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

Muammer KIRICI¹*, Mahinur KIRICI², Muhammed ATAMANALP³

¹Bingöl University, Faculty of Agriculture, Department of Fisheries, Bingöl, TURKEY.
²Bingöl University, Faculty of Arts and Science, Department of Chemistry, Bingöl, TURKEY.
³Atatürk University, Faculty of Fisheries, Department of Aquaculture, Erzurum, TURKEY.

*Corresponding Author Tel.: +90 536 891 71 50
E-mail: muammerkirici@hotmail.com
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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⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺) on fish kidney glutathione reductase were investigated. Kᵢ constants and I₅₀ values for metal ions were determined by Lineweaver-Burk graphs and plotting activity % vs. [I], respectively. I₅₀ values were 0.00078, 0.622, 0.722, 0.073 and 0.519 mM, and Kᵢ 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⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺, 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 Saşlaştırılan Glutatyon Redüktaz Enzim Aktivitesine Bazı Metallerinin vitro İnhibisyon Etkileri

Bu çalışmada, glutatyon redüktaz (EC: 1.8.1.7; GR) içi bahçelardan Capoeta trutta böbrek dokusundan saşştırılmıştır. Saştırma işlemi, homojenat hazırlanması, amonyum sulfat dağıtılması ve 2',5'-ADP Sepharose 4B afinite kromatografisi olmak üzere 3 basamakta gerçekleştirilmiştir. Bu yöntemle, 11.91 EÜ/mg protein spesifik aktiviteye %35.4 verimle 794 kat saştırılmıştır. Saştırma sonucu elde edilen saf enzim saflık kontolü sodyum dodesil sulfat poliakrilamid jel elektroforezi (SDS-PAGE) ile yapıldı ve enzimlerin tek band olduğu görülmuştur. Enzimin molekül kütle yaklaşıkları 55 kDa olarak hesaplanmıştır. Ayrıca, bu böbrek dokusundan saştırılan glutatyon redüktaz enzimi üzerine bazı metallerin (Ag⁺, Co²⁺, Ni²⁺, Cu²⁺ ve Zn²⁺) in vitro inhibisyon etkileri incelenmiştir. Metallerin Kᵢ sabiti ve IC₅₀ değerleri sırasıyla Lineweaver-Burk ve Yüzde (%) Aktivite-[Metal] grafikleri ile hesaplanmıştır. Bu grafiklerden, Ag⁺, Co²⁺, Ni²⁺, Cu²⁺ ve Zn²⁺ metalleri için IC₅₀ değerleri sırasıyla 0.00078, 0.622, 0.722, 0.073 ve 0.519 mM, ve Kᵢ 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.

Anahtar Kelimeler: Glutatyon redüktaz, Capoeta trutta, böbrek, inhibitör, saştırma.
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 defense consists of low molecular weight antioxidant compounds (e.g., glutathione and vitamin sC and E), and the second defense mechanism comprises antioxidant enzymes (Blahova 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 maintenance 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 Tandogan, 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 mMTris–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.

Preparation of the Hemolysate; Fish samples (n= 10; 190 ± 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 (1×10 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.](image)

**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\textsubscript{50} values were obtained from activity (%) vs. metal ion concentration plots (Figure 2) (Tekman et al., 2008).

In order to determine K\textsubscript{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 1mL of total reaction volume. Lineweaver-Burk graphs were drawn by using 1/V vs. 1/[S] values and K\textsubscript{i} constant were calculated from these graphs (Figure 3). Regression analysis graphs were drawn for IC\textsubscript{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\textsubscript{f} values were calculated for standart proteins and GR according to Laemmli’s procedure (1970) from R\textsubscript{f}-LogMW graph. Molecular weights of kidney GR enzymes were 55 kDa.

*n vitro* inhibitory activities of Ag\textsuperscript{+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} as metal ions were evaluated on fish kidney GR enzyme. IC\textsubscript{50} values were found as 0.00078, 0.622, 0.722, 0.073 and 0.519 mM, and K\textsubscript{i} constants were found as 0.000394 ± 0.0002, 0.235 ± 0.027, 0.279 ± 0.048, 0.026 ±
Figure 2. Activity %-[Metal] regression analysis graphs for fish kidney GR in the presence of five different metal concentrations.

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<tr>
<th>Table 1. Purification scheme of GR from <em>C. trutta</em> kidney</th>
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<td><strong>Purification step</strong></td>
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<tr>
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<tr>
<td>Hemolysate</td>
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<td>Ammoniumsulfateprecipitation (40-70%)</td>
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<td>2', 5'-ADP Sepharose 4B affinitychromatography</td>
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<th>Table 2. IC$_{50}$ K$_1$ values and inhibition types for five inhibitors for <em>C. trutta</em> kidney GR</th>
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<td><strong>Metal ions</strong></td>
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</tr>
<tr>
<td>Ag$^{+}$</td>
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<tr>
<td>Co$^{3+}$</td>
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<td>Cu$^{2+}$</td>
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<td>Ni$^{2+}$</td>
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<td>Zn$^{2+}$</td>
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Figure 3. Lineweaver–Burk graph in 5 different substrate concentrations and in 3 different metal (Ag⁺, Co²⁺, Cu²⁺, Ni²⁺ and Zn²⁺) concentrations for determination of $K_v$.

0.008 and 0.382 ± 0.024 mM for Ag⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺, 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 (NH₄)₂SO₄. 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⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺ were chosen to investigate their inhibitory effects on fish liver GR. IC₅₀ values and Kᵢ constants are the most suitable parameters for seeing inhibitory effects. As shown in Table 2, IC₅₀ values were 0.00078, 0.622, 0.722, 0.073 and 0.519 mM, and Kᵢ 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⁺, Co²⁺, Ni²⁺, Cu²⁺ and Zn²⁺, respectively. IC₅₀ values and Kᵢ 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 gill's 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 need 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 revers cause a great danger for the creatures living in watery areas. The required precautions should be taken immediately.

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