

In vitro Inhibition Effects of Some Metal Ions on Glutathione Reductase Purified from *Capoeta trutta* Kidney

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Abstract

Glutathione reductase (EC: 1.8.1.7; GR) was purified from the kidney of the teleost fish *Capoeta trutta*. The purification procedure consisted of three steps: preparation of homogenate, ammonium sulfate fractionation and affinity chromatography on 2',5'-ADP Sepharose 4B. The enzyme was purified 794-fold with a yield of 35.4% and a specific activity of 11.91 U/mg proteins. In order to control enzyme purity, SDS-PAGE was done and showed a single band for enzyme. A single band was obtained approximately at 55 kDa. In addition, inhibitory effects of metal ions (Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} and Zn^{+2}) on fish kidney glutathione reductase were investigated. K_i constants and IC_{50} values for metal ions were determined by Lineweaver-Burk graphs and plotting activity % vs. [I], respectively. IC_{50} values were 0.00078, 0.622, 0.722, 0.073 and 0.519 mM, and K_i constants were 0.000394 ± 0.0002 , 0.235 ± 0.027 , 0.279 ± 0.048 , 0.026 ± 0.008 and 0.382 ± 0.024 mM for Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} and Zn^{+2} , respectively. From these results, we showed that Ag^+ is the most potent inhibitor of glutathione reductase enzyme.

Keywords: Glutathione reductase, *Capoeta trutta*, kidney, inhibitor, purification.

Öz

Capoeta trutta Böbrek Dokusundan Saflaştırılan Glutasyon Redüktaz Enzim Aktivitesine Bazı Metallerinin *in vitro* İnhibisyon Etkileri

Bu çalışmada, glutasyon redüktaz (EC: 1.8.1.7; GR) içsu balıklarından *Capoeta trutta* böbrek dokusundan saflaştırılmıştır. Saflaştırma işlemi, homojen atın hazırlanması, amonyum sülfat çöktürmesi ve 2',5'-ADP Sepharose 4B afinite kromatografisi olmak üzere 3 basamakta gerçekleştirilmiştir. Bu yöntemlerle, enzim 11.91 EÜ/mg protein spesifik aktivite ve %35.4 verimle 794 kat saflaştırılmıştır. Saflaştırma sonucu elde edilen saf enzimin saflık kontrolü sodyum dodesil sülfat poliakrilamid jel elektroforezi (SDS-PAGE) ile yapıldı ve enzimlerin tek band olduğu görülmüştür. Enzimin molekül kütlesi yaklaşık olarak 55 kDa olarak hesaplanmıştır. Ayrıca, balık böbrek dokusundan saflaştırılan glutasyon redüktaz enzimi üzerine bazı metallerin (Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} ve Zn^{+2}) *in vitro* inhibisyon etkileri incelenmiştir. Metallerin K_i sabiti ve IC_{50} değerleri sırasıyla Lineweaver-Burk ve Yüzde (%) Aktivite-[Metal] grafikleri ile hesaplanmıştır. Bu grafiklerden, Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} ve Zn^{+2} metalleri için IC_{50} değerleri sırasıyla 0.00078, 0.622, 0.722, 0.073 ve 0.519 mM, ve K_i sabitleri sırasıyla 0.000394 ± 0.0002 , 0.235 ± 0.027 , 0.279 ± 0.048 , 0.026 ± 0.008 ve 0.382 ± 0.024 mM olarak hesaplanmıştır. Sonuç olarak, Ag^+ metalinin diğer metallerden daha fazla *C. trutta* böbrek GR enziminin hibe ettiği tespit edilmiştir.

AnahtarKelimeler: Glutasyon redüktaz, *Capoeta trutta*, böbrek, inhibitör, saflaştırma.

