

Purification and characterization of NADPH-cytochrome P450 reductase from Lake Van fish liver microsomes and investigation of some chemical and metals' effects on the enzyme activity

Müslüm KUZU¹, Mehmet ÇİFTÇİ^{2,3,*}

¹Faculty of Pharmacy, Ağrı İbrahim Çeçen University, Ağrı, Turkey

²Faculty of Science, Atatürk University, Erzurum, Turkey

³Faculty of Science and Letters, Bingöl University, Bingöl, Turkey

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Abstract: NADPH-cytochrome P450 reductase was purified from Lake Van fish liver microsomes by primary and secondary DEAE-cellulose column chromatograph with 20.46 μ M/min/mg enzyme specific activities, 54.4% purification yield, and purification of 38-fold. The purity of the enzyme was established, and its monomer molecular weight was determined by SDS-polyacrylamide gel electrophoresis. SDS-PAGE results showed a single band and the molecular weight of NADPH-cytochrome P450 reductase was 70 kDa. In addition, optimum ionic strength, optimum pH, optimum temperature, and stable pH values were determined for the enzyme in the kinetic studies performed. K_M and V_{max} were determined for NADPH and cytochrome c. Effects of some metals ions, antibiotics, and some other drugs used in aquarium fisheries on the activity of the enzyme were investigated. IC₅₀ values and K_i values of metals showing an inhibitory effect were calculated.

Key words: NADPH-cytochrome P450 reductase, Lake Van fish, inhibition, metal, antibiotic

1. Introduction

The membrane-bound microsomal monooxygenase enzyme system is localized in the endoplasmic reticulum (ER) of most animal tissues. Although the highest content of microsomal monooxygenase enzyme system components is found in liver cells, this system is also present in other tissues, such as the lungs, kidneys, brain, lymphocytes, vascular smooth muscle, olfactory and intestinal epithelium, and nasal mucosa.¹ The liver microsomal monooxygenase system, which catalyzes the metabolism of many exogenic and endogenous compounds, is divided into 3 components, namely P450, NADPH-cytochrome P450 reductase (CPR), and phospholipids.^{2,3} When the hydrophilic catalytic region of the CPR enzyme, which is a flavoprotein, is on the cytosolic surface of the endoplasmic reticulum, it is bound to the endoplasmic reticulum membrane via the hydrophobic α -helix transmembrane region located on the N-terminal point.⁴⁻⁶ It is known that CPR takes charge in the production of reactive oxygen species that cause genotoxicity and cytotoxicity as well as lipid peroxidation and bioreductive activation of some anticancer drugs and antibiotics.⁷ The basic redox partner of the enzyme in the cell is the cytochrome P450 species constituting the monooxygenase enzyme family.⁸ The enzyme contains FAD and FMN binding domains. From this aspect, the enzyme shows similarity with nitric oxide synthase that is found in mammals,⁹ methionine synthase reductase,¹⁰ and NR1¹¹ enzymes.

*Correspondence: mciftci@bingol.edu.tr

NADPH-cytochrome P450 reductase was first purified from yeast cells in 1940 and was named NADPH-bound cytochrome c reductase, and it contains FMN.¹² The mammalian CPR enzyme was first isolated from pig liver by Horecker in 1950, and it was determined that the enzyme contains a FAD cofactor.¹³

Functional and structural properties of mammalian cytochrome P450 reductase, which was purified from bovine liver,⁷ guinea pig liver,¹⁴ pig liver,^{15,16} sheep liver and lung,^{17,18} human liver,¹⁹ human placental microsomes,²⁰ human neutrophil membranes,²¹ human brain,²² rat liver,^{23,24} and rat brain²⁵ microsomes, were examined in detail and some of their kinetics and structural and functional differences were determined. Regarding fish species, cytochrome P450 reductase was purified and characterized from porgy (*Stenotomus chrysops*) liver,²⁶ rainbow trout liver,²⁷ and leaping mullet liver²⁸ microsomes.

