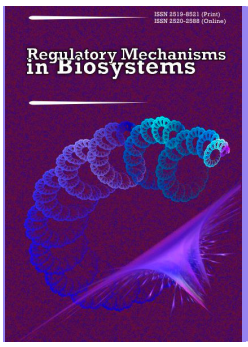

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In vitro effects of some metal ions on glutathione reductase in the gills and liver of *Capoeta trutta*

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Many aquatic environmental problems have arisen in consequence of contamination of water by toxic metals and organic pollutants in the present age of technology. Metals play vital roles in enzyme activities and other metabolic events due to their bioaccumulative and nonbiodegradable properties among aquatic pollutants. The aim of this study was to evaluate the inhibitory effects of some metal ions (Ag^+, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}) on *Capoeta trutta* gill and liver glutathione reductase (EC: 1.8.1.7; GR). For this purpose, initially, GR was purified from *C. trutta* gill and liver. Purification procedure consisted of three steps; preparation of hemolysate, ammonium sulphate precipitation and 2', 5'-ADP Sepharose 4B affinity chromatography. Using this procedure, *C. turtta* gill GR, having the specific activity of 19.111 EU/mg proteins, was purified with a yield of 38.8% and 910.05-fold; *C. trutta* liver GR, having the specific activity of 16.167 EU/mg proteins, was purified with a yield of 21.1% and 734.86-fold. The purity of the enzymes was checked on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and each purified enzyme showed a single band on the gel. In addition, inhibitory effects of some metal ions (Ag^+, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}) on GR from gill and liver were investigated in vitro. K_i constants and IC_{50} values for metal ions which showed inhibition effects were determined by Lineweaver-Burk graps and plotting activity % vs. [I]. In conclusion, IC_{50} values for fish gill GR were 0.000625, 0.153, 0.220, 0.247 and 0.216 mM and K_i constants for fish gill GR were 0.00045 ± 0.00008, 0.128 ± 0.036, 0.182 ± 0.138, 0.482 ± 0.219 and 0.112 ± 0.047 mM for Ag^+, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, respectively. IC_{50} values for fish liver GR were 0.000437, 0.217, 0.185, 0.355 and 0.349 mM and K_i constants for fish liver GR were 0.00025 ± 0.00013, 0.532 ± 0.146, 0.123 ± 0.066, 0.093 ± 0.020 and 0.151 ± 0.084 mM for Ag^+, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, respectively. *In vitro* inhibition rank order was determined as $Ag^+ > Co^{2+} > Zn^{2+} > Ni^{2+} > Pb^{2+}$ for fish gill GR; $Ag^+ > Cu^{2+} > Co^{2+} > Pb^{2+} > Ni^{2+}$ for fish liver GR. From these results, we showed that Ag^+ metal ion is the most potent inhibitor of GR enzyme on gill and liver tissues.

Keywords: *Capoeta trutta*; glutathione reductase; liver; gill; metal toxicity

Introduction

Glutathione reductase (Glutathione: $NADP^+$ oxidoreductase, E.C.1.8.1.7; GR), a key enzyme in glutathione metabolism, is a member of the pyridine-nucleotide disulfide oxidoreductase family of flavoenzymes (Kondo et al., 1980). This flavin enzyme is essential for reduction of glutathione disulfide (GSSG) to the reduced form (GSH), necessary for protection of cells against oxidative stress as an antioxidant (Cooper and Kristal, 1997). GSH has an important role in the synthesis and degradation of proteins, regulation of enzymes, formation of the deoxyribonucleotid precursors of DNA, and protection of cells against free radicals and reactive oxygen species such as H_2O_2, $O_2^{\bullet}$ and $^{\bullet}OH$ (Gul et al., 2000; Isık et al., 2015). Decreased GSH levels have been reported in several diseases, such as acquired immune deficiency syndrome (AIDS) (Akerlund et al., 1997), adult respiratory distress syndrome (Pacht et al., 1991), Parkinson's disease (Jenner and Olanow, 1998), and diabetes (Yoshida et al., 1995). In addition, recent results suggest that GSH is essential for cell proliferation (Poot et al., 1995), and plays a role in the regulation of apoptosis (Van den Dobbelsteen et al., 1996).