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Introduction

The contamination of aquatic environment by metals is the consequence of industrial, agricultural and anthropogenic activities, such as an urban runoff, sewage treatment, and domestic garbage dumps, thus aquatic organisms are exposed to unnaturally high levels of these metals (Sampaio *et al.*, 2008; Qu *et al.*, 2014). Exposure of aquatic organisms to metals can increase reactive oxygen species (ROS) generation, leading to oxidative stress, as has been reported in many aquatic organisms after exposure to sublethal concentrations of some metals, such as Cu, Cd, Pb and Fe (Fernandez *et al.*, 2010; Borkovic-Mitic *et al.*, 2013). ROS are products of electron transport chains, enzymes, and redox cycling and their production may be enhanced by exposure to xenobiotics. Oxidative stress occurs when ROS overwhelm the cellular defenses, causing damage to proteins, membranes, and DNA (Kelly *et al.*, 1998; Adams and Greeley, 2000) and is defined as a disruption of the proantioxidant balance, which leads to potential damage (Yonar and Sakin, 2011). Fish, as many other vertebrates, are endowed with defensive mechanisms to counteract the harmful effects of ROS resulting from the metabolism of various chemicals or xenobiotics. The first line of defence consists of low molecular weight antioxidant compounds (e.g., glutathione and vitamin C and E), and the second defence mechanism comprises antioxidant enzymes (Blahová *et al.*, 2013; Yonar *et al.*, 2014). Enzymatic defense is provided by many enzyme systems such as glutathione reductase (GR), glutathione peroxidase, glutathione S-transferase, superoxide dismutase, catalase, aldo keto reductase and DNA repair enzymes. Particularly, GR is essential for the maintain-

ce of cellular glutathione in its reduced form, which is highly nucleophilic for many reactive electrophils (Carlberg and Mannervik, 1975; Sahin *et al.*, 2012).

GR is the key enzyme of glutathione metabolism and is widespread in all tissues and blood cells. This enzyme catalyses reduction of oxidized glutathione (GSSG) to glutathione (GSH) in the presence of NADPH and maintains a high intracellular GSH/GSSG ratio of about 500 in red blood cells (Kondo *et al.*, 1980). By maintaining a high ratio of [GSH] / [GSSG], the enzyme enables several vital functions of the cell such as the detoxification of ROS as well as protein and DNA biosynthesis (Schirmer *et al.*, 1989). Decreased glutathione levels have also been reported in several diseases, such as acquired immune deficiency syndrome (Akerlund *et al.*, 1997), Parkinson's disease (Jenner and Olanow, 1998) and diabetes (Vijayalingam *et al.*, 1996). High GSSG concentrations inhibit a number of important enzyme systems, including protein synthesis (Deneke and Fanburg, 1989).

GR has been purified from human and different animal tissues, using various purification procedures (Carlberg and Mannervik, 1981; Le Trang *et al.*, 1983; Ulusu and Tandon, 2007; Tekman *et al.*, 2008; Taser and Ciftci, 2012). In addition, effects of many drugs, metals and chemicals on human and animal tissues GR enzyme activities have been investigated so far (Erat *et al.*, 2003; Tekman *et al.*, 2008; Taser and Ciftci, 2012). However, no reports could be found in the literature on the effects of these metals on *C. trutta* fish kidney GR. Therefore, in the present study we have purified GR from *C. trutta* and examined *in vitro* inhibition effects of some metal ions on the enzyme.

Materials and Methods

Chemicals; 2', 5'-ADP Sepharose 4B was obtained from Pharmacia. NADPH, GSSG, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Aldrich Chem. Comp. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Enzyme Assay; GR activity was measured spectrophotometrically at 25°C by the modified method of Carlberg and Mannervik (Carlberg and Mannervik, 1975). The assay system contained 50 mM Tris-HCl buffer pH 8.0, containing 1 mM EDTA, 1 mM GSSG and 0.1 mM NADPH. One enzyme unit was defined as the amount that oxidizes 1 μ mol NADPH per min under the assay conditions.

