Purification of glutathione reductase from some tissues of *Capoeta umbla* and the inhibitory effects of some metal ions on enzyme activity

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**ABSTRACT**

The aim of this study was to determine the *in vitro* inhibitory effects of some metal ions (silver ion (Ag⁺), cadmium ion (Cd²⁺), cobalt ion (Co²⁺), copper ion (Cu²⁺), nickel ion (Ni²⁺), lead ion (Pb²⁺) and zinc ion (Zn²⁺)) on glutathione reductase (GR) enzyme activities that purified from the gill, kidney and liver tissues of *Capoeta umbla*. For this purpose, the enzyme was purified from the gill, kidney and liver of *C. umbla* freshwater fish using ammonium sulfate precipitation and affinity column chromatography methods using 2′,5′-ADP Sepharose 4B. Within this study, the GR enzyme was purified for the first time from the tissues of *C. umbla*. Enzyme purity and molecular weight were determined using the sodium dodecyl sulfate polyacrylamide gel electrophoresis method. In addition, the inhibitory effects of different metal ions (Ag⁺, Cd²⁺, Co²⁺, Cu²⁺, Ni²⁺, Pb²⁺ and Zn²⁺) on GR enzyme activities of the gill, kidney and liver tissue of *C. umbla* were investigated under *in vitro* conditions. The metal ion concentrations inhibiting 50% of enzyme activity (IC₅₀) were obtained by plotting activity percentage versus [I] figures. Finally, the dissociation constants of the enzyme inhibitor complex (Kᵢ), and the inhibition types, were calculated from Lineweaver–Burk plots. *In vitro* inhibition rank order was determined as Ag⁺>Co²⁺>Pb²⁺>Zn²⁺>Cu²⁺ for *C. umbla* gill GR; Ag⁺>Pb²⁺>Co²⁺>Ni²⁺>Zn²⁺ for *C. umbla* liver GR; Ag⁺>Cu²⁺>Co²⁺>Pb²⁺>Ni²⁺ for *C. umbla* kidney GR. From these results, we showed that Ag⁺ metal ion is the most potent inhibitor of GR enzyme on gill, liver and kidney tissues. Our results also demonstrate that these metals might be dangerous at low micromolar concentrations for *C. umbla* GR enzyme.

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Introduction

Glutathione reductase (GR) catalyzes the conversion of oxidized glutathione (GSHG) to its reduced form (GSH) and allows the ratio of GSH/GSSG to stay at a certain level (Kuzu et al., 2016). Glutathione (γ-L-glutamyl-L-cysteinylglycine: GSH) is the most important source of nucleophilic thiol equivalents found in cells. Also, the function of some proteins depend on the stability of thiol: disulfide exchange reactions. GSH / GSSG ratios are very important for cell survival, so, it is absolutely necessary to regulate the pentose phosphate pathway system (Akkemik et al., 2011). In the cell, while an increase in GSSG concentration inhibits many important enzyme systems, a decrease in GSH causes oxidative damage and pathological problems like cancer, apoptosis, aging, AIDS, diabetes, Alzheimer’s and Parkinson’s disease (Thaikovskaya et al., 2005; Fraternale et al., 2009; Simic et al., 2009; Gironi et al., 2011; Raza, 2011; Kirici et al., 2017a).

In this century, our earth is polluted, particularly by human activities like scrap metal disposal and agricultural practices. Therefore, living organisms, especially fish, are intensively exposed to metals in the environment. The toxic effects of metals on aquatic organisms have been investigated by many researchers. In addition, many researchers are attempting to make sense of how metals affect enzymes and the mechanisms at work (Ceyhun et al., 2011; Loro et al., 2012; Qu et al., 2014; Kirici et al., 2016a, b; Kucuk & Gulcin, 2016; Kirici et al., 2017b; Kirici et al., 2017a). Until now, no studies have been found in published literature on the purification of GR from Capoeta umbla gills, liver and kidney tissue, and the effects of Ag⁺, Cd²⁺, Ni²⁺, Pb²⁺ and Zn²⁺ metal ions on GR activity of C. umbla. The purpose of this study was to contribute to the understanding of any possible effects of metal ions on purified C. umbla tissues GR in vitro.

