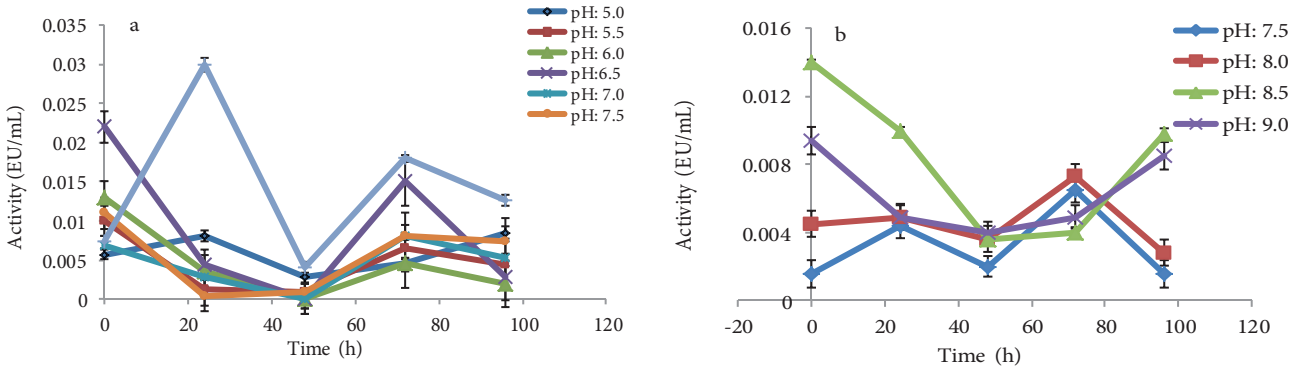

Figure 4. a. Stable pH graph determined with K-phosphate buffers at different pH for mitochondrial TrxR purified rainbow trout liver. b. Stable pH graph determined with Tris-HCl buffers at different pH for mitochondrial TrxR purified rainbow trout liver.

The Lineweaver–Burk graphs are shown in Figures 5a and 5b for NADPH and DTNB. A K_M of 12.65 μM and a V_{max} of 0.513 EU mL^{-1} were obtained for NADPH, and 0.0828 μM and 0.079 EU mL^{-1} were obtained for DTNB. V_0 and k_{cat} values were 44.103 min$^{-1}\mu$M^{-1} and 36.2 $\times$ 10^3 min^{-1} for DTNB respectively (Table 2).[11]

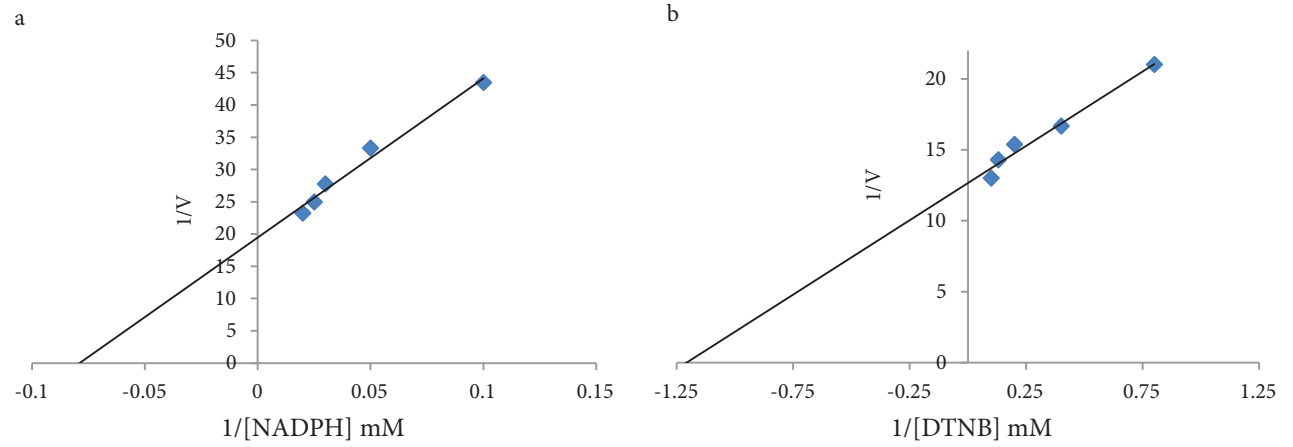

Figure 5. a. Kinetic analysis of rainbow trout liver mitochondrial TrxR activity. The activity was assayed in the ranging from 0.015 mM to 0.15 mM NADPH with 5 mM fixed DTNB. b. Kinetic analysis of rainbow trout liver mitochondrial TrxR activity. The activity was assayed in the ranging from 0.1 mM to 1 mM DTNB with 0.2 mM fixed NADPH.