Metals exist naturally on earth, unlike organic compounds, which are chemically synthesized. These metals are released into water systems through natural means, such as air, surface water, and soil (groundwater) or the activities of humans. In recent years, the aquatic system has been exposed to metal pollution as a result of the rapid development in industry.²⁹ Metals can be hazardous for living organisms at a lethal level since they have the property of spreading in nature and penetrating into living organisms. For instance, metals have the tendency to accumulate in some tissues of the human body, and they have a potential toxic effect, even in low amounts of exposure. Nonetheless, some metals like copper and iron are essential and they have a critical role in some important enzyme systems.³⁰ On the other hand, it was reported that nonessential metals such as cadmium, mercury, aluminum, and thallium have a toxic effect, even in trace amounts. However, it was emphasized that many metals, whether essential or nonessential, can pose a serious risk for human health and exhibit side effects on the ecosystem itself.³¹

An antibiotic is a substance that kills bacteria or stops them from growing.³² Aminoglycoside group antibiotics (gentamicin, kanamycin, etc.) are known to have nephrotoxic, neurotoxic, and ototoxic effects.³³ Tetracycline group antibiotics are broad-spectrum bacteriostatic antibiotics and show their effects by inhibiting protein synthesis as a result of binding to 30S subunit.³⁴

Apart from its function of electron transport in monooxygenase reactions, the enzyme also has the function of catalyzing the reduction reactions of various chemicals that contain therapeutically very important antibiotics, aromatic nitro compounds, pesticides, and other environmental pollutants.^{35–37} The aim of the present study was to purify NADPH-cytochrome P450 reductase enzyme, determine its biological and kinetic properties, give detailed information on the xenobiotic metabolism and liver microsomal monooxygenase enzyme system, and assist in a better understanding of this system. Another aim of this study was to research the in vitro effects of some drugs used for animal health and in aquarium fisheries and metal ions on the enzyme activity, to which all living things in nature are, primarily, including humans, exposed through various ways.

2. Results and discussion

CPR enzyme was purified 38-fold with a yield of 54.4% from Lake Van fish liver microsomes by conducting ion exchange chromatography in 2 stages with 20.46 $\mu\text{M}/\text{min}/\text{mg}$ protein specific activity (Table). SDS-PAGE was conducted to check the enzyme purity (Figure 1). An R_f -log MW graph was drawn and the molecular mass of the enzyme was measured as ~ 70 kDa. The optimal pH, optimal ionic strength, optimal temperature, and stable pH of the enzyme were 8.0 (Figure 2), 1 M K-phosphate, 50 °C (Figure 3), and 7.6, respectively. The K_M constant was 12.82 μM and the V_{max} value was 42.9 $\mu\text{M}/\text{min}/\text{mL}$ for cytochrome c, whereas the K_M constant was 5.208 μM and the V_{max} value was 12.76 $\mu\text{M}/\text{min}/\text{mL}$ for NADPH (Figures 4 and 5). Furthermore, IC50 values of Hg^{2+} , Ag^+ , and Cu^{2+} ions, which exhibited inhibitory effects on enzyme activity, were 2.1936 μM ,

2.9877 μM , and 397.66 μM , respectively. Their K_i constants were $1.36 \pm 0.63 \mu\text{M}$ (competitive, Figure 6A), $1.60 \pm 0.76 \mu\text{M}$ (competitive, Figure 6B), and $279.79 \pm 176.9 \mu\text{M}$ (competitive, Figure 6C), respectively. The effects of 5 drugs on CPR activity were also examined at different concentrations. Kanamycin (0.047–1.17 mM), oxytetracycline (3.87–38.7 μM), and gentamicin (6.34–79.3 μM) exhibited activator behavior on CPR activity, with AC50 values of 103.85, 18.06, and 31.33 μM , respectively. Benzalkonium chloride and acriflavine did not show any effect on CPR activity at 0.75–30 μM or 0.85–17 μM , respectively.

