# Properties and some in vitro studies of 6-phosphogluconate dehydrogenase purified from the liver of *Chalcalburnus tarichi*, the only fish living in Lake Van's highly alkaline water (pH 9.8)

**Muhammet GÜLER**[*], **Vedat TÜRKOĞLU, Mehmet Rıza KIVANÇ**
Department of Biochemistry, Faculty of Science, Yüzüncü Yıl University, Van, Turkey

**Abstract:** In this study, some enzymology parameters and some antibiotics affecting the enzyme activity of 6-phosphogluconate dehydrogenase (6PGD) extracted from t the livers of fish from Lake Van (*Chalcalburnus tarichi*) were investigated because it is an important enzyme-producing NADPH, a reductive power, protecting the cell against the oxidative agents by producing reduced glutathione. The crude enzyme solution was obtained by using the affinity chromatography method. The native molecular weight of the enzyme is ∼90,000 ± 3000 Da and it is composed of two subunits with identical molecular weights of ∼46,000 Da as exhibited on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The pH and temperature for optimal conditions of 6PGD were about 8.5 and 40 °C, respectively, and the enzyme showed optimal activity in 40 mM ammonium sulfate solution. In addition, in vitro effects of clindamycin phosphate, streptomycin sulfate, and lincomycin antibiotics on the enzyme activity were investigated.

**Key words:** Enzyme purification, affinity chromatography, antibiotic, enzyme inhibition

## 1. Introduction

The only fish known to live in Lake Van, Turkey, is *Chalcalburnus tarichi*. In May and June, the fish migrate from the lake to less alkaline water to spawn either near the mouths of the rivers feeding the lake or in the rivers themselves. After spawning season, they return to the lake.

Some studies have been carried out on this fish, such as studies of its systematic[1] and fisheries biology,[2] its gonadal histology and biochemical properties,[3] purification and partial properties of glucose 6-phosphate dehydrogenase from fish liver,[4] and effects of some drugs on hepatic glucose 6-phosphate dehydrogenase activity in the fish.[5]

6-Phosphogluconate dehydrogenase (EC 1.1.1.44; 6PGD), the third enzyme in oxidative reactions of the pentose phosphate metabolic pathway, catalyzes the conversion of 6-phosphogluconate (6PGA) and NADP$^+$ to D-ribulose 5-phosphate and NADPH, which protects the cell against the oxidant by producing reduced glutathione (GSH).[6,7]

There are many parameters that can affect the enzymatic activities, such as pH, temperature, and salt concentrations. Enzyme activity usually varies with pH changes. When an enzyme activity is measured based on the pH, mostly a bell-shaped curve is obtained. Its optimum pH frequently coincides with the physiological pH value (between 7.35 and 7.45) and the activity decreases significantly at both the acid and the alkaline site and drops finally to zero towards the most extreme pH ranges at both sides.

---

[*]Correspondence: mguler@yyu.edu.tr

Enzymes are also very sensitive to temperature changes. Theoretically there is no upper limit for the increase of enzyme activity with increasing temperature; however, above a distinct temperature the rate of enzyme catalysis reactions decreases to zero. This decrease is due to the limited stability, which is a general feature of the enzyme.[8]

Ionic strength is another important parameter affecting enzyme activity. This is especially noticeable where catalysis depends on the movement of charged molecules relative to each other. Thus, both the binding of charged substrates to enzymes and the movement of charged groups within the catalytic 'active' site will be influenced by the ionic composition of the medium.[9]

6PGD has been purified and characterized from many different sources, such as rat liver,[10] sheep liver,[11,12] pig liver,[13] and Lake Van fish liver.[14] However, there are not any studies in the literature on the characterization of fish liver 6PGD and on the in vitro effects of clindamycin phosphate, streptomycin sulfate, and lincomycin on the enzyme activity. Herein, our main purpose is to characterize Lake Van fish liver 6PGD and to investigate the in vitro effect of some antibiotics on the enzyme activity. Thus, 6PGD was purified and the effects of some parameters such as pH, temperature, and ionic strength were investigated. The effects of clindamycin phosphate, streptomycin sulfate, and lincomycin antibiotics on the enzyme activity were also investigated in this paper.

