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RESEARCH ARTICLE

Purification of glucose 6-phosphate dehydrogenase from *Capoeta umbla* gill and liver tissues and inhibition effects of some metal ions on enzyme activity

Muammer Kırıcı[1*] • Muhammed Atamanalp[2] • Mahinur Kırıcı[3] • Şükrü Beydemir[4]

[1] *Bingöl University, Vocational School of Technical Sciences, Department of Veterinary Health, 12000, Bingöl, Turkey*

[2] *Atatürk University, Faculty of Fisheries, Department of Aquaculture, 25240, Erzurum, Turkey*

[3] *Bingöl University, Faculty of Arts and Science, Department of Chemistry, 12000, Bingol, Turkey*

[4] *Anadolu University, Faculty of Pharmacy, Department of Biochemistry, 26470, Eskişehir, Turkey*

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ABSTRACT

In this study, the *in vitro* effects of some metal ions (Ag^{+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+}) on freshwater fish *Capoeta umbla* liver and gill glucose 6-phosphate dehydrogenase (G6PD) have been investigated. For this purpose, *C. umbla* liver and gill G6PD enzymes were purified, with a specific activity of 31.52 and 22.83 EU/mg protein, 3353.2 and 736.5 fold in a yield of 19.28% and 23.15%, respectively. In order to control the enzyme purification SDS-PAGE showed a single band for the enzyme. In addition, *in vitro* effects of metal ions on enzyme activity were researched. As a result, Ag^{+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} inhibited fish liver G6PD; Ag^{+}, Cd^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+} inhibited fish gill G6PD. Besides, it was found that the most effective inhibitor of G6PD enzyme within metal ions used was Ag^{+}. Our results also demonstrate that these metals might be dangerous at low micromolar concentrations for fish G6PD enzyme.

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* Corresponding author
E-mail address: muammerkirici@hotmail.com (M. Kırıcı)

Introduction

Glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49; G6PD) is a key and critical enzyme that is the oxidative compound of the pentose phosphate metabolic pathway (Beutler, 2008). The important physiological mission of G6PD is the production of NADPH and ribose 5-phosphate. Ribose-5-phosphate is involved in making DNA and RNA. NADPH is a coenzyme participating in the synthesis of a number of organic molecules such as proteins, nucleic acids and membrane lipids (Srivastava and Beutler, 1970; Hopa et al., 2015). NADPH is also used as a substrate by the glutathione pathway and cytochrome P450 enzymes, which protect cells against oxidative stress and harmful chemicals (Riganti et al., 2012).

The pollution of aquatic environment by contaminants especially metals has become a matter of great concern over the last few decades not only because of the threat to fresh water supplies but also with the damage caused to the aquatic life (Koca et al., 2005). Metals reach the aquatic resources as a consequence of industrial, agricultural, sewage disposal, soil leaching and rainfall, thus aquatic organisms including fishes are exposed to a significant amount of these pollutants in aquatic environments (Heath, 1987; Atli et al., 2006). Several authors reported that heavy metals cause toxicity in aquatic environments and the heavy metal damage is an important factor in many pathological and toxicological processes. It has been reported that metals can change enzymatic activities by binding the functional groups, comprising the carbonyl, carboxyl and sulfhydryl or by substituting of the metal associated with the enzyme (Sivaperumal et al., 2006; Kaya et al., 2013; Kucuk and Gulcin, 2016). The toxicological effects of metals are usually enzyme inhibition and denaturation (Ekinci and Beydemir, 2010). Generally metal inhibition of enzyme is based on metal binding to the protein. Therefore, human metabolism is affected by metal toxicity. Over consumption of fish causes a variety of diseases such as cancer, diabetes, Alzheimer and Parkinson diseases (Jomova and Valko, 2011; Kirici et al., 2017a; Hunsaker and Franz, 2019).