Material and Methods

**Materials**

Chemicals for electrophoresis, 2′,5′-ADP Sepharose-4B, NADPH, protein assay reagents and GSSG were obtained from Sigma-Aldrich. Silver nitrate (AgNO₃), copper sulfate pentahydrate (CuSO₄.5H₂O), cobalt nitrate hexahydrate (Co(NO₃)₂.6H₂O), nickel chloride hexahydrate (NiCl₂.6H₂O), lead nitrate (Pb(NO₃)₂), zinc chloride (ZnCl₂), cadmium sulfate hydrate (3CdSO₄.8H₂O) and all other chemicals used were of analytical grade and obtained from Merck.

**Preparation of the Homogenates**

Fish samples (n = 10; 190 ± 20 g) were caught from the Murat River (Bingöl, Turkey). The fish were dissected in the abdominal region and their kidney and liver tissues were removed. Then the head region was cut off and their gill tissues were removed. Eight grams of gill, kidney and liver tissue samples were washed three times with 0.9% NaCl solution. Then, with the aid of a scalpel, the tissue samples were cut into small pieces. These pieces were homogenized with the aid of liquid nitrogen and suspended in a 50 mM KH₂PO₄ buffer (at a pH of 7.4) that included 1 mM of phenylmethylsulfonyl fluoride (PMSF), 1 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of 1,4-dithiothreitol (DTT). The suspension was centrifuged (Hettich Universal 320, Tuttlingen, Germany) at 13500 rpm for 2 hours at 4°C, and then the precipitate was thrown away. The supernatant was stored at 4°C (Le Trang et al., 1983).

**GR Activity Assay**

Enzyme activity was measured spectrophotometrically with a Beckman Coulter DU730 UV/Vis Spectrophotometer (Beckman Coulter Inc., California, America), at 25°C using the modified method of Carlberg and Mannervik (Carlberg & Mannervik, 1975). The assay system used to measure the enzyme activity comprised 50 mM of Tris–HCl buffer (at a pH of 8.0), containing 0.1 mM of NADPH, 1 mM of EDTA and 1 mM of GSSG.

**Ammonium Sulfate Fractionation and Dialysis**

The precipitation of ammonium sulfate was carried out according to our previous studies (Kirici et al., 2016a, 2016b, 2017b). The precipitation range of this enzyme was determined to be 30 to 80%, 40 to 80% and 40 to 80% for gill, kidney and liver tissue, respectively, in this study. The precipitate was dissolved in 50 mM of phosphate buffer (pH 7.0). This dissolved precipitate was dialyzed in 1 mM of EDTA and 10 mM of K-phosphate buffer containing 5 mM of β-mercaptoethanol (pH 7.5) for 2 hours with two changes of buffer at 4°C.

**2′, 5′-ADP Sepharose 4B Affinity Chromatography**

The 2′,5′-ADP Sepharose 4B affinity column (1×10 cm) was prepared according to our previous studies (Kiricci et al., 2015, 2016b, 2017a). The column material was balanced with 50 mM of K-phosphate buffer including 1 mM of EDTA and 1 mM of DTT (pH 6.0) with a peristaltic pump. The flow rate was adjusted to 50 ml/h. The previously obtained enzyme sample
was loaded onto the affinity column. Afterwards, the chromatography column was washed with equilibration buffer (50 mL of 50 mM of K-phosphate buffer including 1 mM of EDTA and 1 mM of DTT, at a pH of 6.0) and washing buffers (25 mL of 0.1 M K-phosphate + 0.1 M of K-acetate at a pH of 6.0 and 25 mL of 0.1 M K-phosphate + 0.1 M of K-acetate at a pH of 7.85). The enzyme was eluted with 1 mM of GSH + 0.5 mM of NADPH + 1 mM of EDTA in 50 mM of K-phosphate, at a pH of 7.5. One milliliter samples of the eluates were placed into Eppendorf tubes and the activity was individually measured. Active fractions were collected. All the procedures were performed at 4°C (Le Trang et al., 1983).