Preparation of the Hemolysate; Fish samples (n= 10; 190 \pm 20 g) were caught from Murat River (Bingöl, Turkey). The fish were decapitated and their kidneys were extracted. 10 g kidney samples were washed three times with 0.9% sodium chloride solution. Then, using a scalpel, kidney samples were cut into small pieces. These pieces were homogenized with the aid of liquid nitrogen and suspended in a 50 mM KH₂PO₄ (pH 7.4) buffer that includes 1 mM PMSF, 1 mM EDTA and 1 mM DTT. The suspension was primarily centrifuged at 13,500 rpm for 2 h, and the precipitate was thrown away. Supernatant was used in further studies (Le Trang *et al.*, 1983).

Ammonium Sulfate Fractionation and Dialysis; The hemolysate was subjected to precipitation with ammonium sulfate (kidney: between 40% and 70%). Enzyme activity was determined both in the supernatant and in the precipitate for each respective precipitation. The precipitate was dissolved in phosphate buffer (50 mM, pH 7.0). The resultant solution

was clear and contained partially purified enzyme. This solution was dialyzed at 4°C in 1 mM EDTA + 10 mM K-phosphate buffer (pH 7.5) for 2 h with two changes of buffer (Ak-kemik *et al.*, 2011). Partially purified enzyme solution was kept at 4°C.

2', 5'-ADP Sepharose 4B Affinity Chromatography; 2 g of dry 2',5'-ADP Sepharose 4B was used for a column (1 \times 10 cm) of 10 mL bed volume. The gel was washed with 300 mL of distilled water to remove foreign bodies and air, suspended in 0.1 M K-acetate + 0.1 M K-phosphate buffer (pH 6.0) and packed in the column. After settling of the gel, the column was equilibrated with 50 mM K-phosphate buffer including 1 mM EDTA pH 6.0 with a peristaltic pump. The flow rates for washing and to equilibration were adjusted 20 mL/h. Previously obtained dialyzed sample was loaded onto the 2',5'-ADP Sepharose 4B affinity column and the column was washed with 25 mL of 0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0 and 25 mL of 0.1 M K-acetate + 0.1 M K-phosphate, pH 7.85. Washing was continued with 50 mM K-phosphate buffer including 1 mM EDTA, pH 7.5, until the final difference in the absorbance reached 0.05 at 280 nm. The enzyme was eluted with a gradient mixture of 0 to 0.5 mM GSH + 1 mM NADPH in 50 mM K-phosphate, containing 1 mM EDTA (pH 7.5). Active fractions were collected and dialyzed with equilibration buffer. All procedures were performed at 4°C (Boggaram *et al.*, 1979; Le Trang *et al.*, 1983; Coban *et al.*, 2007).

Protein Determination; Protein concentration was determined at 595 nm according to the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE); to determine the enzyme's Purity, SDS-PAGE was performed

according to Laemmli's method (Laemmli, 1970). The acrylamide concentration of the stacking and separating gels was 3% and 8%, respectively, and 1% SDS was also added to the gel solution. The gel was stained for 2 h in 0.1% Coomassie Brilliant Blue R-250 containing 50% methanol, 10% acetic acid and 40% distilled water. Then the gel was washed with many changes of the same solvent without dye. Cleared protein bands were photographed (Figure 1).

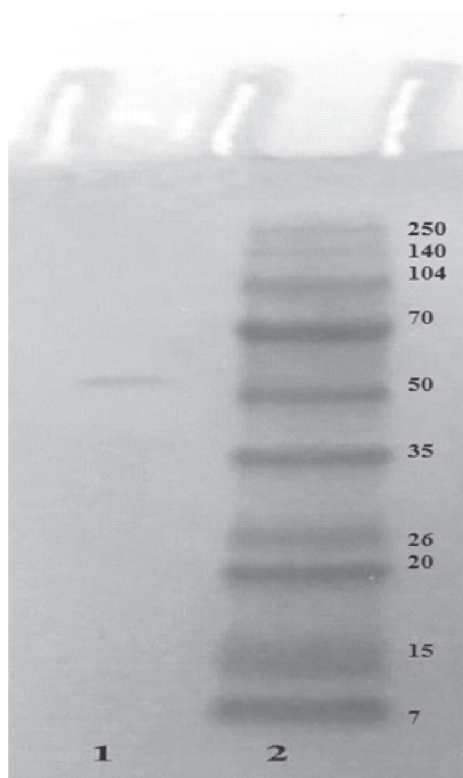


Figure 1. SDS-polyacrylamide gel electrophoresis of purified GR. Lane 1: *C. trutta* kidney GR and Lane 2: Standard proteins.

