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PURIFICATION OF GLUTATHIONE REDUCTASE ENZYME FROM WHITING FISH GILL TISSUE AND INVESTIGATION OF METAL INHIBITION

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Abstract: Pollution in the seas basically accumulates in marine organisms. Heavy metal residues in aquatic ecosystems can pass through food and cause toxic effects and accumulations in human health. Glutathione reductase (GR), which is among the basic enzymes, has an important place in the suppression of stress in the cell. In this study, glutathione reductase enzyme from whiting fish gill tissue was partially purified for the first time in the literature and the effects of heavy metal compounds on enzyme activity were determined. The purification process was carried out in three stages as homogenate preparation, ammonium sulfate precipitation and also dialysis. In conclusion the study, optimum level pH 7.0, optimal substrate concentration 2 mM NADPH and optimum buffer 150 mM KH_2PO_4 were determined. After partial purification, the inhibition effects of Cd^{2+}, Ni^{2+}, Zn^{2+} as heavy metal ions were investigated. The IC_{50} levels of heavy metals were calculated as 20.17 µM, 33.7 µM and 59.31 µM, respectively.

Keywords: Enzyme inhibition, Purification, Glutathione reductase, Heavy metals, *Merlangius euxmus*

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1. Introduction

The whiting fish (*Merlangius euxmus*) is a member of the Gadidae family. Their body shapes whitare slender and elongated. All fins are soft and spineless. Colors may vary. Generally, the back is bluish or yellowish brown, but the abdomen is white or silver (Fisher, 1973). A whiting fish with an average length of 15-20 cm can reach up to 50 cm in length (Slastenenko, 1956). They are widely distributed in the European coastal regions of the Black Sea, Aegean Sea and Mediterranean (Ivanov, 1985). Whiting prefers muddy bottoms and coastal waters at depths up to 30-100 m.

Pollution in the seas basically accumulates in marine organisms. One of the important pollutants in the seas is heavy metals, which are among the most harmful elements. Heavy metal residues in aquatic ecosystems can pass into foods and cause toxic effects and accumulations in human health. For this reason, it passes to humans through the food chain (Tüzen, 2003). Unhealthy diet in humans causes oxidative stress in cells and reactive oxygen species that occur as a result of this stress damage cell components in the body. Reactive oxygen species that cause oxidation the main cause of cell damage and death and is well known to be associated with diseases such as cancer and cardiovascular disease (Valko et al., 2006).

Antioxidants can reduce the stress state caused by reactive oxygen species. Studies have focused on the role of antioxidants in treating and preventing diseases (Perry et al., 2002). Glutathione (GSH), a reducing molecule found in almost all eukaryotes, is effectively involved in many vital functions, including antioxidant defense, detoxification of metabolites, cell cycle regulation, gene expression, and immune function (Lue al., 2009).

Glutathione reductase (EC1.8.1.7; GR), which has an important place in the glutathione mechanism, is an important enzyme containing flavin adenine dinucleotide (FAD) and catalyzes oxidized glutathione (GSSG) to reduced glutathione (GSH) (Kocaoğlu et al., 2019).

GR is converted back to GSH by transferring an electron from NADPH to the disulfide bonds of GSSG. Therefore, this enzyme has an important role in the antioxidant defensive system together with GR. Nicotinamide Adenine Dinucleotide Phosphate (NADPH), which has a reduction potential against reactive oxygen species in the environment, is required for this reaction to take place in the mechanism. Therefore, NADPH protects against free radical damage (Sen et al., 2010). At the same time, GR plays an important role in the reduction and oxidation of intracellular GSH (Toribio et al., 1996). GSH and GR, the deficiency of which causes oxidative damage in cells,

causes many diseases such as cancer, Alzheimer's, diabetes, sickle cell anemia and Parkinson's (Wu et al, 2004).

The primary objective of this study, inhibition kinetics of heavy metals by making partial purification of the GR enzyme from the gill tissue of whiting (*Merlangius euxmus*) and due to its harmful effects on fish, as it has an important mechanism as a therapeutic approach for various diseases.

2. Materials and Methods

2.1. Chemicals

The chemicals used in the experiment process were purchased from Sigma-Aldrich and Merck.

2.2. Glutathione Reductase Enzyme Activity

Enzyme activity was measured in Shimadzu UV-1800 spectrophotometric device at 340 nm for 3 minutes, following the decrease in absorbance caused by the oxidation of NADPH (Calberg and Mannervik, 1985).