Liver is an important and specific tissue for storing of metals. In the studies conducted on various species of fish metal bioaccumulation has been found higher in the liver, when the compared to other organs (Rainbow and White, 1990). The gill is the first tissue contacting with the contaminants in the water. Due to its large surface area and the small diffusion distance between the water and blood, the gills are primarily affected by contaminants such as metals. In general, the gill cells respond rapidly to various chemicals to overcome physiological impairment or tissue damage, and chemicals may have a negative effect on the overall gill function, enhancing fish susceptibility to toxic compounds and potentially leading to fish mortality (Cerqueira and Fernandes, 2002; Demir et al., 2016).

It is well known that almost all metals show their effects on various enzymes in the metabolism. Particularly, some enzymes are target for this substances such as carbonic anhydrase, glutathione reductase, G6PD and paraoxonase (Ekinci et al., 2007; Alici et al., 2008; Ekinci and Beydemir, 2009; Şentürk et al., 2011; Söyüt et al., 2012; Türkeş et al., 2015; Kirici et al., 2016a).

Also, G6PD-metal interactions have been investigated on a variety of fishes *in vitro* for a long time (Cankaya et al., 2011; Hu et al., 2013; Comakli et al., 2015; Kirici et al., 2016a). In our study, we investigated the toxicological effects of some metal ions, including Ag^+, Cd^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+} on the G6PD enzyme purified from the liver and gill of freshwater fish *Capoeta umbla* using the affinity chromatography method under *in vitro* conditions.

Material and Methods

Chemicals

$Pb(NO_3)_2$, $3CdSO_4.8H_2O$, $FeCl_3$, $NiCl_2.6H_2O$, $AgNO_3$, $CuSO_4.5H_2O$, 2',5'-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, glucose 6-phosphate, protein assay reagent and chemicals for electrophoresis were purchased from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals used were of analytical grade and were purchased from Merck (Darmstadt, Germany)

Fish

Capoeta umbla (healthy, adult fish-weighing 150-250 g) were caught from Murat River (Turkey, Bingol). Fish samples were brought in accordance with the cold chain rules (+4°C). The fish were decapitated and their livers and gills were extracted and stored at -80°C. *C. umbla*, Transcaucasian barb, inhabits Euphrates-Tigris River Systems. It is also known as "lake fish or stream fish" locally and it is the most commercially valued fish for the local people around Murat River (Çoban et al., 2013).

G6PD activity assay

Enzyme activity was performed at 37°C in the spectrophotometer in accordance with the Beutler method (Beutler, 1971).

Preparation of homogenate

The frozen liver and gill tissues were thawed and cut into small pieces by using a scalpel. Liver and gill simples (10 g) were washed three times with 0.9% sodium chloride solution. These samples were homogenized gently for about 45 sec. and suspended in standard homogenizator buffer, containing 50 mM KH_2PO_4, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM Ethylenediaminetetraacetic acid (EDTA) and 1 mM Dithiothreitol (DTT). The homogenates were centrifuged for 2 h at 13.500 rpm. The supernatant was collected and kept for analysis (Kirici et al., 2016b).

Ammonium sulfate precipitation

The ammonium sulfate precipitation experiments were performed according to our previous studies (Kirici et al., 2016a; Kirici et al., 2016b; Kirici et al., 2017a). We determined that the ammonium sulfate was precipitated at 40–80% for gill and 40–80% for liver in our present study. The precipitate was obtained after centrifugation at 13500×rpm for 15 min (+4°C) and redissolved in 50 mM KH_2PO_4 buffer (pH 7.2).

Dialysis

Dissolved precipitate as mentioned above was dialyzed at two hours in Dialysis Buffer (65 mM K-phosphate + 50 mM K-acetate) (pH 7.0) (Kirici et al., 2016a).