## 2. Results and discussion

In this study, 6PGD was purified from Lake Van fish liver. Some characteristics of the enzyme and effects of some drugs on the enzyme activity were investigated. 6PGD was purified by $2',5'$-ADP Sepharose 4B affinity gel chromatography, which is an effective one-step method for enzyme purification. Affinity chromatography is a remarkable method used to purify a protein of interest from the pool of proteins. It is carried out according to the advantage of high affinity of protein because of specific chemical groups.[15,16]

The purification method above yielded fish liver 6PGD at 446-fold and a specific activity of 23,173 EU/mg protein. The eluates were plotted by arraying out protein determination and 6PGD activity (Figure 1). SDS polyacrylamide gel electrophoresis was performed after purifying the enzyme. Fish liver 6PGD was loaded on two lines (line A and line B) using the same eluate to display the purity of the enzyme clearly (Figure 2). The electrophoretic pattern and the biochemical reaction catalyzed by the enzyme show that the enzyme was purified from fish liver because the pattern shows that there is only one protein in the eluates after purification. In addition, the chemical reaction catalyzed by the purified enzyme indicates that this protein is 6PGD. To estimate the molecular weight of the enzyme, logMw vs. relative mobility ($R_f$) was plotted using protein molecular weight marker (Fermentas unstained protein ladder SM0671) (Figure 3). The native molecular weight of the enzyme is about 93,000 Da, composed of two subunits with identical molecular weights of 46,000 Da. The molecular weight of 6PGD varies between 80 and 150 kDa according to its source. The molecular weight of the subunit of the enzyme was different from those reported for the enzyme extracted from *Phormidium* sp. (52,000),[17] *Streptococcus faecalis* (55,000),[18] and rat liver (54,000).[19]

The enzyme activity was measured at various pH values of 1 mM Tris-HCl buffer. Activity% vs. pH of the enzyme is shown in Figure 4. The optimum pH was determined as about 8.5, distinct from lamb liver,[20] pig liver,[12] human erythrocytes,[19] and the human brain.[21] More than 50% of the maximum activity was shown in the pH range of 6.0–10.0. In one study, glucose 6-phosphate dehydrogenase, the first enzyme of oxidative reactions of the pentose phosphate metabolic pathway, showed high activity between pH levels of 7.0 and 9.5.[4]
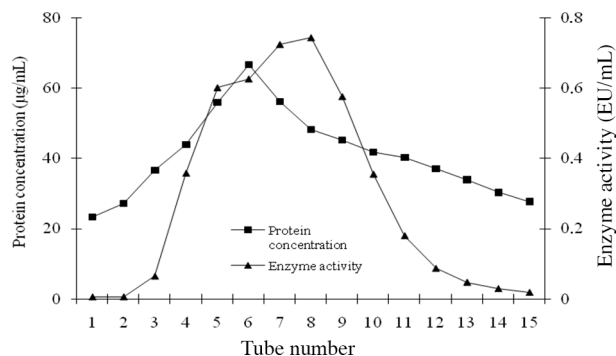
**Figure 1.** Purification of 6PGD enzyme using $2',5'$-ADP Sepharose 4B affinity chromatography column.



**Figure 2.** SDS-PAGE photograph. Lane C: standard proteins (Fermentas unstained protein ladder SM0671). Lanes A and B: purified 6PGD from Lake Van fish liver.
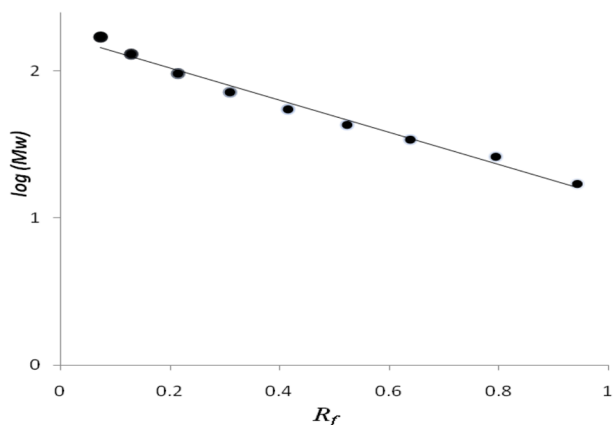


