



## Investigation of the Inhibition Effects of Some Metal Ions on Glutathione Reductase Enzyme From Japanese Quail (*Coturnix coturnix japonica*) Liver

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**Abstract.** In the present study, the inhibition effect of some heavy metal ions on glutathione reductase (GR) enzyme which has important functions in metabolism, was investigated. For this purpose, GR enzyme was purified with 19.33 EU/mg specific activity, a yield of 14.06% and 128.8-fold from quail liver by using ammonium sulphate precipitation and 2', 5'-ADP Sepharose 4B affinity chromatography. To check the purity of GR enzyme, sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) was performed and single band was observed. The activity of the GR enzyme was measured spectrophotometrically at 340 nm according to the method of Carlberg and Mannervik. Then, the inhibitory effects of different heavy metal ions (Co<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup>, Fe<sup>2+</sup>, Cd<sup>2+</sup>, Al<sup>3+</sup> and Fe<sup>3+</sup>) on the activity of GR enzyme were examined under in vitro conditions. For the GR enzyme from quail liver tissue, heavy metal concentrations (IC<sub>50</sub>) were obtained in which 50% of GR enzyme activity was inhibited in vitro conditions. Finally, K<sub>i</sub> values for these metal ions were calculated from the Lineweaver-Burk plots.

**Keywords:** Glutathion Reductase, Enzyme, Purification, Inhibition, Quail, Liver.

## Bazı Metal İyonlarının Japon Bildırıcın (*Coturnix coturnix japonica*) Karaciğer Glutatyon Redüktaz Enzimi Aktivitesi Üzerine İnhibisyon Etkilerinin Araştırılması

**Özet.** Bu çalışmada, bazı ağır metal iyonlarının metabolizmada önemli fonksiyonlara sahip olan glutatyon redüktaz (GR) enziminin aktivitesi üzerine inhibisyon etkileri araştırılmıştır. Bu amaçla, GR enzimi bildırıcın karaciğerinden amonyum sülfat çöktürmesi ve 2', 5'-ADP Sepharose 4B afinite kromatografisi kullanılarak 19.33 EU/mg spesifik aktivite ile % 14.06 verimle 128.8 kat saflaştırılmıştır. GR enziminin saflığını kontrolü için, sodyum dodesil sülfat poliakrilamid jel elektroforezi (SDS-PAGE) yapıldı ve jelde tek bant gözlemlendi. GR enziminin aktivitesi, Carlberg ve Mannervik yöntemine göre 340 nm'de spektrofotometrik olarak ölçüldü. Daha sonra, GR enziminin aktivitesi üzerine farklı ağır metal iyonlarının (Co<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup>, Fe<sup>2+</sup>, Cd<sup>2+</sup>, Al<sup>3+</sup> ve Fe<sup>3+</sup>) inhibisyon etkileri in vitro koşullarda araştırıldı. Bildırıcın karaciğer dokusundan saflaştırılan GR enzim aktivitesinin % 50'sinin in vitro koşullarda inhibe edildiği ağır metal konsantrasyonları (IC<sub>50</sub>) değerleri belirlendi. Son olarak, bu metal iyonları için K<sub>i</sub> değerleri Lineweaver-Burk grafiğinden hesaplandı.

**Anahtar Kelimeler:** Glutatyon Redüktaz, Enzim, Saflaştırma, İnhibisyon, Bildırıcın, Karaciğer.

## 1. INTRODUCTION

Glutathione reductase GR (NADPH: GSSG oxidoreductase, EC 1.8.1.7) belongs to pyridine nucleotide disulfide oxidoreductase family of flavoenzymes,[1] that includes thioredoxin reductase and lipoamide dehydrogenase [2]. The main task of the GR enzyme is to catalyze the reduction reaction of the glutathione in the presence of NADPH.



GR plays an important role in redox homeostasis,[3] and protecting protein oxidation in human cells [4-6]. Reduced glutathione (GSH) is fundamental compound for cell survival, it is also necessary for elimination of reactive oxygen species (ROS), heavy metals and xenobiotics [7]. GSH also participates in the metabolic and biochemical reactions such as amino acid transport, prostaglandin synthesis, protein synthesis, DNA synthesis and repair, and enzyme activation [8]. It has also been found that the activity of enzymes purified from different sources is related to the toxicity of various chemicals and metals [9-15].