As a result of investigating the in vitro effects on the enzyme activities of the Se^{4+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, and Al^{3+} metal ions, it was detected that Se^{4+} metal ion activated the enzyme; however, all of the

other metals inhibited the enzyme noncompetitively (Figure 6; Table 3). For all of the metals that showed an inhibitory effect, Activity% - [I] and Lineweaver–Burk graphs were drawn and IC_{50} values were calculated as 0.150, 0.186, 0.350, 0.930, and 3.270 mM, respectively; K_i constants were calculated as 0.14 ± 0.026, 0.17 ± 0.033, 0.34 ± 0.025, 0.85 ± 0.045, and 1.60 ± 0.285 mM (Figures 7a and 7b).[12]

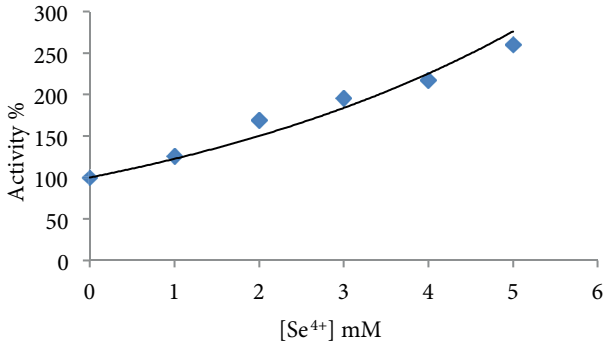

Figure 6. Effect of Se^{4+} on activity of rainbow trout liver mitochondrial TrxR.

3. Discussion

In this investigation, the metal ions studied showed high inhibitory effects on rainbow trout liver TrxR enzyme activity. Nowadays, the amounts of industrial wastes and metal refuses are rising in rivers, lakes, and seas worldwide. The undesirable side effects of these metal ions on TrxR activity, body metabolism, and several physiological and biochemical functions can be reduced for fish and fish eaters by focusing on these K_i and IC_{50} values obtained. Therefore, TrxR enzyme was purified from rainbow trout liver mitochondria and characterized, and some of the kinetic features were investigated. It was thought that the contact between metal wastes and water resources as a result of current technological developments might result in dangerous outcomes, and the effect of some metal ions on pure enzyme was investigated.

In the purification of different TrxR enzymes, heat and acidic procedures, ammonium sulfate precipitation, dialysis, and ion exchange and affinity chromatography were used. For instance, DEAE ion exchange, Sephadex G-50, and CM Cellulose 2',5'-ADP Sepharose 4B affinity chromatography techniques were used previously in order to purify rat liver TrxR enzyme.[13–15] Once again, rat liver cytosolic TrxR enzyme was purified 4000-fold in five steps with 18% purity.[14] In another study, Rigobello et al. purified mitochondrial TrxR enzyme from rat liver in six steps.[15] In another study, TrxR enzyme was purified 1000-fold from calf liver and thymus in 4 steps with 10% yield.[13]

In our study, the enzyme, which had 11.9 EU/mg protein specific activity, was purified 672-fold in a single step with 2',5'-ADP Sepharose 4B affinity chromatography with 2.38% yield in line with the literature information and some modifications (Table 1).

Thus, the enzyme was purified in a single step within a very short time. Although the yield of purified protein was low with this method, it had the advantages of being not time-consuming and being low cost. The purity of the enzyme was checked in SDS-PAGE and a single band was obtained as seen in Figure 1 in the 2nd and 3rd wells. In our study, the native molecular mass of the enzyme was found to be approximately 151 kDa with gel filtration chromatography and subunit molecule mass was found to be approximately 70 kDa with SDS-PAGE.[16] There is some information on *Danio rerio* (zebrafish) TrxR such as sequence of protein, cDNA, and nucleotide levels (GenBank accession number 18307). *Danio rerio* TrxR protein has 602 amino acids and

its cDNA has 2975 base pairs, for a calculated molecular mass of approximately 70 kDa. This result supports our results for rainbow trout liver TrxR. It can be said that the active form of the enzyme is homodimeric due to the fact that the native molecular mass is almost twice the mass of the subunit molecule. These results are in parallel with those for calf liver and thymus and rat liver cytosolic TrxR enzymes. On the other hand, they are different than those for *E. coli* TrxR enzyme.[17]