**Figure 3.** Standard $R_f$–log MW graph of fish liver 6PGD using SDS-PAGE.
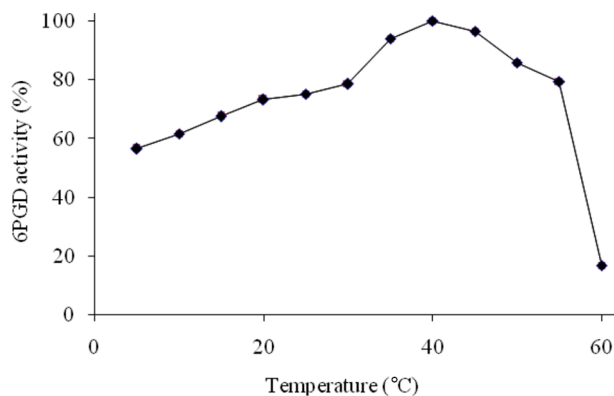


**Figure 4.** Effect of temperature on the enzyme activity. Temperature was only changed from 5 to 70 °C. Readings were taken at 1-min intervals for 5 min. The experimental cuvette contained 1 M Tris-HCl buffer (pH 8.0), 0.1 M $MgCl_2$, 6 mM 6PGA, 2 mM $NADP^+$, and 50 $\mu$L of purified 6PGD in a final volume of 1.0 mL.

The influence of temperature on the enzyme activity is displayed in Figure 5. The optimum temperature for the 6-phosphogluconate dehydrogenase activity was 40 °C. The enzyme activity reduced sharply above 55 °C. The enzyme exhibited more than 50% of maximum activity between 5 and 55 °C.

The results presented in Figure 6 show variations in enzyme activity with varying $(NH_4)_2SO_4$ in Tris-HCl buffer (pH 8.0). The enzyme exhibited maximum activity in 40 mM $(NH_4)_2SO_4$ solution. The activity reduced to the control activity after adding 50 mM $(NH_4)_2SO_4$. This result is in agreement with the literature studies.[22−24] In these studies, it has been reported that the enzyme shows maximum activity in lower salt concentrations.
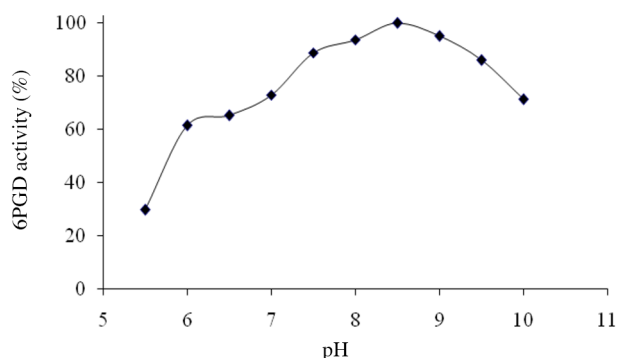