Metals are natural trace components of the aquatic environment, but their levels have increased due to industrial, agricultural and mining activities. All metals are potentially harmful to aquatic organisms at a certain level of exposure and absorption (Kalay and Canli, 2000). This situation may be hazardous for living systems, especially aquatic living systems, including specific enzymes. It is well-known that enzymes catalyze almost all chemical reactions in the metabolisms of living systems. These chemical substances, including pollutants, pesticides, drugs and metal ions, influence metabolisms at low concentrations by decreasing or increasing enzyme activities (Ekinci et al., 2007). Fish are widely used to evaluate the health of aquatic ecosystems because pollutants build up in the food chain. Because of this, in recent years numerous metal toxicity studies have been performed on fish by many scientists worldwide (Kalyoncu et al., 2011; Yi and Zhang, 2012; Yousafzai et al., 2012; Squadrona et al., 2013). There is no report available on the purification of GR enzyme from the gills and liver of *C. trutta*. Therefore, the aim of this study was to purify GR enzyme, the metabolic importance of which has long been acknowledged, from the gills and liver of *C. trutta* and to examine the *in vitro* effects of certain metals upon enzyme activity.

Materials and methods

Chemicals. NADPH, GSSG, protein assay reagents and chemicals for electrophoresis were obtained from Sigma Aldrich Chem. Comp. 2′,5′-ADP Sepharose-4B was obtained from Pharmacia. $AgNO_3$, $CuSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $Pb(NO_3)_2$, $ZnCl_2$ and all other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of the hemolysate. Fish samples (n = 10; 190 ± 20 g) were caught from Murat River (Bingöl, Turkey). The fish were decapitated and their gills and livers were extracted. 8 g gill and liver samples were washed three times with 0.9% sodium chloride solution. Then, using a scalpel, the gill and liver samples were cut into small pieces. These pieces were homogenized with the aid of liquid nitrogen and suspended in a 50 mM KH_2PO_4 (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).

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 µmol NADPH per min under the assay conditions.

Ammonium sulfate fractionation and dialysis. The hemolysate was subjected to precipitation with ammonium sulfate (liver: between 30% and 70%; gill: between 20% 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 (Akkemik 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 (1×10 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. The 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 (Le Trang et al., 1983).

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. The cleared protein bands were photographed (Fig. 1).

***In vitro* effects of metal ions.** In order to determine the effects of the metal ions on fish liver and gill 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. In order to determine K_i constants in the media with inhibitor, the substrate (GSSG) concentrations were 0.3, 0.8, 1.4, 2.0 and 3.0 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. Regression analysis graphs were drawn for IC_{50} using inhibition % values by a statistical package (SPSS-for Windows; version 17.0) on a computer (Student t-test; n = 3).

Results

In this study, *C. trutta* gill and liver GR enzyme were first isolated. Purification procedure was carried out by the preparation of hemolysate, ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity chromatography. As a result of the three consecutive steps, *C. trutta* gill GR, having the specific activity of 19.111 EU/mg proteins, was purified with a yield of 38.8% and 910.05-fold (Table 1); *C. trutta* liver GR, having the specific activity of 16.167 EU/mg proteins, was purified with a yield of 21.07% and 734.86-fold (Table 2). Purity of the enzyme was determined by SDS-PAGE and showed a single band on the gel (Fig. 1). Fig. 1 exhibits the results of SDS-PAGE which was performed for determination of the purity and molecular weight of the enzyme. Rf values were calculated for both standard proteins and GR; Rf-Log MW graph was obtained according to Laemmli's (Laemmli, 1970) procedure, and the molecular mass of *C. trutta* gill and liver GR was nearly 50 and 55 kDa.