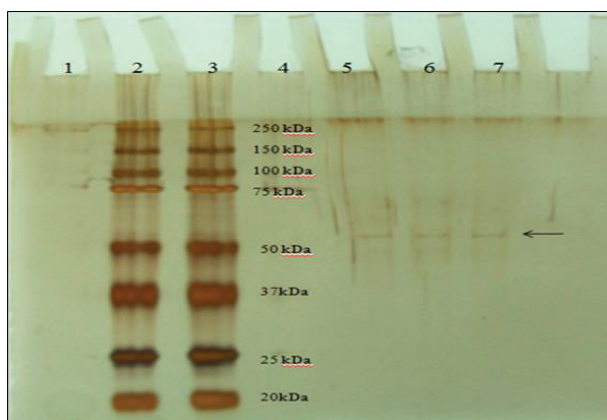


Figure 1. SDS-PAGE patterns of purified Lake Van fish liver NADPH-cytochrome P450 reductase. Lines 2 and 3, eight reference proteins (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, Precision Plus Protein Kaleidoscope Standards #161-0375), Lines 5, 6, and 7, cytochrome P450 reductase obtained from secondary DEAE-cellulose column.

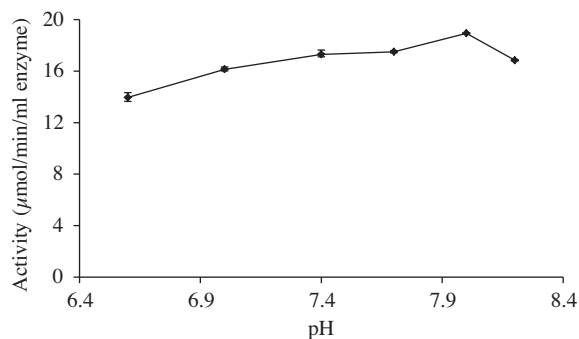


Figure 2. Effect of pH on activity of Lake Van Fish NADPH-cytochrome P450 reductase. The buffers used were 0.3 M K-phosphate buffer (pH 6.6–8) and 0.3 M Tris–HCl buffer (pH 8.2).

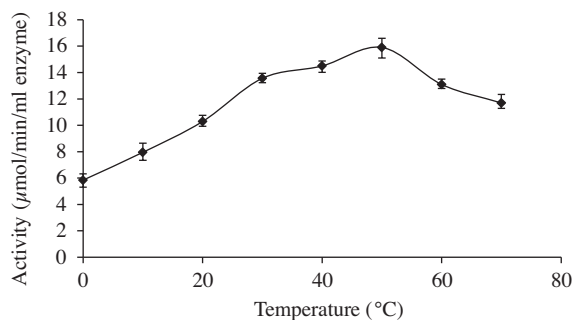


Figure 3. Effect of temperature on activity of Lake Van fish liver NADPH-cytochrome P450 reductase activity. The activity was assayed in 0.3 M K-phosphate buffer (pH 8.0).

NADPH-cytochrome P450 reductase enzyme contains FAD and FMN prosthetic groups, and it is an enzyme that falls under the flavoprotein family. Many members of the cytochrome P450 family require this enzyme in the oxidative metabolisms of most drugs and endogenous compounds. It is also known that the enzyme functions as a restrictor in reactions that are catalyzed by the cytochrome P450 enzymes.³⁸

The Lake Van fish *Chalcalburnus tarichi* (pearl mullet) used as the source in this study is a member of the cyprinidae family, and it is an endemic species that lives only in the basin of Lake Van. Lake Van has a different ecosystem and it is the largest known soda lake on Earth. Its water has a high level of alkaline (pH

9.8) and its rate of salinity is 19%. This alkaline and salty water mix prevents all fish species apart from Lake Van fish from living in the lake.³⁹

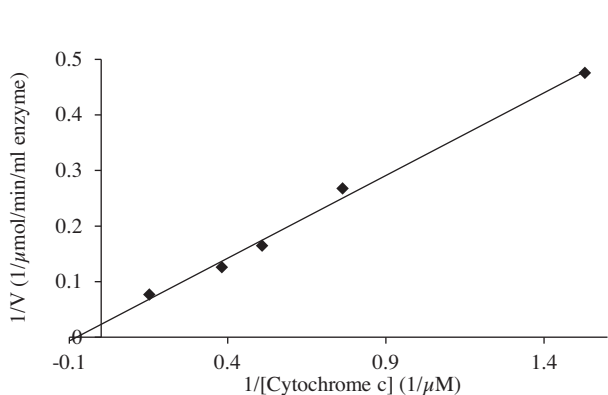


Figure 4. Kinetic analysis of Lake Van fish liver NADPH-cytochrome P450 reductase activity. The activity was assayed in the range from 0.65 μM to 6.55 μM cytochrome c with 0.09 mM fixed NADPH.