**Figure 5.** Effect of pH on the enzyme activity. Readings were taken at 1-min intervals for 5 min. Tris-HCl buffer was used from 5.5 to 9.5. The complete system contained 1 M Tris-HCl buffer, 0.1 M $MgCl_2$, 6 mM 6PGA, 2 mM $NADP^+$, and 50 $\mu$L of purified 6PGD in a final volume of 1.0 mL.
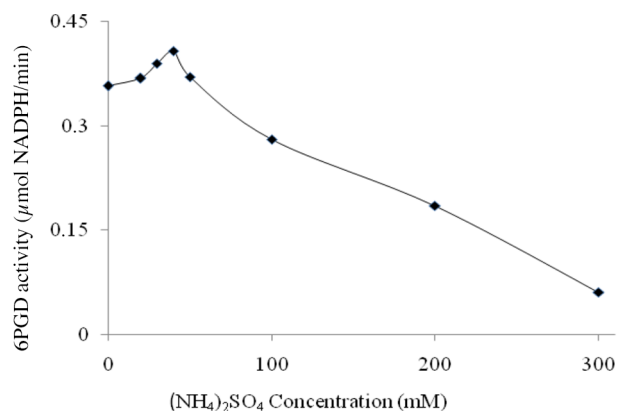
**Figure 6.** Effect of ionic strength on the enzyme activity. The assay system was identical to that described in Figure 4, except that $(NH_4)_2SO_4$ was used from 0.02 to 0.3 M.
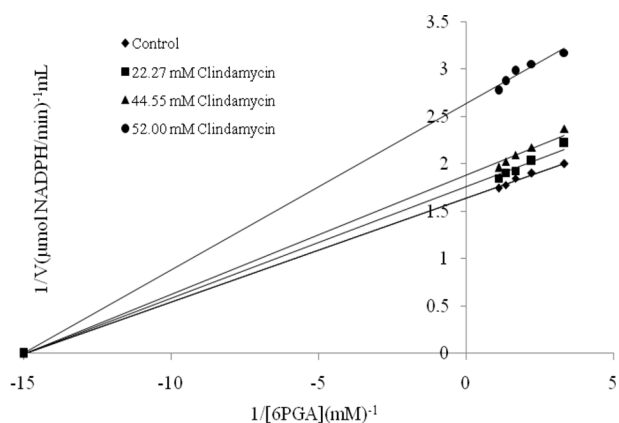


**Figure 7.** Lineweaver–Burk graphs with five different substrate concentrations (6PGA) and three different clindamycin phosphate concentrations used for the determination of $K_i$.
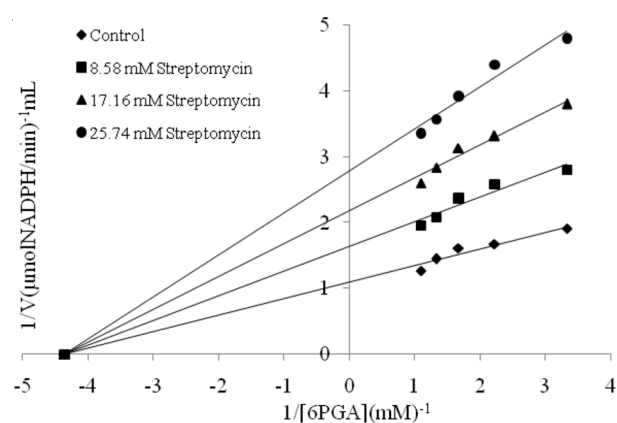
**Figure 8.** Lineweaver–Burk graph with five different substrate concentrations (6PGA) and three different streptomycin sulfate concentrations for determination of $K_i$. The assay system was the same as that described in Figure 7 except that three different concentrations of streptomycin sulfate (8.58, 17.16, and 25.74 mM) were used for the experimental cuvette in place of clindamycin phosphate.

In this study, clindamycin phosphate, streptomycin sulfate, and lincomycin antibiotics showed noncompetitive inhibition on the in vitro enzyme activity (Table). In order to determine quantitative inhibitory effects