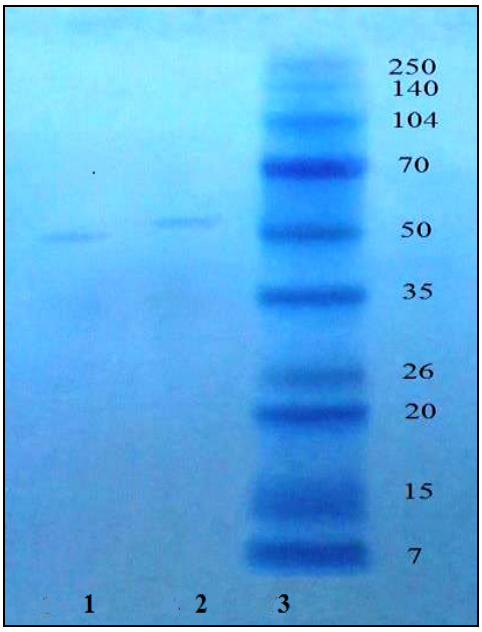

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified GR: Lane 1: *C. trutta* gill GR; Lane 2: *C. trutta* liver GR; Lane 3: standard proteins

In this study we investigated the *in vitro* effects of Ag^+, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} on fish gill and liver GR activity. As shown in Table 3, IC_{50} values for fish gill GR were 0.000625, 0.153, 0.220, 0.247 and 0.216 mM and K_i constants for fish gill GR were 0.00045 ± 0.00008, 0.128 ± 0.036, 0.182 ± 0.138, 0.482 ± 0.219 and 0.112 ± 0.047 mM for Ag^+, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, respectively (Fig. 2). As shown in Table 4, IC_{50} values for fish liver GR were 0.000437, 0.217, 0.185, 0.355 and 0.349 mM and K_i constants for fish liver GR were 0.00025 ± 0.00013, 0.532 ± 0.146, 0.123 ± 0.066, 0.093 ± 0.020 and 0.151 ± 0.084 mM for Ag^+, Co^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}, respectively (Fig. 3). It is clear that Ag^+ is the most potent inhibitor for *C. trutta* gill and liver GR enzymes.