In blood, lead inhibits the  $\delta$ -aminolevulinic acid dehydratase ( $\delta$ -ALAD) causing accumulation of  $\delta$ -aminolevulinic acid ( $\delta$ -ALA) which produces its oxidant product, 4,5-dioxovaleric acid that disturbs the GSH/GSSG balance by binding to the thiol group –SH in both reduced glutathione GSH and oxidized glutathione GSSG. Reduction in cell glutathione level causes to rise the number of Reactive Oxygen Species (ROS) and that will be addition burden on enzymatic antioxidant defense system which include GR, CAT, SOD and GPx enzymes [16].

Iron and cobalt ions with two contiguous oxidation states work as a prooxidant when it is present in excess,[17] they convert endogenous hydrogen peroxide through Fenton reaction into hydroxyl and hydroperoxyl radicals which are members of ROS.[18] Cadmium inhibit GR by binding to sulfhydryl groups of proteins and

causes glutathione depletion [19]. Also zinc is involved in antioxidant defence system as a co-factor of superoxide dismutase (SOD) as well as more than 300 mammalian protein and the deficiency of zinc has the passive effect on DNA repairing processes [20]. In addition to the role of GR as anti-oxidant enzyme through controlling the ratio of GSH /GSSG it's consider a target enzyme for anticancer and antimalarial drug development [9]; so understanding the kinetic mechanism of inhibition by metal ion may add something in this direction.

Japanese Quail; This is a type of quail that is full of flesh and sound, which is higher than others. These quails, which are more popular among quail producers, are more fleshy and partly similar to chicken. Selection of Japanese quails is also rare. Despite the lack of success in selection, a variety of breeds were produced, such as british range, manchurian golden, english white and tuxedo.

The GR enzyme has been purified from different sources (These sources include pig erythrocytes, bovine erythrocytes, rat liver, cattle liver, sheep brain, sheep liver like mammalian tissues, rainbow trout and turkey liver tissue, fungi, cyanobacteria microorganisms, wheat, corn, peas and spinach such as plant resources) and effects of metal ions, many drugs, and chemicals on enzyme activities have been investigated so far [21-38].

Our work consists of two steps. In the first step of this work is to purify the glutathione reductase enzyme from the quail liver tissue, which regulates intracellular glutathione metabolism, protects the cell from damage to the oxime molecules, and plays an important role in amino acid transport in DNA and protein synthesis. In the second step, we investigated the effect of some heavy metals on the activity of the purified enzyme.

## 2. MATERIALS and METHODS

### 2.1. Chemicals

NADPH, GSSG, GSH and standard protein, used in electrophoresis studies, were provide from Sigma Chem. Co. The others were provided from

either Sigma-Aldrich (St. Louis, MO, USA) or Merck (Kenilworth, NJ, USA).

## 2.2. Preparation of Homogenate

The quails were obtained from the farm of Bingol University Faculty of Agriculture. Fresh quail's liver were washed in isotonic (0,16 M KCl) saline containing 1mM EDTA, and 5 g was first cut into small pieces, then suspended with 15 mL, of 50 mM potassium phosphate buffer (pH: 7.0) containing 1 mM EDTA, 1 mM PMSF, and 1 mM DTT by homogenizer. The precipitate was removed after centrifugation at 15 000 rpm and 4 °C for one hour. The supernatant was brought to 20-60% saturation with solid ammonium sulfate (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the precipitate gathered by centrifugation at 13500 rpm and 4 °C for 15 min. Precipitate was dissolved in potassium phosphate buffer pH 7.5 including 1 mM EDTA, and then dialyzed at 4 °C in the same buffer [39].

## 2.3. 2',5'-ADP Sepharose-4B Affinity Chromatography

2 g dry, 2', '5' ADP sepharose affinity gel was weighed for 10 mL column size. The affinity gel was washed three times with 400 mL of deionized water, to eliminate air and foreign bodies, and suspended in 0.1M K-acetate/0.1M K-phosphate buffer solution (pH 6.0) and packed in the column. After the packaging, the column was equilibrated with 50 mM K-phosphate buffer solution containing 1 mM EDTA (pH 6.0). The column flow rates were adjusted to 20 mL/h during washing and equilibration processes. The dialyzed sample was applied to the 2', 5'-ADP Sepharose-4B affinity column and washed successively with 25 mL 0.1 M K-acetate + 0.1 M K-phosphate (pH 6.0), and 25 mL 0.1 M K-acetate + 0.1M K-phosphate, pH 7.85. The washing process was maintained with 50 mM K-phosphate + 1mM EDTA buffer solution, pH 7.0 until the absorbance difference was 0.05 at 280 nm. 1 mM GSH and 0.5 mM NADPH in 50 mM K-phosphate + 1 mM EDTA (pH 7.0) buffer solution was used the enzyme elution. Active fractions were combined and dialyzed with 50 mM K-

phosphate + 1 mM EDTA (pH 6.0) buffer solution. All process were carry out at + 4 °C [40-42].