2.3. Preparation of Homogenate

Gill tissues were taken from whiting fish samples (Figures 1 and 2). 5.5 g of gill tissues were weighed and subjected to physical disintegration in liquid nitrogen in a mortar. After crushing, gill tissues were taken into a 50 ml falcon tube and 0.1 M KH_2PO_4 (pH7.6) buffer containing 1mM EDTA + 0.15 M KCl was added to it and made up to 35 ml. Then, centrifugation was performed at +4 °C at 15000 rpm for 60 minutes. After centrifugation, the supernatant and precipitate were separated from the filter paper by filtration and the enzyme activity was examined.

Figure 1. Whiting (*Merlangius euxmus*).

2.4. Ammonium Sulphate Precipitation and Dialysis Process

One of the methods used to increase the concentration of the protein of interest in the protein purification process is to separate that protein from the proteins of interest. The solubility of proteins is related to the distribution of hydrophilic and hydrophobic parts of that protein. Thanks to these features, precipitation is done in accordance with our purpose. The most preferred of these precipitation processes is precipitation with high salt concentrations. For the homogenate prepared from the gill tissue of whiting, precipitation processes were carried out at different intervals of 0-20%, 20-40%, 40-60% during the ammonium sulfate treatment. For this process, solid $(NH_4)_2SO_4$ was added piece by piece and transferred to the homogenate in ice with the help of magnetic stirrer. As a result of the precipitation, the range in which the enzyme was active was determined by reaching saturation in the range of 40-60%. The precipitate was dissolved in 150 mM KH_2PO_4 (pH 7.0) buffer. After the interval obtained, dialysis was done to desalinate the protein sample. Dialysis was performed for 3 hours in 15 mM KH_2PO_4 (pH 7.0) buffer.

Figure 2. Whiting gill tissue.

2.5. Characterization Study of GR Enzyme from Whiting Gill Tissue

2.5.1. Determination of optimum ionic strength

In order to determine the optimum ionic strength for the activity of the GR enzyme from whiting gill tissue, solutions of KH_2PO_4 buffer were prepared at 50, 100, 150, 200, 300, 400, 500, 600, 700, 800 mM concentrations and their activity measurements were carried out. As a result of the measurements, the optimum level ionic strength was determined in 150 mM KH_2PO_4 buffer.

2.5.2. Determination of optimum pH values

As a result of the optimization of the KH_2PO_4 buffer, pH optimization was performed at the concentration with the highest activity. It was prepared for optimization at pH values of 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9 in 150 mM KH_2PO_4 buffer and activity measurements were examined. As a result of the measurements, 150 mM KH_2PO_4 buffer was determined as the optimum ionic strength.

2.5.3. Determination of optimum amount of substrate

For the determination of the optimum substrate amount, the activity was determined using the optimum pH and optimum buffer determined from whiting gill tissue and 50, 60, 70, 80, 90 and 100 µl NADPH as substrate. As a

result of the measurements, the optimum substrate of the GR enzyme of the gill tissue was determined as 100 µl using 100 mM KH_2PO_4 (pH 7.0) buffer.

2.6. *In vitro* Enzyme Inhibition

Using heavy metals at various concentrations, their inhibition effects on the GR enzyme of whiting gill tissue were evaluated. A heavy metal-free assay was used as a control (100% activity). Effects of different heavy metal constituents of $Ni(NO_3)_2$, $Cd(NO_3)_2$ and $ZnCl_2$ heavy metals on the GR enzyme activity of gill tissue were measured spectrophotometrically. Using typical polynomial regression software, a graph of inhibitory concentration versus percent activity was plotted for each heavy metal. Heavy metal concentrations (IC_{50}) that inhibit enzyme activity by 50% were determined and shown in Table 1.

Table 1. Whiting (*Merlangius euxmus*) GR enzyme inhibition data with heavy metal components