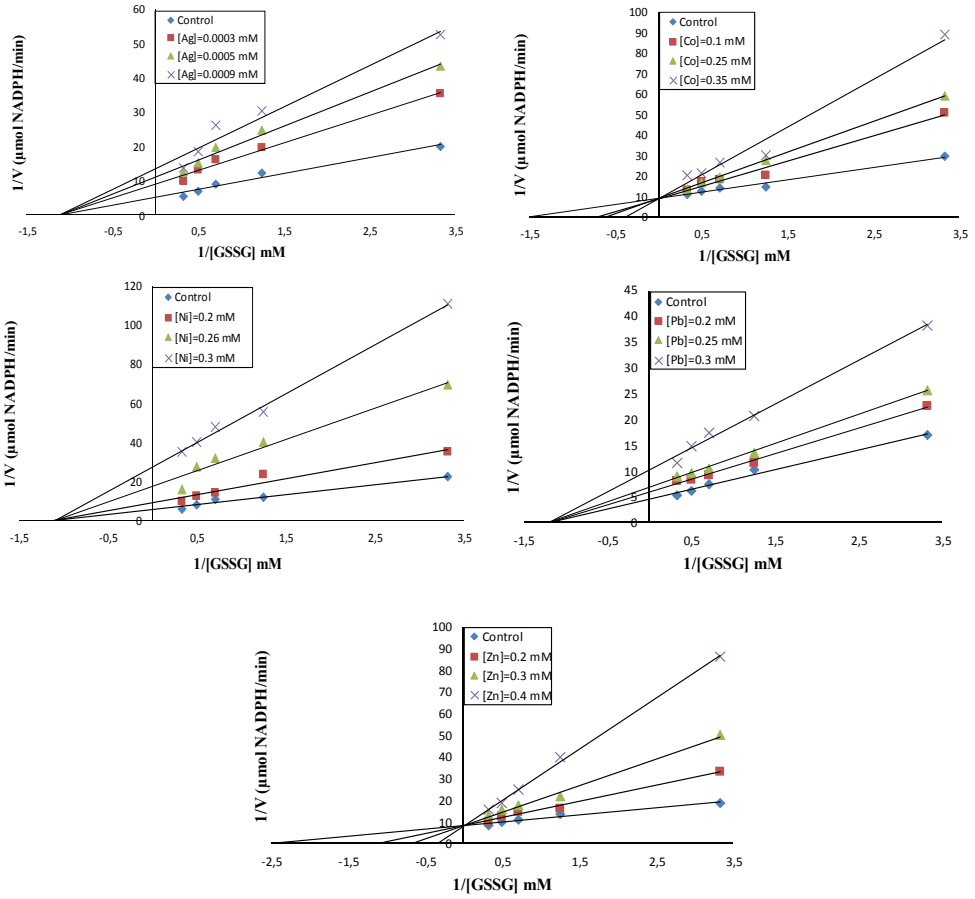

Fig. 2. Activity%-[Metal] regression analysis graphs for *C. trutta* gill GR in the presence of three different metal concentrations

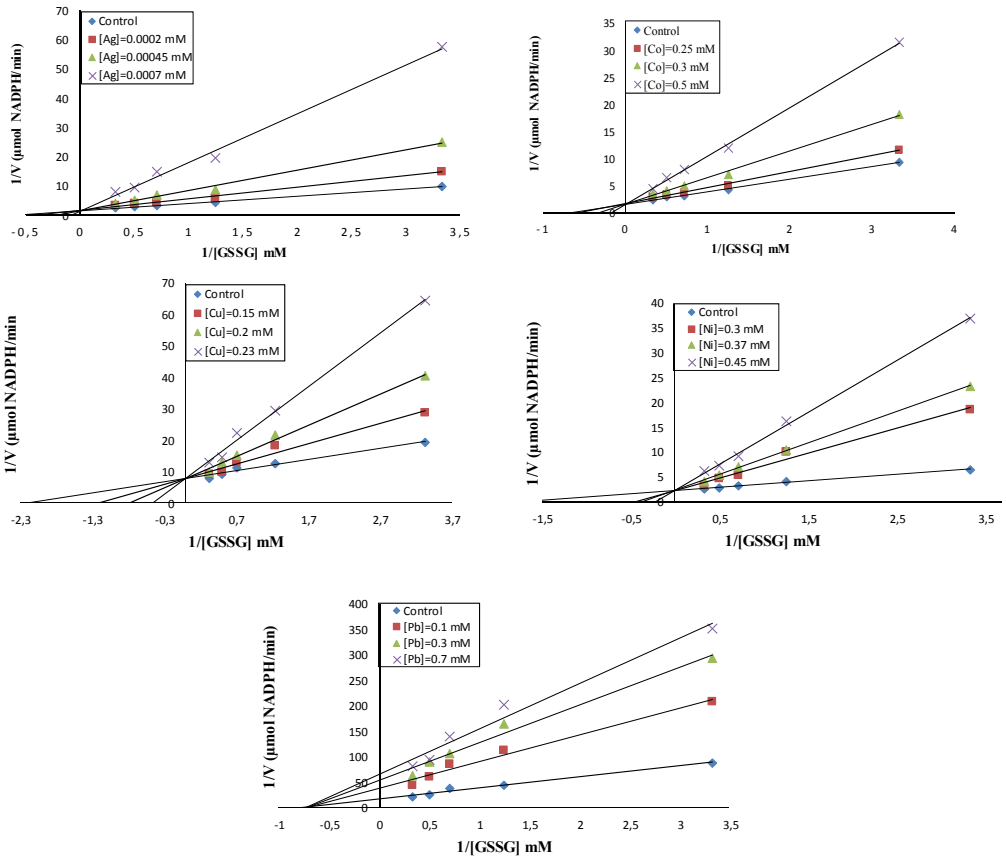

Fig. 3. Activity%-[Metal] regression analysis graphs for *C. trutta* liver GR in the presence of three different metal concentrations

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

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	0.197	9.250	27.0	5.319	249.750	0.021	1.00	100.00
Ammonium sulfate precipitation (20–70%)	0.329	5.730	6.5	2.139	37.245	0.057	2.73	40.21
2', 5'-ADP Sepharose 4B affinity chromatography	0.516	0.027	4.0	2.064	0.108	19.111	910.05	38.80