Affinity chromatography

2', 5'-ADP Sepharose 4B affinity column (10 cm×1 cm) was prepared according to our previous studies (Kirici et al., 2016a; Kirici et al., 2016b; Kirici et al., 2017a). The column material was equilibrated with buffer (0.1 M K-acetate + 0.1 M K-phosphate, pH 6.0) by means of a peristaltic pump (flow rate: 50 ml/h). After the dialyzed enzyme solution was loaded on the column and the flow rate was regulated to 20 ml/h. The column was respectively 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). Eventually, the enzyme was eluted with a solution of 80 mM K-phosphate + 80 mM KC1 + 0.5 mM NADP+ + 10 mM EDTA (pH 7.8). The enzyme activity was measured, and the activity-containing tubes were collected together (Ninfali et al., 1990).

Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method for liver and gill G6PD enzymes (Bradford, 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The control of enzyme purity was carried out using Laemmli's procedure in 3% and 8% acrylamide concentrations for running and stacking gel, respectively (Laemmli, 1970). Samples were applied as 15 µL to the electrophoresis. Gel was stained with Coomassie Brilliant Blue R-250.

In vitro inhibition studies

In order to determine the effects of the metal ions (Ag^+, Cd^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+}) on fish liver and gill G6PD, 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_{50} values were obtained from activity (%) vs. metal ion concentration plots. In order to determine K_i constants in the media with inhibitor, the substrate (G6P) concentrations were 0.03, 0.06, 0.09, 0.15 and 0.27 mM. Inhibitor solutions 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 K_i constant were calculated from these graphs. Regression analysis graphs were drawn for IC_{50} using inhibition % values by a statistical package (SPSS 17) (Student t-test; n = 3).

Results

G6PD which is an important and a key enzyme for the pentose phosphate metabolic pathway, was purified from *Capoeta umbla* liver and gill by simple chromatographic method and the inhibitory effects of some metal ions (Ag^+, Cd^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+}) were examined on the enzyme activities in the present study.

G6PD was purified by using preparation of the hemolysate, ammonium sulfate fractionation (30–80% for liver; 40–80% for gill) and 2 ', 5'-ADP Sepharose 4B affinity chromatography (Table 1 and Figure 1). The overall purification, fish gill G6PD was obtained with a yield of 23.15% a specific activity of 22.83 EU/mg proteins, and this enzyme was purified approximately 736.5 fold (Table 1); fish liver G6PD was obtained with a yield of 19.28% a specific activity of 31.52 EU/mg proteins, and this enzyme was purified approximately 3353.19 fold (Table 1).

In order to show inhibitory effects, while the most suitable parameter is the K_i constant some researchers use the IC_{50} value (Kirici et al., 2016c; Kucuk and Gulcin, 2016; Kirici et al., 2017b; Caglayan et al., 2019). K_i constant was calculated from Lineweaver-Burk graphs and IC_{50} value was calculated from the regression graphs. Therefore, in this study, both the K_i and IC_{50}

parameters of these metal ions for G6PD were determined. The effects of metal ions on G6PD enzyme from *C. umbla* gill and liver were determined as millimolar levels.

Table 2 and Figure 2 shows the *in vitro* effects and inhibition types of metal ions Ag^{+}, Cd^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+} on gill G6PD activity. IC_{50} values of Ag^{+}, Cd^{2+}, Fe^{2+}, Ni^{2+} and Pb^{2+} were determined as 0.035, 0.376, 0.755, 1.487 and 0.096 mM; K_i constants were determined as 0.019, 0.318, 0.402, 0.152 and 0.036 mM for gill G6PD, respectively. Ag^{+} and Cd^{2+} showed noncompetitive inhibition, while Fe^{2+} and Pb^{2+} inhibited the enzyme in a competitive manner and Ni^{2+} inhibited the enzyme in an uncompetitive manner.