Metal components	IC_{50} (µM)
Cd^{2+}	20.17
Ni^{2+}	33.7
Zn^{2+}	59.31

3. Results and Discussion

GSH is a tripeptide composed of glutamic acid, glycine and cysteine, especially produced in the liver and many tissues. Moreover, GSH is a powerful intracellular antioxidant and has a protective effect on antioxidant vitamins C and E (Blokhina et al., 2003). Besides all this, GSH, which is responsible for amino acid transport, peroxide metabolism, bone and muscle integrity, regulation of many enzyme functions, is effective in DNA synthesis and repair of damaged parts, and its deficiency leads to cell death (Macmillan and Cruthirds, 2001). GR is one of the essential enzymes of the antioxidant system. GR is an enzyme that maintains proper function and is required in humans, encoded by the GSR gene.It plays an important role in the prevention of cellular oxidative stress (Angelucci et al., 2008).It is a low molecular weight thiol that protects the organism from the harmful effects of intracellular oxidized molecules (Angelucci et al., 2008). It catalyzes the reduction of oxidized glutathione in the presence of NADPH (Williams et al. 1976). In the NADPH-dependent reaction with GSSG, glutathione reductase acts as a reducing agent that reduces the activity of GSH (Carlberg et al., 1985).The most important issue in the reaction catalyzed by the enzyme is the maintenance of the GSH/GSSG ratio in the cellular environment (Toribio et al., 1996). In addition, for this reason, it helps to maintain important cellular functions such as detoxification of reactive oxygen species (ROS) (Çakmak et al., 2011; Şentürk and Şentürk 2020).

Heavy metal accumulation, which is one of the biggest environmental pollution problems in the world, is exposed to serious accumulation in seas, lakes and streams. As a result of this negative effect, heavy metals have a toxic effect on fish, which is an important food source for human life. As a result of heavy metals, which cause accumulation on fish as a result of their intake of food, they cause inhibition of antioxidant enzymes in the cell and some problems occur in living organisms due to oxidative damage. The GR enzyme, which has an important place among antioxidant enzymes, constitutes a defense mechanism against the damage caused by oxidative stress.

In this experiment carried out, the glutathione reductase enzyme was partially purified from whiting fish gill tissue. After determining their characteristic properties, heavy metal inhibition kinetics were investigated. After partial purification process, homogenate preparation, ammonium sulfate precipitation process, dialysis, determination of characteristic properties, the study was completed by determining the inhibition kinetics with heavy metal application.

The purification process was first performed by the homogenate preparation process. The prepared gill tissue homogenates were precipitated between 0-100% ammonium sulphate. In the precipitation process, it was determined that the GR enzyme precipitated in the range of 40-60%. Erat (2002) determined the ammonium sulfate range for GR enzyme from bovine and human erythrocytes as 30-70%, Acan and Tezcan (1989) determined the ammonium sulfate range of GR enzyme for sheep brain as 35-55%, Ulusu et al. (2005) found it in the range of 0-60% from sheep liver. After ammonium sulfate precipitation, dialysis was performed to remove undesirable ions in the environment. Işık and Soydan (2023a) found the ammonium sulfate range of GR enzyme from gill tissue of Scorpion fish is between 60-80%.

In another study by Işık and Soydan (2023b), the ammonium sulfate range of the GR enzyme from the muscle tissue of Scorpion fish was found to be in the range of 60-80%.

Ni^{2+}, Zn^{2+} and Cd^{2+} heavy metals were applied on the partially purified enzyme. The IC_{50} values of applied heavy metals were calculated as 33.7 µM, 59.31 µM, and 20.17 µM, respectively. Graphs of IC_{50} values are given in Figure 3-5 and Table 1.

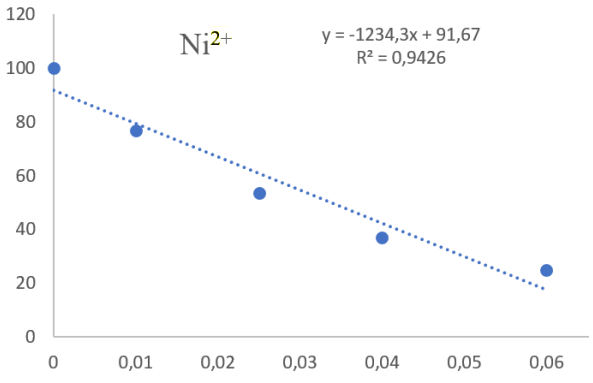

Figure 3. Effect of [Ni^{2+}] GR enzyme activity from whiting fish gill tissue.

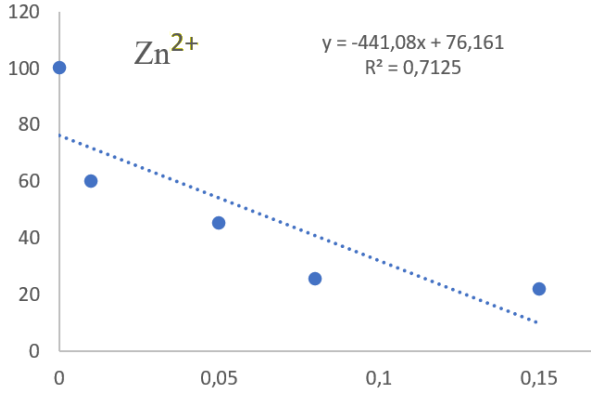

Figure 4. Effect of [Zn^{2+}] GR enzyme activity from whiting fish gill tissue.