**Protein Determination**

Protein concentration was determined at a wavelength of 595 nm using the Bradford method (Bradford, 1976). Bovine serum albumin was used as the standard during this process.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was carried out using the Laemmli method to check the purity of the enzyme (Laemmli, 1970). Ten milliliters of the sample were placed on each electrophoresis gel stick. The acrylamide concentration of the stacking and the separating gels were 3% and 8% containing 1% SDS, respectively. The gel image obtained after electrophoresis was photographed (Figure 1).

**In vitro Effects of Metal Ions**

The inhibitory effects of Ag⁺, Cd²⁺, Co²⁺, Cu²⁺, Ni²⁺, Pb²⁺ and Zn²⁺ were investigated at five different concentrations for the fish gill, kidney and liver GR enzyme activities. The control cuvette with 100% enzyme activity did not contain any metal ions. GR activity was measured in the presence of different metal ion concentrations. The IC₅₀ values were obtained from activity percentage versus metal ion concentration plots (Figure 2).

GSSG was used as a substrate for the analysis of the GR enzyme activities. In this study, substrate concentrations were determined to be 0.3, 0.8, 1.4, 2 and 3 mM. Metal ions were added to the reaction medium to provide inhibitors at three different constant concentrations. Lineweaver–Burk (Lineweaver and Burk, 1934) plots for fish gill, kidney and liver GR enzyme activities were drawn by using three different metal ion concentrations and five different substrate (GSSG) concentrations. After this, the Ki values and inhibition types were calculated using the Lineweaver–Burk curves (Figure 2).

**Results**

In this research, GR enzyme was first purified from C. umbla gill, kidney and liver tissue. The purification procedure consisted of three steps: preparation of the homogenate, ammonium sulfate precipitation and finally, affinity gel chromatography. The purity of the enzymes were determined by SDS-PAGE and showed single bands on the gel (Figure 1). Rf values were calculated for standard proteins and GR according to Laemmli’s procedure from Rf-LogMW plots. Molecular weights for the gill, kidney and liver GR enzymes were 50, 53 and 55 kDa, respectively.

![Figure 1. SDS-PAGE photograph SDS-PAGE analysis of purified liver G6PD. Lane 1: molecular-mass markers (kDa): Escherichia coli β-galactosidase (116), rabbit phosphorylase B (97.4), bovine serum albumin (66), chicken ovalbumin (45) and bovine carbonic anhydrase (29) (Sigma: MW-SDS-200) (Lane 1: Gill; Lane 2, 5: Standard proteins; Lane 3: Kidney; Lane 4: Liver).](image-url)
Table 1. Purification scheme of GR enzyme from gill, kidney and liver tissues of *C. umbla*

| Tissue        | Purification step                                      | Activity (U/mL) | Protein (mg/mL) | Total volume (ml) | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purification factor | Yield (%) |
|---------------|--------------------------------------------------------|-----------------|-----------------|-------------------|-------------------|-------------------|-------------------------|---------------------|-----------|
| Gill          | Hemolysate                                             | 0.193           | 16.64           | 29.5              | 5.694             | 490.9             | 0.012                   | 1                   | 100       |
|               | Ammonium sulfate precipitation                         | 0.328           | 4.27            | 9.6               | 3.149             | 40.99             | 0.077                   | 6.621               | 55.30     |
|               | 2', 5'-ADP Sepharose 4B affinity chromatography        | 0.555           | 0.041           | 5                 | 2.775             | 0.205             | 13.54                   | 1167                | 48.74     |
| Kidney        | Hemolysate                                             | 0.276           | 7.28            | 25                | 6.9               | 182               | 0.038                   | 6.621               | 55.30     |
|               | Ammonium sulfate precipitation                         | 0.334           | 1.06            | 6.5               | 2.171             | 6.89              | 0.315                   | 8.31                | 31.46     |
|               | 2', 5'-ADP Sepharose 4B affinity chromatography        | 0.531           | 0.036           | 3                 | 1.593             | 0.108             | 14.75                   | 389.2               | 23.09     |
| Liver         | Hemolysate                                             | 0.463           | 43.81           | 31                | 14.35             | 1358              | 0.011                   | 1                   | 100       |
|               | Ammonium sulfate precipitation                         | 0.609           | 33.46           | 11.3              | 6.882             | 378.1             | 0.018                   | 1.72                | 47.95     |
|               | 2', 5'-ADP Sepharose 4B affinity chromatography        | 0.825           | 0.049           | 3                 | 2.475             | 0.147             | 16.84                   | 925.1               | 17.24     |

Figure 2. Activity (%) ±[Metal] regression analysis graphs for some fish tissues GR in the presence of five different metal concentrations.