## 2.4. Activity Determination

The GR enzyme activity was measured spectrophotometrically (Shimadzu Spectrophotometer UV-1800 at 25 °C) according to the method of Carlberg and Mannervik [43].

## 2.5. Protein Determination

Quantitative proteins amount in homogenate and eluates was determined spectrophotometrically according to the Bradford method, bovine serum albumin was used as a standard [44].

## 2.6. SDS-PAGE

The SDS PAGE method was used for the control of enzyme purity, recommended by Laemmli [45].

## 2.7. In vitro evaluation the effect of metal ions

The metal ions were added to the reaction medium at different concentrations (0.01–3 mM), to examine the effect of the metal ions on the GR enzyme activity. Primarily metal ion concentrations versus % activity graphs were prepared to determine metal concentrations inhibiting 50% of enzyme activity. Then Lineweaver-Burk plots were prepared using 3 different inhibitor concentrations to determine K<sub>i</sub> constants and inhibition type [46].

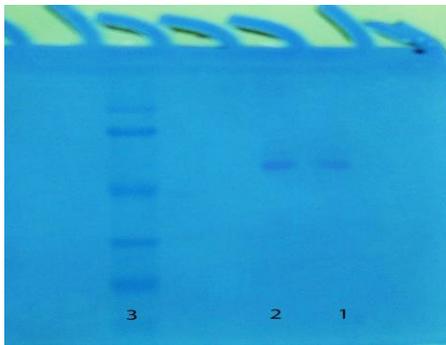
## 3. RESULTS AND DISCUSSION

In this study, GR enzyme from quail (*Coturnix coturnix japonica*) liver was purified from quail liver and investigated the inhibition effect of different heavy metal ion on enzyme activity. The enzyme purification processes consist of three steps; preparation of the homogenate, ammonium sulfate precipitation and 2', 5'-ADP Sepharose-4B affinity chromatography. As a result the enzyme was purified with 19.33 EU/mg specific activity with a yield of 10.25% and 128.8 purification fold (Table 1).

**Table 1.** Purification steps of GR enzyme from quail liver tissue.

Purification steps	Total volume (mL)	Activity EU/mL	Protein (mg/mL)	Total activity (EU)	Total protein (mg)	Specific activity (EU/mg)	Yield (%)	Purification fold
Homogenate	12	1.105	7.1	13.26	85.2	0.15	100	1
2',5'-ADP Sepharose 4B-Affinity chromatography	3.5	0.386	0.02	1.35	0.07	19.33	10.25	128.8

After the purification procedures were completed, the enzyme purity was checked according to the SDS-PAGE method (Figure 1). The molecular weight of the subunits of the GR enzyme purified from the quail liver was estimated to be about 59 kDa, using the SDS-PAGE method.

**Figure 1.** SDS-polyacrylamide gel electrophoresis of purified GR. Lane 1 and 2: affinity chromatography; 3: standard protein ( $\beta$ -galactosidase from E-coli 120 kDa, Bovine serum albumin 85 kDa, chicken ovalbumin 50 kDa, Bovine carbonic anhydrase 35 kDa, bovine milk 25 kDa).

$\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Al}^{3+}$ , and  $\text{Fe}^{3+}$  heavy metals were selected to determine the inhibitory effect on quail GR activity.  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Al}^{3+}$ , and  $\text{Fe}^{3+}$  inhibited the activity of the enzyme.  $\text{IC}_{50}$  values for these inhibitors ranged from 0.021 to 2.47 mM, and the  $\text{K}_i$  constants ranged from 0.014 to 1.605.  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Al}^{3+}$  competitively inhibit the enzyme activity while others cause non-competitive inhibition [47-49].