Table 2
Purification scheme of GR from *C. trutta* liver

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	0.317	14.250	30.5	9.669	434.630	0.022	1.00	100.00
Ammonium sulfate precipitation (30–70%)	0.423	9.730	8.5	3.595	82.705	0.043	1.95	37.18
2', 5'-ADP Sepharose 4B affinity chromatography	0.679	0.042	3.0	2.037	0.126	16.167	734.86	21.07

Table 3
Ki and IC_{50} values obtained from regression analysis graphs for fish gill GR in the presence of different metal ion concentrations

Metal ions	IC_{50}, mM	K_i, mM	Inhibition type
Ag^+	0.000625	0.00045 ± 0.00008	non-competitive
Co^{2+}	0.153	0.128 ± 0.036	competitive
Ni^{2+}	0.220	0.182 ± 0.138	non-competitive
Pb^{2+}	0.247	0.482 ± 0.219	non-competitive
Zn^{2+}	0.216	0.112 ± 0.047	competitive

Table 4
K_i and IC_{50} values obtained from regression analysis graphs for fish liver GR in the presence of different metal ion concentrations

Metal ions	IC_{50}, mM	K_i, mM	Inhibition type
Ag^+	0.000437	0.00025 ± 0.00013	competitive
Co^{2+}	0.217	0.532 ± 0.146	competitive
Cu^{2+}	0.185	0.123 ± 0.066	competitive
Ni^{2+}	0.355	0.093 ± 0.020	competitive
Pb^{2+}	0.349	0.151 ± 0.084	non-competitive

Discussion

In the developing world, heavy metal pollution is a significant environmental problem. Almost all living things are affected negatively by toxic substances, including heavy metals (Raspanti et al., 2009). In general, heavy metals produce their toxicity by forming complexes with organic compounds. For example metal complexes of sulfur, oxygen and nitrogen are the most common groups. If the metals are bound to these groups, they may become inactive enzyme forms because, metals bond with SH groups of the cysteine residues and thus, mercaptans are formed. Enzymes are the bio-catalysts in nature which regulate the rate and direction of biochemical reactions. Inhibition of enzyme activities by toxic compounds such as metal, drugs, pesticides and gases may cause a hazardous situation for living organisms. Therefore, the number of toxicology studies on the effects of metals on enzyme activities have increased in recent years (Alim et al., 2014). Fish as the most important aquatic food source are indicator organisms for heavy metal pollution of their environment and as such they are a potential risk for human consumption (Farkas et al., 2001).

For this reason, we investigated the effects of Ag+, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} on gill and liver GR enzyme activity of the fish species *C. trutta*. GR was purified from *C. trutta* gill and liver by using preparation of hemolysate, ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity chromatography. GR has been purified from many different sources (Calberg and Mannervik, 1981; Le Trang et al., 1983; Akkemik et al., 2011; Taser and Ciftci, 2012; Yadav et al., 2013) using various purification procedures. All reported purification procedures involve several chromatographic steps, such as, DEAE-Sephadex, Sephadex G-100, hydroxyapatite (Calberg and Mannervik, 1981), 2',5'-ADP Sepharose 4B (Madamanchi et al., 1992), Sephadex G-75, CM-Cellulose, Sephacryl S-200 (Calberg et al., 1981), Reactive Red-120-Agarose, Sephacryl S-300 (Garcia-Alfonso et al., 1993), fast protein liquid chromatography (FPLC)-anion Exchange and FPLC-hydrophobic interaction chromatography (Madamanchi et al., 1992).

Figure 1 exhibits the results of SDS-PAGE which was performed for determination of the purity and molecular weight of the enzyme. The molecular mass of *C. trutta* gill and liver GR was nearly 50 and 55 kDa. GRs of different origins have similar molecular masses as follows; rat liver GR is 60 kDa (Calberg and Mannervik, 1975), bovine brain GR is 55 kDa (Gutterer et al., 1999), turtle liver GR is 55 kDa (Willmore and Storey, 2007), rainbow trout liver GR is 53 kDa (Tekman et al., 2008), turkey liver GR is 65 kDa (Taser and Ciftci, 2012).