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**GILL**

- Activity (%) ±[Ag] (mM)
  - $y = 100e^{-71.62x}$
  - $R^2 = 0.9799$

- Activity (%) ±[Pb] (mM)
  - $y = 100e^{-0.702x}$
  - $R^2 = 0.9837$

- Activity (%) ±[Ni] (mM)
  - $y = 100e^{-8.345x}$
  - $R^2 = 0.9811$

**KIDNEY**

- Activity (%) ±[Ag] (mM)
  - $y = 100e^{-71.62x}$
  - $R^2 = 0.9799$

- Activity (%) ±[Pb] (mM)
  - $y = 100e^{-0.702x}$
  - $R^2 = 0.9837$

- Activity (%) ±[Ni] (mM)
  - $y = 100e^{-8.345x}$
  - $R^2 = 0.9811$

**LIVER**

- Activity (%) ±[Ag] (mM)
  - $y = 100e^{-71.62x}$
  - $R^2 = 0.9799$

- Activity (%) ±[Pb] (mM)
  - $y = 100e^{-0.702x}$
  - $R^2 = 0.9837$

- Activity (%) ±[Ni] (mM)
  - $y = 100e^{-8.345x}$
  - $R^2 = 0.9811$
The sequence of the inhibitors was Ag⁺ > Co²⁺ > Pb²⁺ > Zn²⁺ > Cu²⁺ (Table 2 and Figure 2). While Cu²⁺ and Pb²⁺ showed competitive inhibition, Ag⁺, Co²⁺ and Zn²⁺ inhibited fish gill GR in a non-competitive manner.

Table 2. IC₅₀ values, Ki constants and inhibition types of some metal ions GR obtained from C. umbla gill

| Metal ions | IC₅₀ (mM) | Ki (mM) | Inhibition type |
|------------|-----------|---------|-----------------|
| Ag⁺        | 0.0097    | 0.006 ± 0.001 | Non-competitive |
| Co²⁺       | 0.844     | 0.921 ± 0.196 | Non-competitive |
| Cu²⁺       | 11.18     | 4.493 ± 0.806 | Competitive   |
| Pb²⁺       | 0.944     | 0.533 ± 0.377 | Competitive   |
| Zn²⁺       | 1.313     | 1.850 ± 1.034 | Non-competitive |

C. umbla kidney GR enzyme was purified by using 2',5'-ADP Sepharose 4B affinity chromatography and was found to have a specific activity of 14.75 U/mg proteins, a 389.2-fold purity improvement, and a yield of 23.1% (Table 1). Afterwards, the in vitro inhibitory activities of Ag⁺, Cd²⁺, Co²⁺, Pb²⁺ and Zn²⁺ metal ions were evaluated for fish kidney GR enzyme. IC₅₀ values were found to be 0.00087, 0.559, 0.569, 0.083 and 0.487 mM for Ag⁺, Cd²⁺, Co²⁺, Pb²⁺ and Zn²⁺, respectively, and their Ki constants were 0.0009 ± 0.0005, 0.824 ± 0.124, 1.203 ± 0.210, 0.043 ± 0.017 and 0.124 ± 0.018 mM, respectively. From the results obtained, the sequence of the inhibitors was Ag⁺ > Pb²⁺ > Cd²⁺ > Co²⁺ > Ni²⁺ > Zn²⁺ (Table 3 and Figure 2). Ag⁺ and Pb²⁺ showed competitive inhibition, while Ni²⁺, Co²⁺ and Zn²⁺ inhibited fish kidney GR in a non-competitive inhibition manner.