1053

of these antibiotics on the enzyme activity, both the $K_i$ and the IC50 parameters of these drugs were calculated. The $K_i$ values of clindamycin phosphate (Figure 7), streptomycin sulfate (Figure 8), and lincomycin (Figure 9) were calculated from Lineweaver–Burk graphs. The IC50 values of clindamycin phosphate (Figure 10), streptomycin sulfate (Figure 11), and lincomycin (Figure 12) were estimated from activity (%)–drug concentration graphs. In conclusion, the enzyme has more affinity to streptomycin sulfate than clindamycin phosphate and lincomycin taking into consideration both the $K_i$ and IC50 values.
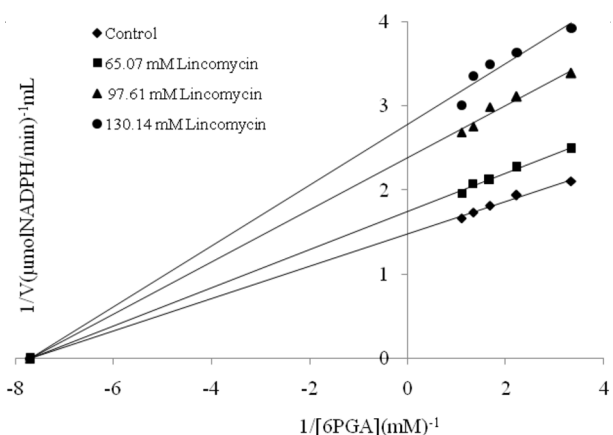


**Figure 9.** Lineweaver–Burk graph with five different substrate concentrations (6PGA) and three different lincomycin concentrations used for the determination of $K_i$. The measurement system was the same as that described in Figure 8, except that three different concentrations of lincomycin (32.50, 65.00, and 97.50 mM) were used for the experimental cuvette in place of clindamycin phosphate.
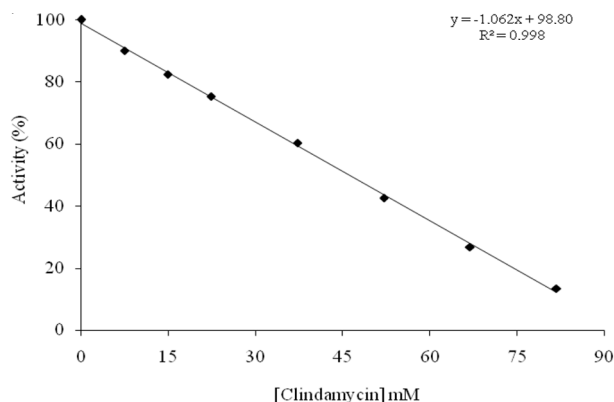
**Figure 10.** Activity% vs. clindamycin analysis graph for fish liver 6PGD. Readings were taken at 1-min intervals for 5 min. The control cuvette contained 1 M Tris-HCl buffer (pH 8.0), 0.1 M $MgCl_2$, 6 mM 6PGA, 2 mM $NADP^+$, and 50 $\mu$L of purified 6PGD in a final volume of 1.0 mL. The experimental cuvette was identical to the control cuvette, except that seven different concentrations of clindamycin phosphate (7.43, 14.85, 22.27, 37.13, 52.00, 66.83, and 81.68 mM) were used.

**Table.** The effect of clindamycin phosphate, streptomycin sulfate, and lincomycin on 6PGD activity purified from *Chalcalburnus tarichi* liver.

| | Inhibition type | $K_i$ (mM) | IC50 (mM) |
|---|---|---|---|
| Clindamycin phosphate | Noncompetitive | $34.69 \pm 4.01^*$ | 51.05 |
| Streptomycin sulfate | Noncompetitive | $16.98 \pm 3.102$ | 27.63 |
| Lincomycin | Noncompetitive | $223.78 \pm 32.16$ | 94.29 |

*Each value is the average of three measurements.