Table 1. Purification scheme of G6PD from *C. umbla* gill and liver

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	0.286	9.13	30	8.58	273.9	0.031	1	100
	Ammonium sulfate precipitation	0.613	19.56	6	3.678	117.6	0.0313	1.01	42.87
	2', 5'-ADP Sepharose 4B affinity chromatography	0.662	0.029	3	1.986	0.087	22.83	736.5	23.15
Liver	Hemolysate	0.535	57.07	33	17.655	1883.3	0.0094	1	100
	Ammonium sulfate precipitation	0.747	55.76	8	5.976	446.08	0.013	1.38	33.85
	2', 5'-ADP Sepharose 4B affinity chromatography	0.851	0.43	4	3.404	0.108	31.52	3353.2	19.28

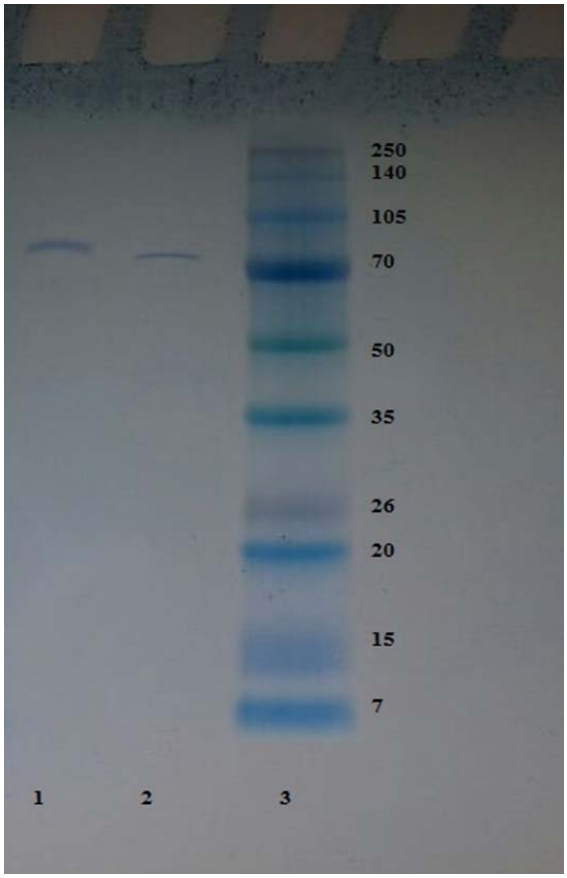

Figure 1. SDS-PAGE photograph (Lane 1: *C. umbla* liver G6PD; Lane 2: *C. umbla* gill G6PD; Lane 3: Standard proteins)

Table 3 and Figure 3 show the *in vitro* effects and inhibition types of metal ions Ag^{+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} on liver G6PD activity. IC_{50} values of Ag^{+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} were determined as 0.00038, 0.423, 8.887, 1.819 and 0.118 mM; K_i constants were determined as 0.00014, 0.335, 4.287, 1.828 and 0.327 mM for liver G6PD, respectively. Ag^{+} and Cd^{2+} showed competitive inhibition, while Cu^{2+}, Ni^{2+} and Pb^{2+} inhibited the enzyme in a competitive manner.

Table 2. IC_{50} values, K_i constants and inhibition types of some metal ions G6PD obtained from *C. umbla* gill

Metal ions	IC_{50} (mM)	K_i (mM)	Inhibition type
Ag^{+}	0.035	0.019 ± 0.00308	Noncompetitive
Cd^{2+}	0.376	0.318 ± 0.00612	Noncompetitive
Fe^{2+}	0.755	0.402 ± 0.102	Competitive
Ni^{2+}	1.487	0.152 ± 0.048	Uncompetitive
Pb^{2+}	0.096	0.036 ± 0.0099	Competitive

Table 3. IC_{50} values, K_i constants and inhibition types of some metal ions G6PD obtained from *C. umbla* liver

Metal ions	IC_{50} (mM)	K_i (mM)	Inhibition type
Ag^{+}	0.000377	0.00014 ± 0.000042	Competitive
Cd^{2+}	0.423	0.335 ± 0.167	Competitive
Cu^{2+}	8.887	4.287 ± 0.197	Noncompetitive
Ni^{2+}	1.819	1.828 ± 1.142	Noncompetitive
Pb^{2+}	0.118	0.327 ± 0.054	Noncompetitive