Table 3. IC₅₀ values, Ki constants and inhibition types of some metal ions GR obtained from C. umbla kidney

| Metal ions | IC₅₀ (mM) | Ki (mM) | Inhibition type |
|------------|-----------|---------|-----------------|
| Ag⁺        | 0.00087   | 0.0009 ± 0.0005 | Non-competitive |
| Cd²⁺       | 0.559     | 0.824 ± 0.124  | Non-competitive |
| Co²⁺       | 0.569     | 1.203 ± 0.210  | Non-competitive |
| Pb²⁺       | 0.083     | 0.043 ± 0.017  | Competitive   |
| Zn²⁺       | 0.487     | 0.124 ± 0.018  | Competitive   |

There are many chemicals that have harmful or beneficial effects on the metabolic reactions that occur in the living body, especially enzymes. The toxicological effects of metals are known as usually enzyme denaturation and inhibition (Ekinci & Beydemir, 2010). There are some enzymes that play a crucial role in metabolic pathways. Some chemicals, especially metals, cause some metabolic diseases by increasing or decreasing the activity of these enzymes, such as Alzheimer's, diabetes and Parkinson's disease (Tchaikovskaya et al., 2005; Gironi et al., 2011; Raza, 2011). Usually, it inhibits the enzyme by binding to the metal and protein. Inhibition of enzymes can be deadly for the metabolism of all living organisms, especially fish (Innocenti et al., 2010).

The harmful effects of metals on the living body are prevented by enzymatic and non-enzymatic antioxidant defense systems. The enzymatic antioxidant defense system consists of many enzymes such as glutathione reductase (GR), superoxide dismutase, glutathione peroxidase, catalase and glutathione s-transferase. On the other hand, the non-enzymatic antioxidant defense system consists of different agents such as vitamins, transferrin, lactoferrin, taurine and glutathione. In particular, one of the most important protective systems in cells is glutathione metabolism (Knapen et al., 1999). GR is especially required for the protection of reduced cellular glutathione, which is highly nucleophilic for many reactive electrophiles. The GR enzyme protects many vital functions such as detoxification of free radicals and reactive oxygen species in the cell (such as H₂O₂, O₂• and •OH), by maintaining a high ratio of GSH/GSSG (Schirmer et al., 1989).

GR has been purified from many different animal, plant and microorganism sources using various procedures: techniques like affinity chromatography, size-exclusive chromatography,
activity in the sequence Ag⁺ in inhibition of liver GR enzymes of demonstrated the in vitro rainbow trout by metal ions (Al³⁺, Cd²⁺, Cu²⁺, Hg²⁺, Fe³⁺ and Pb²⁺). It was reported that Al³⁺, Cd²⁺, Cu²⁺, Hg²⁺, Fe³⁺ and Pb²⁺ inhibited the fish gill glutathione s-transferase enzyme. They found that Cd²⁺, Cu²⁺, Zn²⁺ and Ag⁺ inhibit the Chalcalburnus tarichi fish gill glutathione s-transferase enzyme. In vitro results showed that metal ions inhibit fish gill glutathione s-transferase activity in the sequence Ag⁺ > Cu²⁺ > Cd²⁺ > Zn²⁺.

**Conclusion**

In this study, GR enzyme was purified from C. umbla gill, kidney and liver tissue by preparing the homogenate and using ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity column chromatography methods. Enzyme purity and molecular weight were determined with SDS-PAGE. The inhibitory effects of metal ions (Ag⁺, Cd²⁺, Co²⁺, Cu²⁺, Ni²⁺, Pb²⁺) on GR activity were investigated. Kᵢ constants, IC₅₀ values and inhibition types for Ag⁺, Cd²⁺, Co²⁺, Cu²⁺, Ni²⁺, Pb²⁺ and Zn²⁺ were determined by plotting activity percentage versus [I] and Lineweaver–Burk plots.

**Compliance with Ethical Standards**

**Authors’ Contributions**

MK and MK performed the research, analyzed the data and helped to draft the manuscript; MK, MA and SB conceived and designed the work and wrote the manuscript. All authors contributed to and approved the final manuscript.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Ethical Approval**

For this type of study, formal consent is not required.

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