**Table 2.**  $\text{K}_i$  constants and  $\text{IC}_{50}$  values obtained from regression analysis graphs for GR in the Presence of different metal ions.

Inhibitors	$\text{IC}_{50}$ (mM)	$\text{K}_i$ (mM)	Type of inhibition
$\text{Fe}^{2+}$	3.66	$1.52 \pm 0.552$	non competitive
$\text{Co}^{2+}$	0.58	$0.57 \pm 0.416$	non competitive
$\text{Cd}^{2+}$	0.07	$0.0806 \pm 0.02$	non competitive
$\text{Fe}^{3+}$	2.26	$0.417 \pm 0.05$	non competitive
$\text{Pb}^{2+}$	0.39	$0.105 \pm 0.024$	competitive
$\text{Zn}^{2+}$	0.02	$0.014 \pm 0.002$	competitive
$\text{Al}^{3+}$	2.47	$1.605 \pm 0.899$	competitive

Intracellular to maintain the normal function of cellular and redox homeostasis has two antioxidant defense system against oxidative stress to neutralize ROS, enzymatic and nonenzymatic defense system [47]. Enzymatic antioxidants, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR) [48]

nonenzymatic antioxidant, involving ascorbic acid (Vitamin C), reduced glutathione (GSH), flavones, and anthocyanins, vitamin A,  $\beta$ -Carotene and Vitamin E [49]. Reduced glutathione is required for all antioxidant systems to work effectively.

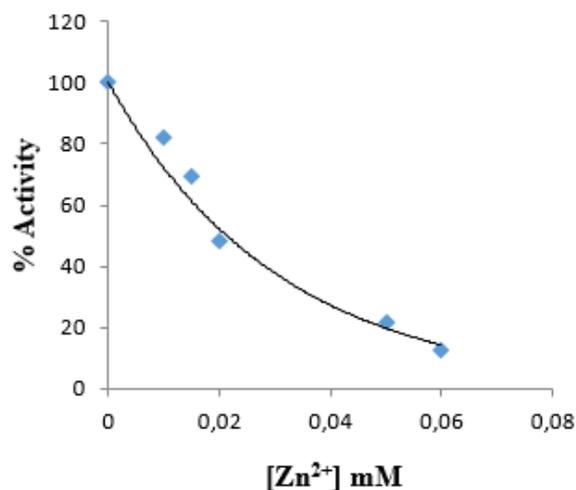
Heavy metals are not eliminated in nature, cause bioaccumulation and create a toxic effect on the organism. A previous study reported that heavy metals such as mercury and cadmium show synergistic toxic effects with salinity [50-52].

According to the results given in Table 2,  $Zn^{2+}$  is the heavy metal ion that most strongly inhibits GR enzyme activity ( $IC_{50}$  values 0.02). It is also seen that other metal ions inhibit enzyme activity even at low concentrations. When inhibition types are analyzed,  $Pb^{2+}$ ,  $Zn^{2+}$ , and  $Al^{3+}$  are competitive inhibitors and other heavy metals show non-competitive inhibition. In vivo and in vitro effects of heavy metals on different enzyme activities have been investigated in previous studies. Similar results were obtained with studies on enzymes such as human erythrocyte GR, human carbonic anhydrase isozymes (CA-I and CA-II), sparus aurata fish; liver GR, blood catalase, liver catalase and glutathione peroxidase [50-54]. In contrast to our result Tandogan and Ulusu were found that zinc ion has the same effect as inhibitor to yeast GR at concentration up to (0.1–2mM) and work as activator above this concentration and they found that zinc ion works as a noncompetitive inhibitor [45]. Like  $Zn^{2+}$ ,  $Pb^{2+}$  and  $Al^{3+}$  ions behave as a competitive inhibitor, reduce GR activity by 76.04%, 72.31% at concentration of 0.8 mM, 4 mM respectively. While  $Fe^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ , and  $Fe^{3+}$  ions work as noncompetitive inhibitors, reduce GR activity by 76.967%, 76.237%, 82.56%, 73.24% at concentration of 6, 0.14, 1.5, 0.415 mM respectively.