In vitro Effects of Metal Ions; In order to determine the effects of the metal ions on fish kidney GR, different concentrations of metal ions were added to the reaction medium. The enzyme activity was measured and an experiment in the absence of inhibitor was used as control (100% activity). The IC_{50}

values were obtained from activity (%) vs. metal ion concentration plots (Figure 2) (Tekman *et al.*, 2008).

In order to determine K_i constants in the media with inhibitor, the substrate (GSSG) concentrations were 0.3, 0.8, 1.4, 2 and 3 mM. Inhibitor solutions (metal salts) were added to the reaction medium, resulting in 3 different fixed concentrations of inhibitors in 1 mL of total reaction volume. Lineweaver-Burk graphs were drawn by using $1/V$ vs. $1/[S]$ values and K_i constant were calculated from these graphs (Figure 3). Regression analysis graphs were drawn for IC_{50} using inhibition % values by a statistical package (Figure 2) (SPSS-for windows; version 17.0) on a computer (student t-test; $n= 3$) (Tekman *et al.*, 2008).

Results

In this study, *C. trutta* kidney GR enzyme was first isolated. Purification procedure was carried out by the preparation of the homogenate, ammonium sulfate precipitation and affinity chromatography on 2',5'-ADP Sepharose 4B. As a result of the three consecutive steps, the enzyme was purified 794-fold with a yield of 35.4% and a specific activity of 11.91 U/mg proteins (Table 1).

The purity of the enzymes were determined by SDS-PAGE and showed single bands on the gel (Figure 1). R_f values were calculated for standard proteins and GR according to Laemmli's procedure (1970) from R_f -LogMW graph. Molecular weights of kidney GR enzymes were 55 kDa.

In vitro inhibitory activities of Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} and Zn^{+2} as metal ions were evaluated on fish kidney GR enzyme. IC_{50} values were found as 0.00078, 0.622, 0.722, 0.073 and 0.519 mM, and k_i constants were found as 0.000394 ± 0.0002 , 0.235 ± 0.027 , 0.279 ± 0.048 , $0.026 \pm$