## 3. Experimental

$2',5'$-ADP Sepharose 4B was obtained from Amersham Biosciences. 6PGA, protein assay reagents, and chemicals for electrophoresis were purchased from AppliChem GmbH. $NADP^+$ and NADPH were purchased from Sigma Chemical Company. All other chemicals used were purchased from either AppliChem or Merck. The antibiotics were used as received without further purification.
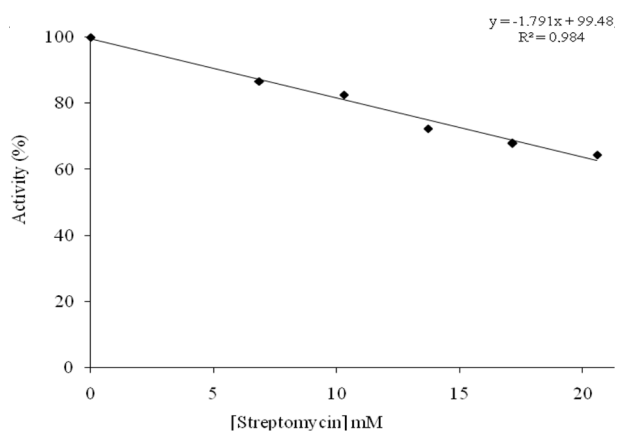
**Figure 11.** Activity% vs. streptomycin sulfate analysis graph for fish liver 6PGD. The control and experimental cuvettes were identical to that described in Figure 10, except that five different concentrations of streptomycin sulfate were used for the experimental cuvette in place of clindamycin phosphate.
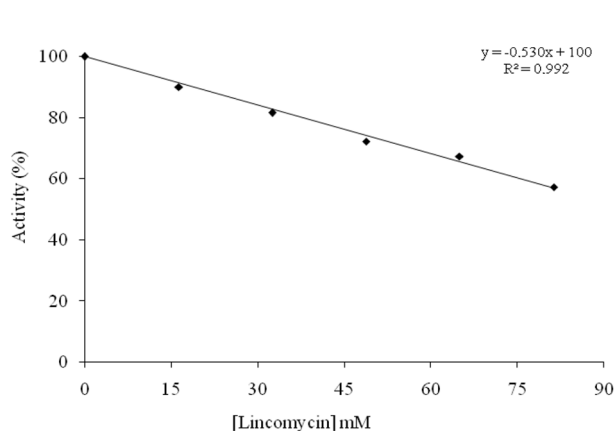


**Figure 12.** Activity% vs. lincomycin analysis graph for fish liver 6PGD.

## 3.1. Homogenate preparation

Ten grams of fresh fish liver was cut into small pieces and added into 0.02 M phosphate buffer (pH 7.4) containing 8.25 g of sucrose. The mixture was homogenized in a Waring blender, and then the homogenate was centrifuged at 12,000 rpm and 4 °C for 60 min and the precipitate was removed. This last process was repeated three times.

## 3.2. Preparation of 2′,5′-ADP Sepharose 4B Affinity Chromatography Column

The 2′,5′-ADP Sepharose 4B Affinity Chromatography Column was prepared by the following sequence: i) 2 g of dried 2′,5′-ADP Sepharose 4B gel was washed with distilled water, to remove impurities and air, and suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0); ii) the gel was packed into a column; iii) the column was equilibrated with 50 mM K-phosphate buffer (pH 6.0) containing 1 mM EDTA by a peristaltic pump; iv) the dialyzed sample obtained previously was loaded into the column; v) the column was 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), respectively; vi) the latter washing was continued with 0.1 M K-acetate/0.1 M K-phosphate (pH 7.85), until the last absorbance became 0.05 at 280 nm; vii) the enzyme was eluted using 5 mM NADP$^+$ containing 80 mM K-phosphate, 80 mM KCl, and 10 mM EDTA (pH 7.85). Active fractions were collected for further studies.[25,26]

## 3.3. Protein determination

The Bradford protein assay method was used for the determination of total protein concentrations in both the dialyzed sample previously prepared and the active fractions depending upon the change in absorbance at 595 nm based on the proportional binding of the dye Coomassie Blue G-250 to proteins. For this purpose, a standard graph of absorbance vs. bovine serum albumin concentration was drawn and then protein concentrations in the samples were estimated by this graph.[27]

### 3.4. Enzyme activity determination

The enzyme activity was measured according to the Beutler method.