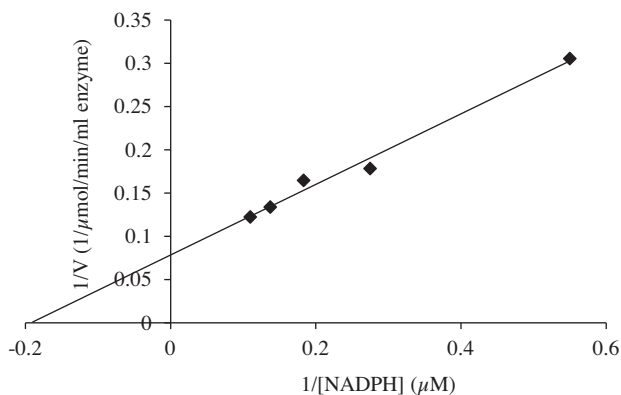


Figure 5. Kinetic analysis of Lake Van fish liver NADPH-cytochrome P450 reductase activity. The activity was assayed in the range from 1.82 μM to 9.09 μM NADPH with 0.03 mM fixed cytochrome c.

Table. Purification scheme of NADPH-cytochrome P450 reductase from Lake Van fish liver microsomes.

Fractions	Activity ($\mu\text{M}/\text{min}/\text{mL}$)	Volume (mL)	Protein (mg/mL)	Total activity	Specific activity ($\mu\text{M}/\text{min}/\text{mg}$)	Yield %	Purification fold
Solubilized microsomes	19.87	28.5	3.685	566.3	5.39	100	1
DEAE-cellulose-1	8.67	39.58	0.1417	343.2	61.19	60.6	11.35
DEAE-cellulose-2	11.93	25.81	0.0583	307.9	204.63	54.4	38

As seen in the Table, the enzyme, which had 20.46 $\mu\text{M}/\text{min}/\text{mg}$ enzyme specific activity, was purified 38-fold with a yield of 54.4%. The molecular mass of the enzyme was calculated as ~ 70 kDa using SDS-PAGE. This value shows similarity with the values that were calculated for rainbow trout liver,²⁷ leaping mullet (*Liza saliens*) liver,²⁸ and human liver⁴⁰ CPR enzymes.

The optimal pH value obtained in our study was very close to the pH values calculated for CPR purified from sheep liver¹⁸ and rabbit peritoneal neutrophils.⁴¹

The optimal ionic strength obtained had a value close to the ionic strength of rat liver and lung CPR⁴² enzyme, whereas this value was higher in comparison with some species.²⁸ The reason for this may be the fact that the electrostatic interaction among enzyme-loaded redox partners was neutralized.⁴³

The optimal temperature value for the enzyme was taken as 50 $^{\circ}\text{C}$ because its activity was highest at this temperature. It was found that the enzyme remained stable with a maximum duration in 0.3 M phosphate buffer at pH 7.6. Therefore, the stable pH of the enzyme was determined as 7.6.

It was observed that the specific activity value, which was calculated in the kinetic studies that were conducted for the enzyme, was close to that of the African clawed frog (*Xenopus laevis*) liver⁴⁴ CPR enzyme and a little lower compared to the rainbow trout liver²⁷ CPR enzyme.