Recently, many studies have been conducted on the relationship between metals and toxicity. Fresh water and marine fish are affected by metal contamination. It is reported that metal toxicity causes irregular metallothionein protein synthesis, renal damage and disruption of bone structure in humans and wildlife (Sato and Kondoh, 2002; Lavery et al., 2009). Due to the fact that metals cause leakage of phosphates, calcium, glycogen and proteins (proteinuria) from the kidney, renal damage can be fatal in mammals (Lavery et al., 2009). Indeed, some metals are known to be extremely toxic to mammals, fish, and other fauna and flora. For instance, mercury is a toxic element which causes environmental problems. Some metals can be found in the form of the free metal ion such as Cd^{2+} (Hisar et al., 2009). Due to the important abovementioned approaches in this subject, in the present study we investigated the *in vitro* effects of Ag^+, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} on fish gill and liver GR activity. K_i and IC_{50} parameters of these metals were determined (Table 3 and 4).

Metals ions inhibited enzyme activity at low concentrations. K_i constants and IC_{50} values are the most suitable parameters for seeing inhibitory effects. As shown in Table 3, IC_{50} values for fish gill GR were 0.000625, 0.153, 0.220, 0.247 and 0.216 mM and K_i constants for fish gill GR were 0.00045 ± 0.00008, 0.128 ± 0.036, 0.182 ± 0.138, 0.482 ± 0.219 and 0.112 ± 0.047 mM for Ag^+, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, respectively (Fig. 2). As shown in Table 4, IC_{50} values for fish liver GR were 0.000437, 0.217, 0.185, 0.355 and 0.349 mM and K_i constants for fish liver GR were 0.00025 ± 0.00013, 0.532 ± 0.146, 0.123 ± 0.066, 0.093 ± 0.020 and 0.151 ± 0.084 mM for Ag^+, Co^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}, respectively (Fig. 3). It is clear that Ag^+ is the most potent inhibitor for *C. trutta* gill and liver GR enzymes.

Our results agree well with other reports in the literature. For example, Alim et al. (2014) examined the effects of some metal ions (Ag^+, Cu^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} and Co^{2+}) on the carbonic anhydrase activity of Tuna gill. Their results showed that all metal ions inhibited the enzyme and that Ag^+ is the most potent inhibitor of carbonic anhydrase enzyme. In another study, Kaya et al. (2013) examined the effects of Ag^+, Ni^{2+}, Cd^{2+} and Cu^{2+} on the carbonic anhydrase of gilthead sea bream liver. Their result showed that Ag^+ had the highest inhibition rate. The inhibition order of the metals was $Ag^+ > Ni^{2+} > Cd^{2+} > Cu^{2+}$. These results confirm our present study. Additionally, in a different study Soyut et al. (2008) investigated the effects of Ag^+, Cu^{2+}, Zn^{2+}, Cd^{2+} and Co^{2+} on the carbonic anhydrase activity of rainbow trout brain

in vitro. They found that *in vitro* inhibition rank order was determined as $Co^{2+} > Zn^{2+} > Cu^{2+} > Cd^{2+} > Ag^{+}$. In another study, Akkemik et al. (2012) investigated the effects of Ag^{+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, Mn^{2+} and Hg^{2+} on turkey liver glutathione S-transferase activity. *In vitro* studies showed that the enzyme activity was inhibited by Ag^{+}, Cu^{2+} and Hg^{2+}. They found that *in vitro* inhibition rank order was determined as $Cu^{2+} > Hg^{2+} > Ag^{+}$.

Conclusion

Today, metal pollution levels are increasing in the aquatic environment. This is a highly significant risk factor for all living organisms including fish and humans. Fish in fresh water and the sea have been consumed by man as an important food source until now and will continue to be consumed in the future. Fish provide one of the most valuable food sources in terms of protein and omega-3 fatty acid for humans. In this study, we purified GR from *C. trutta* gill and liver for the first time. In addition, inhibitory effects of some metal ions (Ag^{+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}) on enzyme activity were reported. The most effective metal ion is Ag^{+}. Ag^{+} inhibits the enzyme at very low doses. GR enzyme assay may be considered as a biomarker for the identification of pollution in aquatic environments.

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