In a recent study, the inhibitor effects of  $Ag^+$ ,  $Cd^{2+}$ ,  $Fe^{3+}$ ,  $Cu^{2+}$  and  $Zn^{2+}$  ions on mitochondrial thioredoxin reductase enzyme which is purified

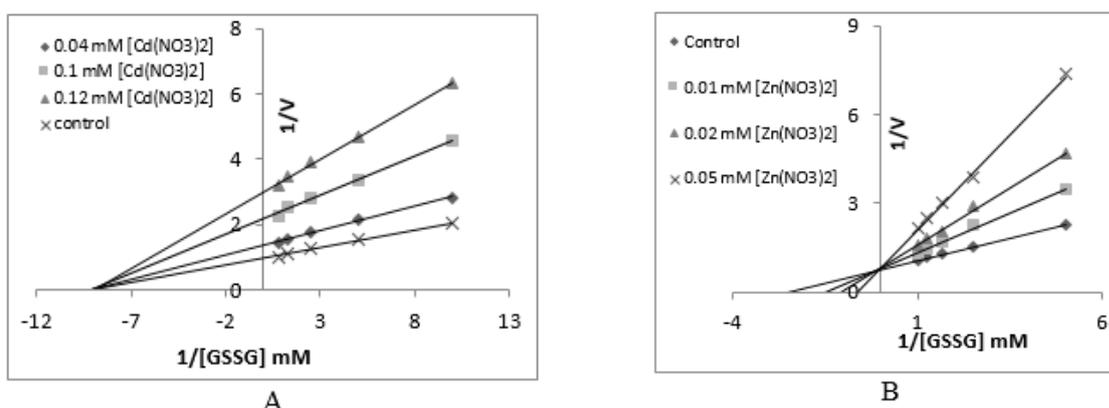
from turkey liver and  $Ag^+$ ,  $Cd^{2+}$ ,  $Pb^{2+}$ , and  $Zn^{2+}$  ions on rat erythrocytes glucose-6-phosphate dehydrogenase enzyme were addressed [10,11]. Furthermore, it was reported in a different study that  $Ag^+$ ,  $Cd^{2+}$ ,  $Cr^{2+}$ , and  $Mg^{2+}$  ions had a noncompetitive inhibitory effect on glutathione S-transferase enzyme which is purified from rainbow trout [55]. In a recent study, the GR enzyme was purified and characterized from the erythrocyte of the quail [56]. Kırıcı et al. determined that  $Ag^+$ ,  $Cu^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Pb^{2+}$  and  $Zn^{2+}$  ions inhibited the gills and liver of *Capoeta trutta* [57].

Young and Cnn have found that the wheat-germ GR is so sensitive to heavy metals. They found that zinc, lead, and cadmium ions reduced the activity of enzyme 50% or bit more at a concentration of 0.01 mM while the iron, aluminum and cobalt ions need to 1mM of concentration to inhibit the enzyme 50% of activity [58].



**Figure 2.** % Activity vs  $[Zn^{2+}]$  regression analysis graphs for quail's liver GR in the presence of 5 Different Zinc concentrations.





**Figure 3.** Lineweaver-Burk graph with 5 different substrate (GSSG) concentrations and 3 different (A)  $\text{Cd}^{2+}$ ; and (B)  $\text{Zn}^{2+}$  Concentrations for determination of  $K_i$ .

In this study it was determined that the selected heavy metal ions inhibited the GR enzyme activity, an important enzyme of the intracellular glutathione antioxidant system, at very low concentrations.

Heavy metal pollution is one of the most important problems today. Especially in industrial areas, heavy metals are mixed with soil, water and air through industrial wastes. These contaminations result in causing bioaccumulation of heavy metals in living organisms. The bioaccumulation is over certain concentration cause toxic effects and lead to various metabolic disorders.

For this reasons, the toxic effects of these metal ions on GR enzyme activity, body metabolism and GSH synthesis can be reduced for quail and quail consumers.

#### 4. CONCLUSION

This work consists of two phases. The GR enzyme was first purified from the liver of the

quail (*Coturnix coturnix japonica*) with a specific activity of 19.33 EU / mg, yielding 10.25% yield and 128.8 fold purification. In the second step, the inhibitory effect of heavy metal ions on the purified enzyme was investigated. As a result of this study, it has been found that  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Al}^{3+}$  metal ions show a high inhibitory activity on the purified quail liver GR enzyme. Today, the number of industrial waste and metal rejection is increasing worldwide. Because of the inhibitory effects on GSH metabolism, quail producers and consumers should be careful these metal ions that cause GR enzyme inhibition.

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