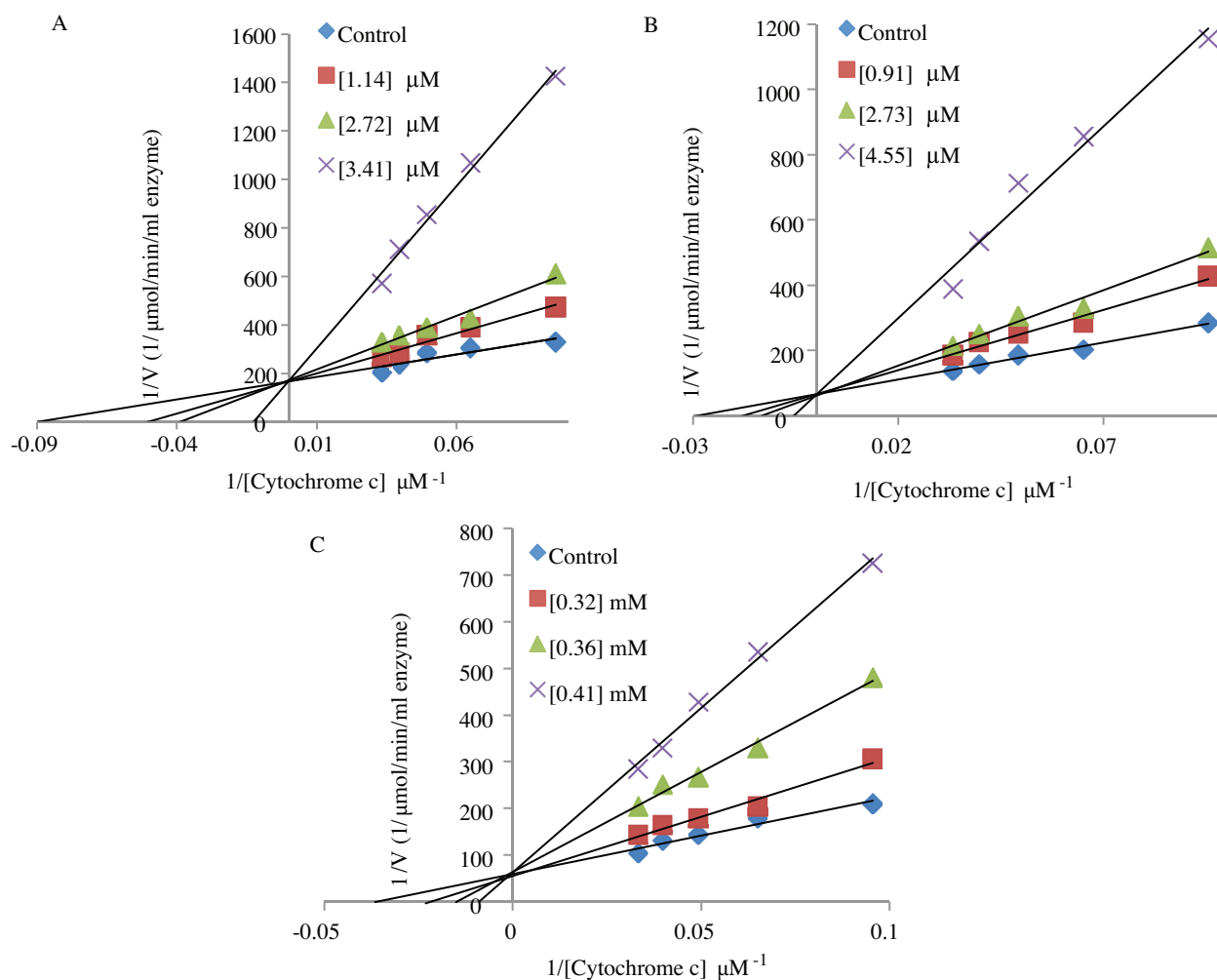


Figure 6. Lineweaver–Burk graphics: **A)** Graph of 5 different cytochrome c concentrations for the determination of K_i using 3 different Hg^{2+} concentrations; **B)** graph of 5 different cytochrome c concentrations for the determination of K_i using 3 different Ag^{+} concentrations; **C)** Graph of 5 different cytochrome c concentrations for the determination of K_i using 3 different Cu^{2+} concentrations.