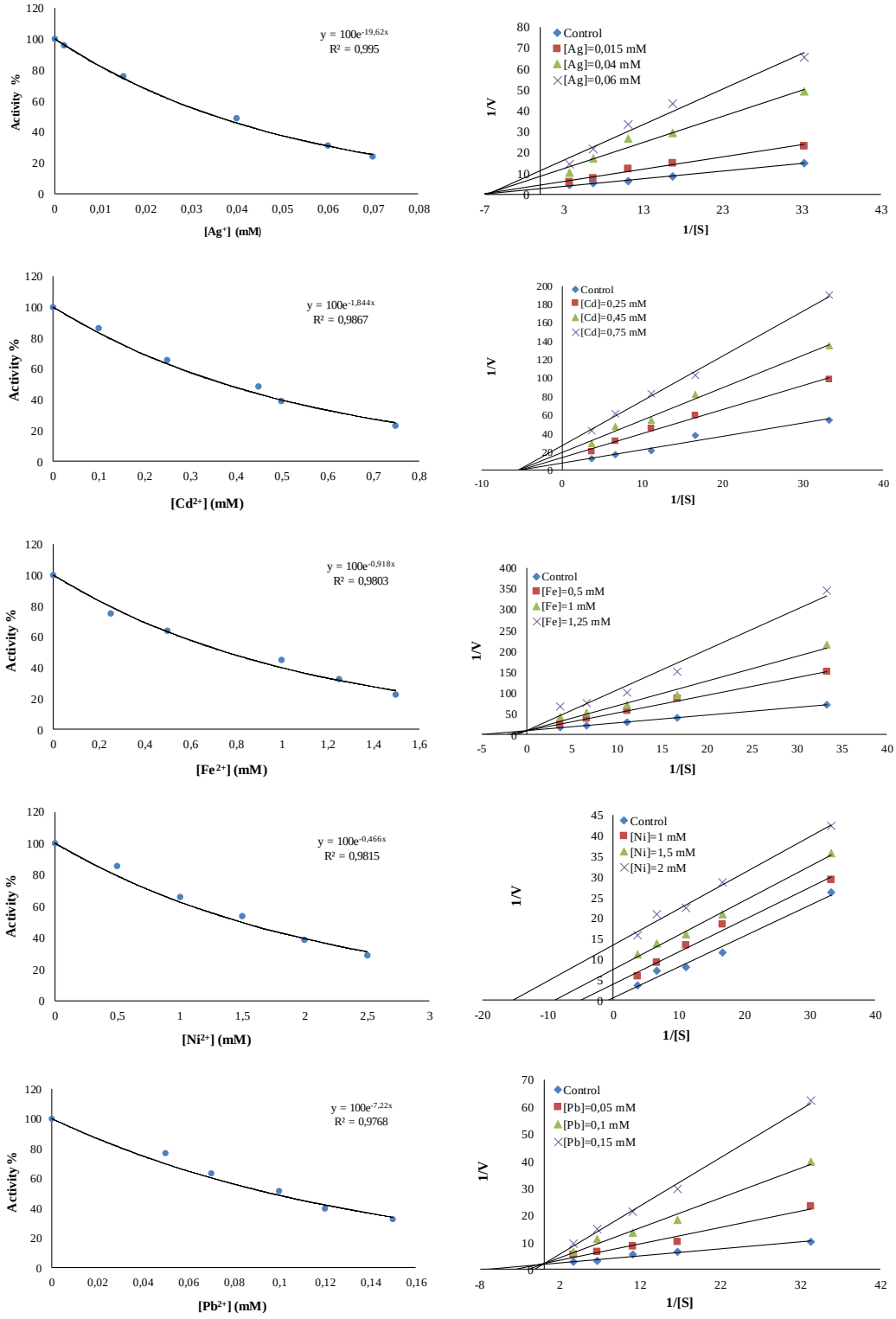

Figure 2. IC_{50} and K_i graphics for *C. umbla* gill G6PD

Discussion

G6PD is widespread in all tissues and blood cells, catalyzing the conversion of glucose 6-phosphate to 6-phosphogluconolactone in the presence of $NADP^+$. The conversion of glucose-6-phosphate to 6-phsophogluconolactone is important in the generation of NADPH, an important coenzyme and reducing agent in lipid and nucleic acid synthesis (Patrinostro et al., 2013). G6PD inhibition within chemical compounds directly affects the increase of reactive oxygen species reducing the amount of intracellular NADPH and GSH, involve to protect cells against reactive oxygen species (Riganti et al., 2012; Hopa et al., 2015).