As is obvious from Figure 3a, the point where the enzyme activity is the highest was obtained at pH 7.5 with phosphate buffer in the activity measurements performed with pure enzyme. Therefore, the optimum pH of the enzyme was 7.5. As can be seen from Figures 4a and 4b, the pH at which enzyme activity was the longest and highest was pH 8.0 with phosphate buffer and the stable pH was 8.0. As can be seen from Figure 3b, 0 °C is the temperature at which enzyme activity was the highest. Therefore, the optimum enzyme temperature was 0 °C. The fact that optimum temperature is 0 °C is a significant result in terms of the adaptation of trout living in cold waters. Similarly, optimum enzyme ionic strength was calculated as pH 7.5 in 0.5 M phosphate buffer (Figure 3c).

In the kinetic studies performed on the enzyme, K_M constant was 0.828 μM and V_{max} value was 0.079 EU/ml for DTNB substrate, and K_M constant was 12.65 μM and V_{max} value was 0.513 EU/mL for NADPH. It can be inferred that the affinity of the enzyme with DTNB is higher since the K_M obtained for the DTNB substrate of enzyme was lower than the K_M obtained for NADPH. These results are in parallel with the results obtained for the calf liver TrxR and rat liver TrxR enzymes. Moreover, for DTNB, the V_0 value was 44 $\times$ 10^3 $min^{-1}\mu M^{-1}$ and k_{cat} value 36.2 $\times$ 10^3 min^{-1} (Table 2).

Table 2. V_{max}, K_M, K_{cat}, and V_0 values of rainbow trout liver mitochondrial thioredoxin reductase.

Substrate	K_M (μM)	V_{max} (EU/mL)	K_{cat} (min^{-1})	V_0 (μM^{-1} min^{-1})
DTNB	0.828	0.0790	36.2 $\times$ 10^3	44 $\times$ 10^3
NADPH	12.650	0.5136	-	-

With regard to the effect of metal ions on the TrxR enzyme in the literature, it is reported that thiols and selenols can form complexes with heavy ions such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} very easily, and bivalent metal ions such as Cd^{2+}, Ni^{2+}, and Zn^{2+} can show inhibitory effects on mammalian or GR enzyme. Taking the structural similarities of GR and TrxR into consideration, it was touched upon that 2+ bivalent metal ions can also inhibit TrxR.[18]

In our study, the in vitro effects of Se^{4+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, and Al^{3+} metal ions on TrxR enzyme activity were investigated. While Se^{4+} ion activated the enzyme, other metal ions inhibited the enzyme noncompetitively (Figure 6; Table 3). Activity% - [I] and Lineweaver–Burk graphs were drawn for Al^{3+}, Co^{2+}, Fe^{3+}, Cu^{2+}, and Ni^{2+} metal ions, and IC_{50} values were calculated as 0.150, 0.186, 0.350, 0.930, and 3.270 mM, while K_i constants were calculated as 0.14 $\pm$ 0.026, 0.17 $\pm$ 0.033, 0.34 $\pm$ 0.025, 0.85 $\pm$ 0.045, and 1.60 $\pm$ 0.285 mM respectively (Figures 7a and 7b). As can be seen from these values, Al^{3+} ion has the lowest IC_{50} value and K_i constant. The activity order following Al^{3+} is Co^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, and Se^{4+}. It is particularly striking that Al^{3+} and Co^{2+} are the most efficient inhibitors among these metal ions. A novel result has been found not previously cited in the literature for TrxR.

Table 3. K_i constants and IC_{50} values obtained from regression analysis graphs for rainbow trout liver mitochondrial thioredoxin reductase in the presence of different metal ion concentrations.

Metal ions	IC_{50} (mM)	K_i (mM)	Inhibition type
$[Al^{3+}]$	0.150	0.140 ± 0.026 mM	Noncompetitive
$[Co^{2+}]$	0.186	0.174 ± 0.033 mM	Noncompetitive
$[Fe^{3+}]$	0.350	0.340 ± 0.025 mM	Noncompetitive
$[Cu^{2+}]$	0.930	0.850 ± 0.045 mM	Noncompetitive
$[Ni^{2+}]$	3.270	1.600 ± 0.285 mM	Noncompetitive

4. Experimental

4.1. Chemicals

The chemicals used in the study were purchased from Sigma while 2',5'-ADP Sepharose 4B was purchased from Pharmasia.