K_M values were $5.208 \mu\text{M}$ and $12.82 \mu\text{M}$, respectively, for NADPH and cytochrome c substrates, whereas V_{max} values were $12.76 \mu\text{M}/\text{min}/\text{mL}$ and $42.9 \mu\text{M}/\text{min}/\text{mL}$, respectively. The K_M value was $7.69 \mu\text{M}$ and the V_{max} value was $47.6 \mu\text{M}/\text{min}/\text{mL}$ for cytochrome c substrate of leaping mullet liver CPR enzyme.⁴⁵ The K_M value was $15 \mu\text{M}$ for cytochrome c of CPR enzyme obtained from rabbit peritoneal neutrophils. The K_M value was $1.9 \mu\text{M}$ for NADPH.⁴¹ As seen, these values are different from those obtained in our study.

It was observed that kanamycin, oxytetracycline, and gentamicin considerably increased the enzyme activity, whereas benzalkonium chloride and acriflavine did not have any effect on enzyme activity.

Pillai and Mehvar⁴⁶ found that the production of reactive oxygen species (ROS) increased via P450 induction, whereas it was decreased via its inhibition. It was reported by Portal et al.⁴³ that CPR participated in the rate-limiting step in reactions that were catalyzed by P450s. In the light of this information, it is possible that kanamycin, oxytetracycline, and gentamicin will increase ROS production in the enzyme activity, thereby causing cell damage.

It was found in our study that Pb^{2+} , Fe^{3+} , Ni^{2+} , Cd^{2+} , Zn^{2+} , and Tl^{+} did not have any effect on enzyme activity; Co^{2+} , Ca^{2+} , As^{5+} , and Mn^{2+} activated the enzyme; Hg^{2+} , Ag^{+} and Cu^{2+} ions competitively inhibited the enzyme; and Al^{3+} partially inhibited the enzyme. Kim et al.⁴⁷ found similar results for Zn^{2+} on recombinant rat liver CPR enzyme and Bozcaarmutlu and Arinç³¹ found similar results for Zn^{2+} on leaping mullet liver CPR enzyme. It was stated by Bozcaarmutlu and Arinç³¹ that Ni^{2+} and Cd^{2+} inhibited leaping mullet liver CPR enzyme. In other conducted studies, it was determined that Tl^{3+} inhibited leaping mullet liver CPR enzyme in vitro,²⁹ whereas Pb^{2+} , Cd^{2+} , and Ni^{2+} inhibited bovine liver CPR enzyme⁴⁸ in vitro. In the conducted studies, it was reported that Al^{3+} and Hg^{2+} noncompetitively inhibited leaping mullet liver CPR enzyme,^{29,31} Cu^{2+} ion competitively inhibited leaping mullet liver CPR enzyme, and Cu^{2+} inhibited and affected liver P450 monooxygenase enzyme system, P450s, and CPR enzyme.⁴⁷

Levesque et al. reported that constant exposure to heavy metal ions below the lethal level caused growth disorders and change in the lipid metabolism in fish. It was also stated that changes occurred in gonad size and hormone levels of fish living in lakes that were exposed to metal contamination.⁴⁹ Since the P450-bound monooxygenase enzyme system takes part in the synthesis of fatty acids and steroids and since CPR enzyme is an essential component of this system, the inhibition of this enzyme may inhibit the synthesis of the mentioned endogenous compounds. It was reported that a decrease in the synthesis of steroids and lipids could affect growth and reproduction in fish. Apart from these, it was stated that the inhibition of CPR enzyme could cause the regulation of toxic chemicals in fish, thereby resulting in population changes. It was also emphasized that the inhibition of CPR could lengthen the durations of the effect of chemicals and consequently increase or decrease the chemical toxicity and affect the metabolism of endogenous compounds since P450 isoforms are responsible for the oxidative metabolism of the majority of drugs and chemicals in humans.³¹

As a result, within this study CPR enzyme from the liver tissue of Lake Van fish was purified 38-fold in electrophoretic purity with 54.4% efficiency, and some of its biochemical features were determined. For the enzyme the optimum conditions were specified and the effects of some heavy metals and drugs on enzyme activity were analyzed. It was determined that while Hg^{2+} , Ag^{+} , and Cu^{2+} ions inhibit the enzyme competitively, kanamycin, oxytetracycline, and gentamicin activate the enzyme.