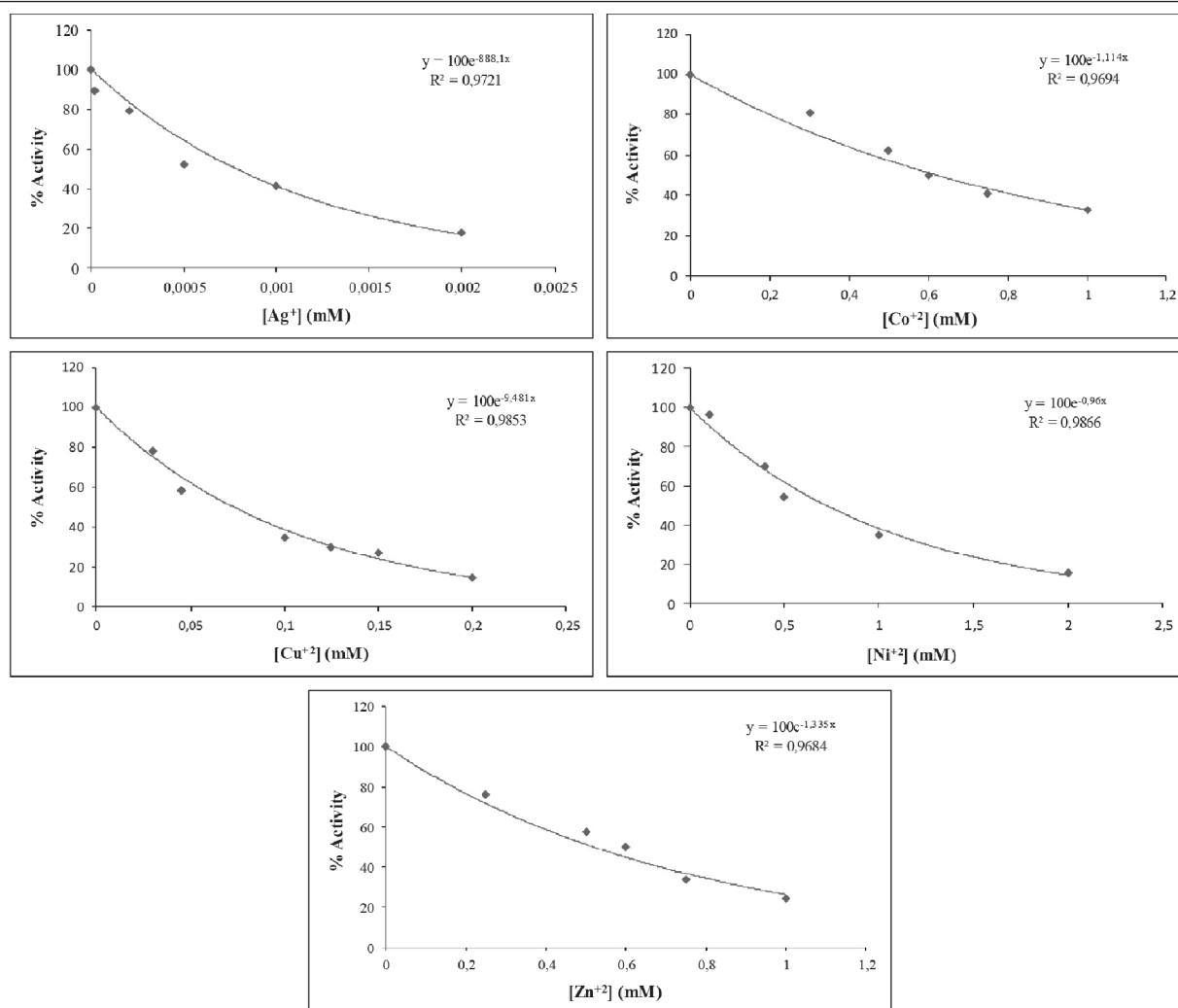


Figure 2. Activity %-[Metal] regression analysis graphs for fish kidney GR in the presence of five different metal concentrations.

Table 1. Purification scheme of GR from *C. trutta* kidney

Purification step	Activity (U/mL)	Protein (mg/mL)	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Hemolysate	0.207	13.69	26	5.382	355.94	0.015	1	100
Ammonium sulfate precipitation (40-70%)	0.295	8.2	9.5	2.803	77.9	0.036	2.4	52.08
2', 5'-ADP Sepharose 4B affinity chromatography	0.381	0.032	5	1.905	0.16	11.91	794	35.4

Table 2. IC₅₀, K_i values and inhibition types for five inhibitors for *C. trutta* kidney GR

Metal ions	IC ₅₀ (mM)	K _i (mM)	Inhibition type
Ag ⁺	0.00078	0.000394 ± 0.0002	Noncompetitive
Co ²⁺	0.622	0.235 ± 0.027	Noncompetitive
Cu ²⁺	0.073	0.026 ± 0.008	Uncompetitive
Ni ²⁺	0.722	0.279 ± 0.048	Noncompetitive
Zn ²⁺	0.519	0.382 ± 0.024	Competitive