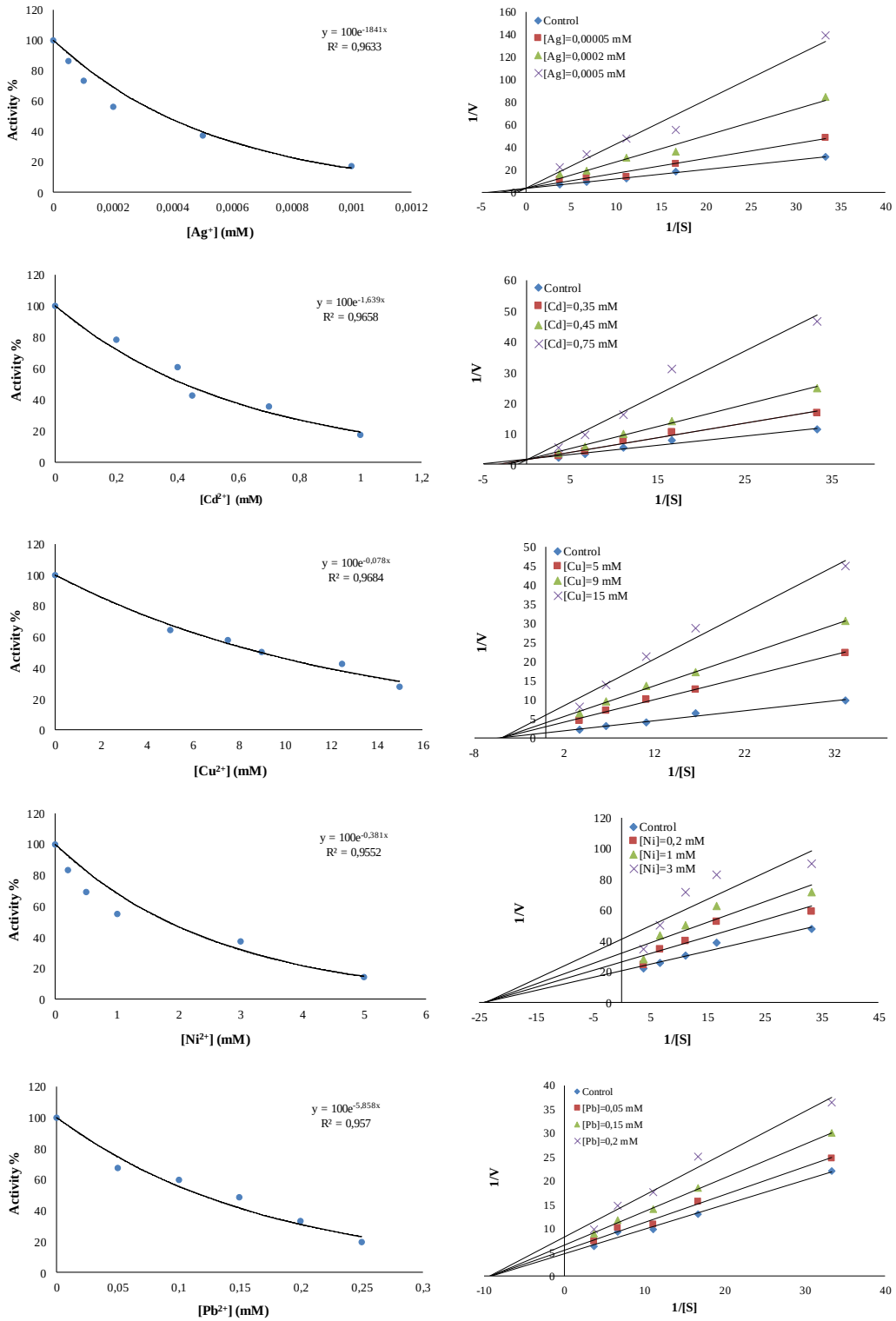

Figure 3. IC_{50} and K_i graphics for *C. umbla* liver G6PD

Some metals such as iron, zinc, copper, calcium, function in the active site of the enzymes as ligands (Alim and Beydemir, 2012). Many enzymes contain Zn^{2+} with in their structures, such as carbonic anhydrase and sorbitol dehydrogenase. Moreover, some enzymes, such as paraoxonase contain multiple metal ions in their three-dimensional structure. The toxicological effects of metals are usually enzyme inhibition and denaturation (Ekinci and Beydemir, 2010; Caglayan et al., 2019). Generally, the mechanism underlying the metal inhibition of the enzyme is based on metal binding to the protein. Metals particularly, bind sulfhydryl groups in proteins and produce mercaptan (Jomova and Valko, 2011; Hunsaker and Franz, 2019).

In this study, the G6PD enzyme was purified from the liver and gill of the *C. umbla* and the toxicological effects of metal ions on the activity of this enzyme were calculated. This metalo-

enzyme is quite common among all organisms. G6PD was first isolated from human erythrocytes by Yoshida and Huang (Yoshida and Huang, 1986). Up to now, G6PD enzymes have been purified from many various sources (Bautista et al., 1988; Özer et al., 2002; Yilmaz et al., 2002; Beydemir et al., 2003; Türkoğlu et al., 2003; Şentürk et al., 2009; Adem and Ciftci, 2012; Comakli et al., 2015; Adem and Ciftci, 2016).

Many chemicals effect the metabolism via changing normal enzyme activity, particularly inhibition of a specific enzyme and the effects can be dramatic and systemic (Adem and Ciftci, 2012). The effects of different chemical substances, drugs and metal ions on G6PD enzyme have been investigated in many *in vitro* studies, performed with various organisms. For example; Şentürk et al. (2009) investigated the *in vitro* effects of deltamethrin, cypermethrin and propoxur on rainbow trout erythrocyte G6PD. They found that deltamethrin and