3. Experimental

3.1. Chemicals

Sodium dodecyl sulfate (SDS), sodium chloride, sodium acetate, hydrochloric acid, phosphoric acid, ethanol, methanol, isopropanol, acetic acid, sodium acetate, and potassium chloride were obtained from E. Merck AG. Ethylenediaminetetraacetic acid (EDTA), β -mercaptoethanol, silver nitrate, and formaldehyde were obtained from Fluka. Precision Plus Protein Kaleidoscope Standards was obtained from Bio Rad. Sodium hydroxide and potassium phosphate were obtained from Riedel de Haen. DE52 diethylaminoethyl ion exchange gel was obtained from Whatman. Glycine was obtained from ICN Biomedicals Inc. and the other chemicals used in the study were obtained from Sigma Chemical Co.

3.2. Fish samples

Lake Van fish samples, obtained from the shores of Edremit, were brought to the laboratory environment in compliance with cold chain rules. The fish were decapitated and their livers were extracted and stored at $-80\text{ }^{\circ}\text{C}$.

3.3. Preparation of liver microsomes

Microsomes were prepared using approximately 12–15 fish livers. Liver samples, which were taken from the storage at $-80\text{ }^{\circ}\text{C}$, were washed with 1.5% cold KCl solution containing 2 mM EDTA. All subsequent studies were conducted at temperatures ranging from $0\text{ }^{\circ}\text{C}$ to $4\text{ }^{\circ}\text{C}$. Homogenate was prepared from liver pieces that were downsized in an adequate amount using a homogenizer in 1.5% KCl solution containing 2 mM EDTA, 0.25 mM PMSF, and 0.25 mM ε -ACA. Then the homogenate was centrifuged at $18,000 \times g$ for 40 min. Microsomes were precipitated by centrifuging the obtained supernatant at $210,000 \times g$ for 60 min using an ultracentrifuge. The obtained microsomes were solved with 1.5% KCl solution containing 2 mM EDTA and precipitated again at $210,000 \times g$ for 60 min. The obtained supernatant was removed again. Microsomes, which were obtained that way, were solved with 10% glycerol solution containing 2 mM EDTA. Microsomal solution was rendered into soluble form using 20 mM potassium phosphate buffer (pH 7.5) containing 25% glycerol, 2 mM EDTA, 20 μM BHT, 0.1 mM DTT, 0.25 mM PMSF, 0.25 mM ε -ACA, 0.5% emulgen 913, and 0.5% Na-cholate. Then the suspension, which contained the microsomes that were broken down with detergent, was centrifuged at $210,000 \times g$ for 60 min. Clear yellow supernatant was taken and the pellet was removed.

3.4. Primary and secondary DEAE-cellulose ion exchange chromatography

The obtained microsomal solution was applied to the DEAE-cellulose ion exchange column balanced with 20 mM potassium phosphate buffer (buffer A) (pH 7.7) containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 0.25 mM PMSF, 0.25 mM ε -ACA, 0.5% emulgen 913, and 0.2% cholate. The flow rate was set to 45 mL/h. The column was washed with 300 mL of buffer A.

The elution process was started after the washing process had been completed. The elution process was conducted with buffer A containing 100 mL of 0.05 M KCl, 100 mL of 0.1 M KCl, 100 mL of 0.15 M KCl, 100 mL of 0.2 M KCl, and 100 mL of 0.3 M KCl. The fractions, which contained NADPH-cytochrome P450 reductase enzyme that exhibited high amounts of activity and that was obtained from the primary DEAE-cellulose column, were diluted by adding 2 buffers at each section and applied to the secondary DEAE-cellulose ion exchange column balanced with buffer A. After the sample had been applied to the column, the column was washed with buffer A. Following this process, the elution process was conducted with buffer A solution containing 0.075 M KCl, 0.15 M KCl, and 0.3 M KCl. It was observed that NADPH-cytochrome P450 reductase enzyme emerged again after buffer A solution containing 0.15 M KCl had been applied. The fractions taken with buffer A solution containing 0.15 M KCl were placed in dialysis bags and dialyzed against buffer A twice for 2 h.