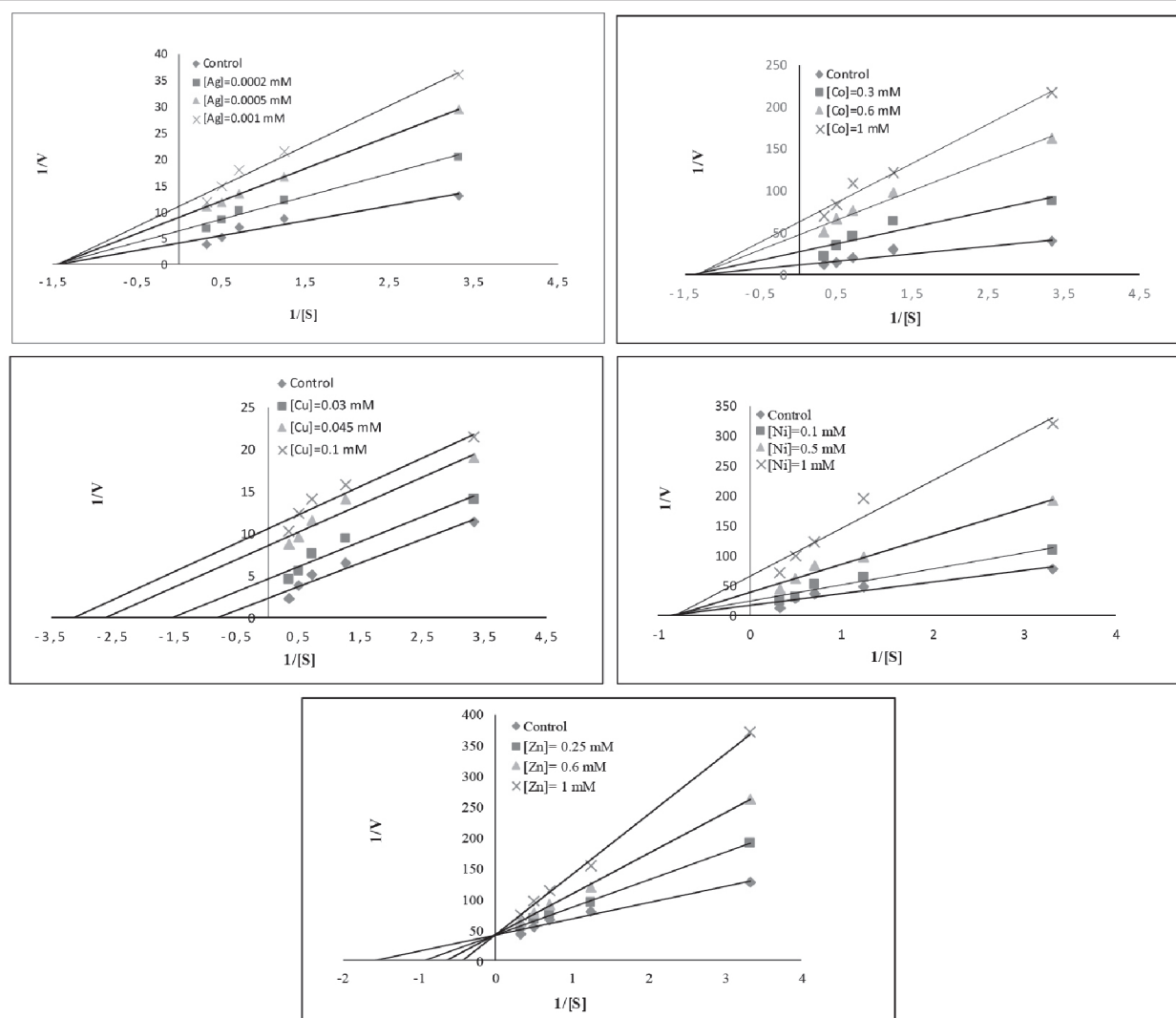


Figure 3. Lineweaver–Burk graph in 5 different substrate concentrations and in 3 different metal (Ag^+ , Co^{+2} , Cu^{+2} , Ni^{+2} and Zn^{+2}) concentrations for determination of K_i .

0.008 and 0.382 ± 0.024 mM for Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} and Zn^{+2} , respectively (Table 2).