4.2. Preparation of the homogenate

The rainbow trout liver, which was preserved at –20 °C, was firstly washed with homogenate buffer in order to be purified. The liver was cut into small pieces in order to prepare homogenate. It was then homogenized with an ULTRA-TURRAX. It was suspended in 0.05 M Tris-HCl + 0.1 M DTT (pH 7.5). Gradient centrifugation was performed in order to obtain liver mitochondria organelles. Precipitate was put into the sonicator three times every 20 s. The precipitate, which was sonicated, was centrifuged again. The supernatant obtained after centrifuging was preserved at +4 °C to be used in the following purification steps.

4.3. Purification of the rainbow trout liver mitochondrial thioredoxin reductase enzyme with 2',5'-ADP Sepharose 4B affinity chromatography

The homogenate prepared was put into a 2',5'-ADP Sepharose 4B column that had been just prepared and balanced. The column was washed with equalizing buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.4) until the absorbance difference in 280 nm became 0.05 after sample transition was performed. After the multistage washing procedure was completed, a multistage elution procedure was performed using 2 mM $NADP^+$, 4 mM $NADP^+$, 6 mM $NADP^+$, 8 mM $NADP^+$, and 10 mM $NADP^+$ solutions consecutively. The eluates that showed activity were collected and their enzyme activity was measured.

4.4. Measurement of thioredoxin reductase enzyme activity

The method determined by Holmgren was used when the activity of thioredoxin reductase enzyme was measured. This method was based on the fact that thioredoxin reductase enzyme catalyzed the reduction of disulfide bonds in DTNB in a NADPH dependent way.[13]

4.5. Determination of protein

The quantitative protein amounts in homogenate and enzyme were determined according to Bradford's method.[19]

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

After the enzyme was purified, 3%–10% discontinuous SDS-PAGE was performed according to Laemmli's method[20] and the purity degree of the enzyme was determined. Cleared protein bands were photographed (Figure 1). An R_f-log MW graph was obtained based on the SDS-PAGE photo (Figure 2a).

4.7. Molecular weight determination

The natural molecular mass of the enzyme was determined by gel filtration chromatography.[21] Firstly, Blue Dextran was passed through a gel filtration column. Later, standard proteins (horse heart cytochrome-c 12.4 kDa, bovine erythrocyte carbonic anhydrase 29 kDa, bovine serum albumin 66 kDa, alcohol dehydrogenase 150 kDa, and β-amylase 200 kDa (MW-GF-200; Sigma)) and then enzyme solution were passed through a gel filtration column. In the samples eluted, absorbance and TrxR activity were measured at 280 and 412 nm, respectively. Then a log Mw-K_{av} graph was drawn. Based on this graph, natural molecule mass of the enzyme was found (Figure 2b).

4.8. Determination of optimum pH

With the aim of determining the optimum pH of TrxR enzyme purified from rainbow trout liver mitochondria, 0.1 M phosphate buffer with pH between 5.0 and 8.0 and 0.1 M Tris-HCl buffer with pH between 7.5 and 9.0 were prepared. Enzyme activity was measured in each buffer (Figure 3a).

4.9. Determination of optimum ionic strength

Optimal ionic strength was identified by preparing solutions in different concentrations of phosphate buffers with 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mM under the optimum pH upon determining the optimum pH. Enzyme activity was examined in each buffer concentration (Figure 3b).

4.10. Determination of optimum temperature

The mixture that contained the buffer, enzyme, and substrates required for activity measurement was put into a water bath adjusted to the heat determined. Between 0 °C and 80 °C, activity was measured at each 10 °C (Figure 3c).

4.11. Determination of stable pH

With the aim of determining the pH where the enzyme is stable, 0.1 M Tris-HCl with pH between 7.5 and 9.0 and 0.1 M phosphate buffer with pH between 5.0 and 8.0 were used. Next, 1 mL of the buffer solution with the pH levels stated above and 0.5 mL of enzyme solution were mixed and the mixture was preserved at +4 °C. With the activity measurements performed every 24 h for 5 days, it was found that the enzyme was stable (Figures 4a and 4b).