3.5. Activity determination

NADPH-cytochrome P450 reductase activity was determined spectrophotometrically in accordance with the method of Master et al.,⁵⁰ except that the activity measurement was conducted at room temperature using phosphate buffer (pH 8.0). Measurements were repeated 3 times. Enzyme activity was calculated using the extinction coefficient of $19.6\text{ mM}^{-1}\text{ cm}^{-1}$ for the difference in absorbance between the oxidized and reduced forms of cytochrome c at 550 nm.⁵¹

3.6. Protein determination

Protein determination was conducted spectrophotometrically in accordance with the method of Lowry.⁵² Crystalline BSA was used as the standard.

3.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

After the enzyme had been purified, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 3%–10% batch was conducted in accordance with the method of Laemmli. Purity grade and monomer molecular mass of the enzyme were determined.⁵³

3.8. Optimal pH studies

In order to determine the optimal pH of the microsomal NADPH-cytochrome P450 reductase enzyme of the Lake Van fish liver 0.3 M K-phosphate (pH ranging from 6.6 to 8.0) and 0.3 M Tris/HCl (pH 8.2) buffers were prepared. Enzyme activity was determined separately in each buffer using an appropriate substrate solution.

3.9. Optimal ionic strength studies

In order to determine the optimal ionic strength of the microsomal NADPH-cytochrome P450 reductase enzyme of the Lake Van fish liver, cytochrome c solution was prepared using K-phosphate in concentrations ranging from 0.1 M to 1.8 M at optimal pH. Consequently, the optimal ionic strength was determined for the enzyme.

3.10. Optimal temperature studies

The mixture, which contained the necessary buffers and substrates for the activity measurements, was held in a water bath set to the related temperature in a cuvette until it reached to the temperature of the water bath. Activity measurements were conducted between 0 °C and 70 °C with 10-°C intervals.

3.11. Stable pH studies

To determine the stable pH, buffers were prepared from the phosphate buffer (pH 7.2, 7.6, 7.8, and 8.0) and from the Tris-HCl buffer (pH 8.2, 8.4, and 8.8). They were put in Eppendorf tubes as 0.5 mL from the enzyme and 0.5 mL from these buffers. Activity measurements were conducted at the beginning, after 12 h, after 24 h, and for an additional 4 days with 24-h intervals.

3.12. Kinetic studies

With the aim of determining the K_M and V_{max} values for cytochrome c and NADPH substrates, activity measurements were conducted via 5 different concentrations of NADPH in the constant cytochrome c concentration. A Lineweaver–Burk plot was drawn using the obtained values. K_M and V_{max} values for NADPH were determined with the help of this plot. Similarly, activity measurements were conducted via 5 different concentrations of cytochrome c in the constant NADPH concentration. A Lineweaver–Burk plot was drawn. K_M and V_{max} values for cytochrome c were determined.⁵⁴

Drugs and metal ions in different amounts and concentrations were added to the cuvette environment and their activity values were measured in order to determine the effects of drugs and some metal ions on the activity of enzymes. Instead of the phosphate buffer, 0.3 M (the concentration that has consistent results in the studies) HEPES buffer (pH 8.0) was used in the conducted activity measurements. This was because it was reported previously that metal ions like cadmium and nickel precipitated in the phosphate buffer system.³¹ Inhibitory effects of drug and metal lines were determined via preliminary tests in the lowest possible concentrations. A % activity-[I] (inhibitor concentration) graph was drawn in order to determine the IC50 values. For this purpose, activity measurements were conducted in 5 different inhibitor concentrations.

In order to determine the K_i values, activity measurements were conducted via 5 appropriate substrate concentrations in the drug concentration that reduced the activity of enzymes to half as well as constant drug and metal ion concentrations that were below and above this value. Lineweaver–Burk plots were drawn for each inhibitor using the obtained values. K_i values and inhibition types were determined.

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