Discussion

Fish are the major part of the human diet and it is not surprising that numerous studies have been carried out on metal accumulation in different fish species (Kucuksezgin *et al.*, 2001; Lewis *et al.*, 2002; Turkmen *et al.*, 2005). Metals are emitted to the water environment from different sources such as transportation, industrial activities, fossil fuels, agriculture, urbanization and other human activities (Gorur

et al., 2012). Metals in water environment bring about balance disorder in ecosystem by causing structural damage in fish at cellular and molecular level; while they, at the same time, cause heavy metal toxicity in humans through the consumption of fish that constitute an important ring in food chain (Fulladosa *et al.*, 2006; Nisbet *et al.*, 2010). Some metals such as Cu, Fe and Zn are essential to life and play unavoidable roles in some critical enzyme systems (Ceyhun *et al.*, 2011). For example, Cu and Zn are critical elements of SOD while Fe is an integral component of CAT (Fernandez *et al.*, 2010).

In the present study, GR enzyme was puri-

fied from *C. trutta* kidney using preparation of homogenate, ammonium sulfate fractionation and 2',5'-ADP Sepharose 4B affinity column chromatography methods. After preparation of the homogenate, precipitate saturation of the enzyme was determined as 40-70% with solid $(\text{NH}_4)_2\text{SO}_4$. This precipitation interval is similar to that of others obtained from different sources (Boggaram et al., 1979; Carlberg and Mannervik, 1981; Ulusu et al., 2005; Tekman et al., 2008; Akkemik et al., 2011). At the end of the last step, the purity of the enzyme was determined by SDS-PAGE and showed single band on the gel (Figure 1).

In addition to purification of the enzyme, Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} and Zn^{+2} were chosen to investigate their inhibitory effects on fish liver GR. IC_{50} values and K_i constants are the most suitable parameters for seeing inhibitory effects. As shown in Table 2, IC_{50} values were 0.00078, 0.622, 0.722, 0.073 and 0.519 mM, and K_i constants were 0.000394 ± 0.0002 , 0.235 ± 0.027 , 0.279 ± 0.048 , 0.026 ± 0.008 and 0.382 ± 0.024 mM for Ag^+ , Co^{+2} , Ni^{+2} , Cu^{+2} and Zn^{+2} , respectively. IC_{50} values and K_i constants show that Ag^+ was the most potent inhibitor for *C. trutta* kidney GR enzyme.

Similar results were obtained in both *in vitro* and *in vivo* studies for various enzymes, such as rainbow trout liver GR (Tekman et al., 2008), human erythrocyte GR (Coban et al., 2007), *Callinectes sapidus* gill carbonic anhydrase (CA) (Skaggs and Henry, 2002), *Carcinus maenas* gill CA (Skaggs and Henry, 2002), rainbow trout brain CA (Soyut et al., 2008), *Dicentrarchus labrax* liver CA (Ceyhun et al., 2011), rainbow trout kidney CA (Soyut and Beydemir, 2011), *Sparus aurata* liver CA (Kaya et al., 2013), *Capoeta umbla* gill CA (Kirici et al., 2016), rainbow trout liver glucose 6-phosphate dehydrogenase (G6PD) (Comakli et al., 2013), *Ctenopharyngodon idella* hepa-

topancreas G6PD (Hu et al., 2013), turkey liver glutathione S-transferase (Akkemik et al., 2012), yellow catfish hepatic 6-phosphogluconate dehydrogenase (Zhuo et al., 2015), *Chalcalburnus tarichii* gills glutathione S-transferase (Ozaslan et al., 2017).

Consequently, the pollution of the aquatic environment with heavy metals has become a worldwide problem during recent years, because they are indestructible and most of them have toxic effects on organisms (Mac Farlane and Burchett, 2000). Heavy metals pollution affects not only aquatic organisms, but also public health as a result of the bioaccumulation in the food chain (Canpolat, 2013). For this reason, great efforts and cooperation between different authorities are needed to protect the aquatic resources from metal pollution. To avoid the aquatic life loss there is need to use the advanced technologies generating less metal pollution to environment. The rising industrial and settlement areas near the rivers cause a great danger for the creatures living in watery areas. The required precautions should be taken immediately.

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