4.12. Kinetic studies

Activity measurements were performed with 5 different concentrations of NADPH in constant DTNB concentration in order to determine K_M constants and V_{max} values for NADPH substrate. Then a Lineweaver–Burk graph was drawn with the values obtained (Figure 5a). K_M constant and V_{max} values were determined for NADPH via this graph. Likewise, a Lineweaver–Burk graph was formed by performing activity measurements

with 5 different concentrations of DTNB in the constant concentration of NADPH (Figure 5b). K_M constant and V_{max} values were calculated for DTNB. Activity measurements were performed under optimal conditions.[12] Later, the k_{cat} value showing the turnover number for DTNB substrate of the enzyme was calculated by using the K_M constant and V_{max} value obtained. The specificity constant V_0 of the enzyme was also calculated.

4.13. In vitro effects of the metal ions

After enzyme activity, the inhibitory effects of Se^{4+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Fe^{3+}, and Al^{3+} metal ions were investigated under in vitro conditions. Firstly, enzyme activities of the metal ions were measured in at least 5 different concentrations. An activation curve was drawn for Se^{4+}, which showed an activator effect (Figure 6). Activity% - [I] graphs were drawn for the metal ions that showed an inhibitory effect and IC_{50} values were calculated (Figure 7a). In addition, inhibition type and K_i constants of the metals that showed inhibitory effects were ascertained by drawing Lineweaver–Burk graphs upon measuring the activities in three different inhibitors and five different substrate concentrations (Figure 7b).

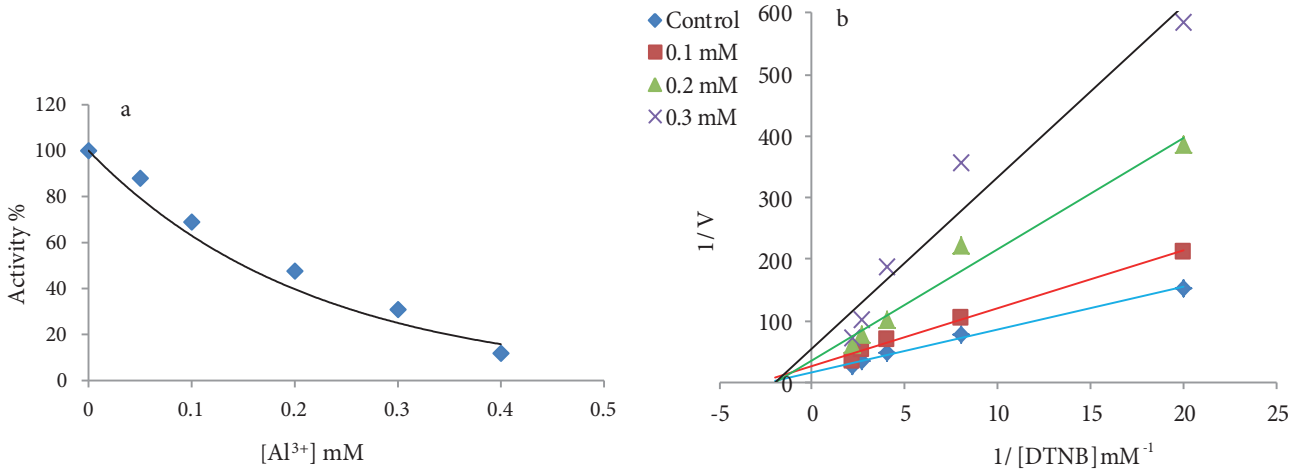

Figure 7. a. Activity% - metal ions regressions analysis graphs for Al^{3+} showing maximum inhibitory effect on rainbow trout liver mitochondrial TrxR. b. Lineweaver–Burk graph for determining K_i constant and inhibition type of Al^{3+} showing maximum inhibitory effect on rainbow trout liver mitochondrial TrxR.

Abbreviations/Definitions

TrxR: Thioredoxin reductase
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
FAD: Flavin adenine dinucleotide
$NADP^+$: Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)
DTNB : 5,5'-dithiobis-2-nitrobenzoic acid
DEAE: Diethylaminoethyl
CM: Carboxymethyl cellulose
EU: Enzyme unit
GR: Glutathione reductase
DTT: Dithiothreitol

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