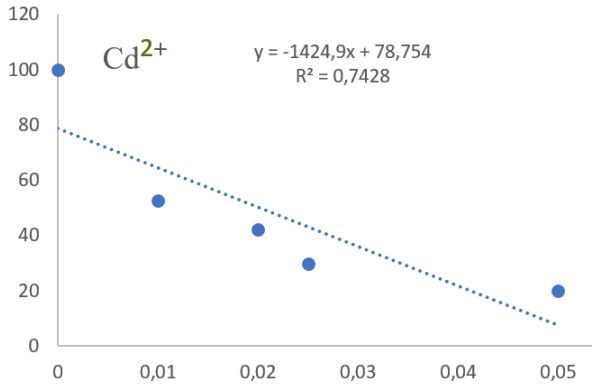

Figure 5. Effect of [Cd^{2+}] GR enzyme activity from whiting fish gill tissue.

The characterization process was carried out in the study. The optimum pH value of the GR enzyme was found to be 7.0, the optimum substrate amount was 100 µl NADPH (2 mM) and the optimum buffer concentration was 150 mM KH_2PO_4.In the literature, the optimum level ionic strength was found to be 435 mM phosphate buffer for bovine erythrocyte GR enzyme and 50 mM Tris for sheep liver (Erat, 2002; Ulusu et al., 2005).In studies conducted with different species, it has been determined that the optimum pH of GR is in the range of 6.5-8.5 (Açan, 1990; Ogus and Ozer, 1998; Özer and Öğüs, 1991; Willmore and Storey, 2007; Tekman et al., 2008).

The damage caused by heavy metals to the environment and especially the excessive exposure of marine organisms show the importance of the study. Because aquatic organisms are exposed to heavy metals, they pass on to humans through food intake. As a result, it causes many diseases by leaving harmful effects on people (Çoban et al., 2007).

Ekinci et al. (2011) investigated the inhibition interaction of GR enzyme isolated from the liver of rainbow trout with heavy metals Co^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Cr^{2+}, Sn^{2+} and Mg^{2+}. The IC_{50} values of heavy metals were found as 42.2 µM, 63.1 µM, 357 µM, 486 µM, 508 µM, 592 µM, and 657 µM, respectively. In a study by Tekman et al. (2008), the inhibition kinetics of heavy metals Cd^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}, Fe^{3+} and Al^{3+} applied on the GR enzyme purified from the liver of rainbow trout (*Oncorhynchus mykiss*) were investigated.IC_{50} values were found as 65.5 µM, 82 µM, 122 µM, 509 µM, 797 µM and 804 µM, respectively. Temel and Çiftçi (2017) determined the inhibition effects of Ni^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+}, Ag^{+} and Al^{3+} heavy metals on the enzyme in the study conducted on the GR enzyme purified from chicken kidney. The IC_{50} values were found to be 337 µM, 191 µM, 168 µM, 187 µM, and 289 µM, respectively. Işık and Soydan (2023) investigated the interaction of GR enzyme isolated from muscle tissue of scorpion fish with Mn^{2+}, Cd^{2+}, Ni^{2+}, and Cr^{3+} heavy metals. The IC_{50} values of heavy metals were found as 2.4 µM, 30 µM, 135 µM and 206 µM, respectively.

4. Conclusion

As a result, in this study, it was determined that the GR enzyme of whiting gill tissue has an inhibitory feature with metal ions even at low concentrations. GR plays an important role in the antioxidant defense system. Inhibition of various heavy metals has a negative effect on the organism. Therefore, it is a factor in the emergence of many pathological disorders. The GR enzyme, which is partially purified from the whiting fish gill tissue, keeps the vitally important GSH/GSSG ratio under control. Care should be exercised in the use of these heavy metals, as they inhibit the GR enzyme and disrupt the balance, and their use should be kept under control. This study was carried out for the first time in the literature in terms of partial purification, characterization and determination of kinetic properties of GR enzyme from whiting fish gill tissue. The findings of our study will contribute to antioxidant enzyme studies.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	S.B.D.	K.I.	B.M.	E.S.	D.E.
C	10	10	20	30	30
D	100				
S		100			
DCP			50	50	
DAI					100
L	20	20	20	20	20
W	20	20	20	20	20
CR	20	20	20	20	20
SR	20	20	20	20	20
PM	20	20	20	20	20
FA	20	20	20	20	20

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval is not required because of this study used commercially caught and sold whiting fish as experimental material.

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