$$6PGA + NADP^+ \xrightarrow{6PGD} \text{Ribulose-5-phosphate} + NADPH + H^+ + CO_2$$
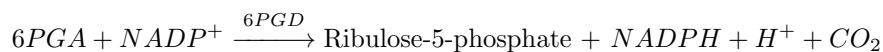
The rate of the formation of NADPH is proportional to 6PGD activity as shown in the above reaction and it is measured spectrophotometrically as an increase in absorbance at 340 nm with a Shimadzu Spectrophotometer UV-1800. The assay system contained 1 mM Tris-HCl buffer (pH 7.5), 5 mM EDTA, 6 mM 6PGA, and 2 mM $NADP^+$ in a total volume of 1 mL. One enzyme unit is defined as the reduction of 1 $\mu$mol of $NADP^+$ per minute under the assay conditions.[28]

### 3.5. Molecular weight determination by SDS-PAGE

To control the purity of the enzyme, SDS-polyacrylamide gel electrophoresis was used according to Laemmli's procedure. The acrylamide concentrations of the stacking and separating gels were selected as 3% and 10%, respectively, and 1% SDS, which provides some functions such as denaturing secondary, tertiary, and quaternary structures by binding to hydrophobic regions of protein, was added to the gel solution. The gel was stabilized in a solution containing 50% propanol, 10% TCA, and 40% distilled water for about 30 min. Then the gel was added to the staining solution containing 0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid, and 39.9% distilled water for about 2 h. Finally, the gel was washed varying the same solvent without dye until protein bands were cleared.[29]

### 3.6. Optimum pH and temperature determination

pH measurements were done at a pH range from 5.5 to 10.0 with 1 M Tris-HCl buffer containing 5 mM EDTA, 0.1 M $MgCl_2$, 2 mM $NADP^+$, and 6 mM 6PGA to determine optimum pH for the enzyme activity and create an activity (%) vs. pH graph.[30]

To estimate optimum temperature for the enzyme activity, measurements were carried out at different temperatures (ranging from 5 to 70 °C) with 1 M Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, 0.1 M $MgCl_2$, 2 mM $NADP^+$, and 6 mM 6PGA, and then the activity (%) vs. temperature graph was drawn.[19]

### 3.7. Effect of ionic strength

Different concentrations of $(NH_4)_2SO_4$ (ranging from 20 to 300 mM) were used to investigate the effect of ionic strength on the enzyme activity. Assays were performed at 37 °C.

### 3.8. In vitro studies

Different concentrations of clindamycin phosphate (48.8–113.9 mM), streptomycin sulfate (8.85–25.74 mM), and lincomycin (32.5–97.5 mM) antibiotics were added to the reaction mixture containing 0.1 M $MgCl_2$, 2 mM $NADP^+$, and 1 mM Tris-HCl buffer (pH 8.0) in order to investigate the effects of the antibiotics on the enzyme activity. The mixture was incubated for 10 min, and then 6PGA was added to the reaction mixture and absorbances at 340 nm were taken at intervals of 5 min. The activity measurement was repeated three times for each control and drug concentration using the same eluate. 6PGD activity was calculated using the following equation:

$$A = (\Delta OD/6.22) \times (Vc/Ve) \times f,$$

where $\Delta$OD is the increase in the absorbance at 340?nm per minute, Vc (mL) is total reaction volume, Ve (mL) is the volume of enzyme solution (diluted sample) added, f is the dilution coefficient, and 6.22 is the millimolar absorption coefficient of NADPH molecules.[28]

So as to find out the $K_i$ constants of the antibiotics, Lineweaver–Burk graphs were drawn. The IC50 (half maximal inhibitory concentration) values of the antibiotics were obtained from activity (%) vs. drug concentration plots. These antibiotics can bind to different sites of the enzyme because the drugs used here are not similar to the substrate of the enzyme at the molecular level.