cypermethrin showed noncompetitive inhibition while propoxur inhibited the enzyme in an uncompetitive manner. Hu et al. (2013) purified G6PD from grass carp (*Ctenopharyngodon idella*) hepatopancreas and determined the inhibition effects of Zn, Mn, Al, Cu and Cd on G6PD activity in *in vitro*. Their results showed that Zn, Al, and Cd showed competitive inhibition, while Cu inhibited the G6PD in a noncompetitive inhibition manner. Besides, the effects of furosemide, digoxin and dopamine on G6PD enzyme, which was purified from rat heart by Adem and Ciftci (2016), were investigated. Their result showed Dopamine inhibited the activity of these enzyme as competitive, whereas furosemide and digoxin inhibited the activity of the enzyme as noncompetitive.

Conclusion

Consequently, accumulation of metals in fish may be considered as an important warning signal for fish health and human consumption. It is a good way to understand the toxic potential of compounds *in vitro* studies. Therefore, we investigated *in vitro* the inhibitory effect on the *C. umbla* liver and gill G6PD enzyme activities of some metal ions. According to these results, Ag^{+} and Cd^{+2} bind to the active sites of the fish liver G6PD enzyme; other metal ions are connected to other regions outside the active region. Besides, Fe^{+2} and Pb^{+2} bind to the active sites of the fish gill G6PD enzyme; other metal ions are connected to other regions outside the active region. IC_{50} values and K_i constants showed that Ag^{+} had the highest inhibitory effect on fish liver and gill G6PD activities.

Compliance with Ethical Standards

Authors' Contributions

MuK and MaK performed the research, analyzed the data and helped to draft the manuscript; MuK, MaK, MA and ŞB 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 there is no conflict of interest.

Ethical Approval

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

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