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

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Introduction

Glutathione reductase (Glutathione: NADPH oxidoreductase, EC 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 regulation of apoptosis (Van den Dobbelsteijn 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 systems 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.

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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⁺, Cu²⁺, Co²⁺, Ni²⁺, Pb²⁺ and Zn²⁺) 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 sulfate precipitation and 2', 5'-ADP Sepharose 4B affinity chromatography. Using this procedure, 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. 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⁺, Cu²⁺, Co²⁺, Ni²⁺, Pb²⁺ and Zn²⁺) on GR from gill and liver were investigated in vitro. Kᵢ constants and IC₅₀ values for metal ions which showed inhibition effects were determined by Lineweaver-Burk graphs and plotting activity % vs. [I]. In conclusion, IC₅₀ values for fish gill GR were 0.000625, 0.153, 0.220, 0.247 and 0.216 mM and Kᵢ constants for fish gill GR were 0.000045 ± 0.000008, 0.128 ± 0.036, 0.182 ± 0.138, 0.482 ± 0.219 and 0.112 ± 0.047 mM for Ag⁺, Cu²⁺, Co²⁺, Ni²⁺, Pb²⁺ and Zn²⁺, respectively. IC₅₀ values for fish liver GR were 0.000437, 0.217, 0.185, 0.355 and 0.349 mM and Kᵢ 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⁺, Cu²⁺, Co²⁺, Ni²⁺, Pb²⁺ and Zn²⁺, respectively. In vitro inhibition rank order was determined as Ag⁺ > Co²⁺ > Zn²⁺ > Ni²⁺ > Pb²⁺ for fish gill GR; Ag⁺ > Cu²⁺ > Co²⁺ > Pb²⁺ > Ni²⁺ 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.

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 regulation of apoptosis (Van den Dobbelsteijn et al., 1996).
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. AgNO₃, CuSO₄·5H₂O, Co(NO₃)₂·6H₂O, NiCl₂·6H₂O, Pb(NO₃)₂, ZnCl₂ 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 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).

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 phosphate buffer (pH 7.0). One enzyme unit was defined as the amount that oxidizes 1 mol NADPH per min under the assay conditions.

Ammonium sulfate fractionation and dialysis. The hemolysate was subjected to precipitation with ammonium sulfate (liver: 30%, gill: 50%) and contained partially purified enzyme. This solution was subjected to precipitation with ammonium sulfate (liver: 30%, gill: 50%) and contained partially purified enzyme. This solution was subjected to precipitation with ammonium sulfate (liver: 30%, gill: 50%). The precipitate was dissolved in phosphate buffer (50 mM, pH 7.0). Its purity and molecular weight of the enzyme were 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.

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.

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 IC₅₀ values were obtained from activity (%) vs. metal ion concentration plots. In order to determine Ki 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 Ki constant were calculated from these graphs. Regression analysis graphs were drawn for IC₅₀ 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. RI values were calculated for both standard proteins and GR; RI-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](Image 334x236 to 511x468)
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.
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 (Raspantii 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 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+, Cu2+, Co2+, Ni2+, Pb2+ and Zn2+ 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, hydroxypatite (Calberg and Mannervik, 1981), 2',5'-ADP Sepharose 4B (Madamanchi et al., 1992), Sepharose 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).

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 Kondo, 2002; 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 Cd2+ (Hisar et al., 2009). Due to the important above-mentioned approaches in this subject, in the present study we investigated the in vitro effects of Ag+, Cu2+, Co2+, Ni2+, Pb2+ and Zn2+ 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 0.0000625, 0.185, 0.532 ± 0.146 and 0.093 ± 0.020 mM for Ag+, Cu2+, Ni2+, Pb2+ and Zn2+ on gill and liver GR activity. K_i values for fish liver GR were 0.0000437, 0.217, 0.532 ± 0.146 and 0.093 ± 0.020 mM for Ag+, Cu2+, Ni2+, Pb2+ and Zn2+ on liver FR activity. These results confirm our present study. Additionally, in a different study Souty et al. (2008) investigated the effects of Ag+, Cu2+, Zn2+, Cd2+ and Co2+ on the carbonic anhydrase activity of rainbow trout brain.
in vitro. They found that in vitro inhibition rank order was determined as Co²⁺ > Zn²⁺ > Cu²⁺ > Cd²⁺ > Ag⁺. In another study, Akkemik et al. (2012) investigated the effects of Ag⁺, Cu²⁺, Zn²⁺, Fe³⁺, Mg²⁺, Ni²⁺, Mn²⁺ and Hg²⁺ on turkey liver glutathione S-transferase activity. In vitro studies showed that the enzyme activity was inhibited by Ag⁺, Cu²⁺ and Hg²⁺. They found that in vitro inhibition rank order was determined as Cu²⁺ > Hg²⁺ > 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⁺, Cu²⁺, Co²⁺, Ni²⁺, Pb²⁺ and Zn²⁺) 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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