## 4. Conclusion

In this study, 6PGD has been purified 446-fold with a specific activity of 23,173 EU/mg protein. This work has demonstrated that 6PGD has two subunits with identical molecular weights.[17−19] The native molecular weight of the enzyme was estimated as 93,000 Da, composed of two exactly equivalent subunits with a molecular weight of approximately 46,000 Da using SDS-PAGE. The enzyme shows its optimal activity at about 8.0–9.0 pH and the lake water is highly alkaline (almost pH 9.8). It can be concluded that the fish has adapted to the lake water. Antibiotics are the most used drugs with the aim of the treatment of different diseases. These medicines have side effects as well as their healing properties. They negatively affect other metabolic pathways such as the pentose phosphate pathway and glycolysis pathway. Among these pathways, the oxidative pentose phosphate pathway is an important metabolic pathway producing NADPH and ribose-5-phosphate. 6PGD is the enzyme that catalyzes the third reaction of this pathway. The antibiotics used in the study inhibited 6PGD noncompetitively, which has been shown by in vitro study. This study can also be supported by in vivo experiments. We think that this work will contribute to similar future works and the literature.

## Acknowledgment

## References

1. Deyrolle, T. *Rev. Mag. Zool. (Paris) 2ieme serie* **1872**, *23*, 401–405.

2. Çetinkaya, O. In *Eastern Anatolia I. and II. Symposium on Fisheries*, Erzurum, Turkey, 1996, pp. 71–83.

3. Ünal, G.; Türkoğlu, V.; Oğuz, A. R. *Fish Physiol. Biochem.* **2007**, *33*, 153–165.

4. Türkoğlu, V.; Altun, M.; Çiftçi, M. *J. Physiol. Biochem.* **2006**, *62*, 155–161.

5. Çiftçi, M.; Türkoğlu, V.; Çoban, T. A. *J. Hazard. Materials* **2007**, *143*, 415–418.

6. Lehninger, A. L.; Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*; W.H. Freeman: New York, NY, USA, 2000.

7. Bianchi, D.; Bertrand, O.; Haupt, K.; Coello, N. *Enzyme Microb. Technol.* **2001**, *28*, 754–759.

8. Bisswanger, H. *Enzyme Kinetics: Principles and Methods*; Wiley: Weinheim, Germany, 2008.

9. Chaplin, M. F.; Buche, C. *Enzyme Technology*; Cambridge University Press: New York, NY, USA, 1990.

10. Maglysh, S. S.; Gorbach, Z. V.; Ostrovskii, I. U. M. *Biokhimiia* **1982**, *47*, 2035–2041.

11. Villet, R. H.; Dalziel, K. *Biochem. J.* **1967**, *104*, 38–39.

12. Somers, D. O.; Hajdu, J.; Adams, M. J. *Protein Expr. Purif.* **1991**, *2*, 385–389.

13. Toews, M. L.; Kanji, M. L.; Carper, W. R. *J. Biol. Chem.* **1976**, *251*, 7127–7131.

14. Güler, M.; Kıvanç, M. R.; Türkoğlu, V.; Basi, Z.; Kıvrak, H. *Bull. Environ. Contam. Toxicol.* **2013**, *9*, 560–564.

15. Prath, R. J.; Ernback, S. *Nature* **1967**, *214*, 1302–1304.

16. Cuatrecases, P.; Anfinsen, C. B. *Methods in Enzymology*; Academic Press: New York, NY, USA, 1971.

17. Sawa, Y.; Suzuki, K.; Ochiai, H. *Agric. Biol. Chem.* **1985**, *49*, 2543–2549.

18. Bridges, R. B.; Palumbo, M. P.; Wittenbreger, C. L. *J. Biol. Chem.* **1975**, *250*, 6093–6100.

19. Beydemir, B.; Çiftçi, M.; Yılmaz, H.; Küvrevioğlu, O. I. *Turk J. Vet. Anim. Sci.* **2004**, *28*, 707–714.

20. Carne, A. *Anal. Biochem.* **1982**, *121*, 227–229.

21. Weisz, K. S.; Schofield, P. J.; Edwards, M. R. *J. Neurochem.* **1985**, *44*, 510–517.

22. Procsal, D.; Holten, D. *Biochem.* **1972**, *11*, 1310–1314.

23. Dyson, J. E.; D'Orazio, R. E.; Hanson, W. L. T. *J. Biol. Chem.* **1973**, *248*, 5428–5435.

24. Pearse, B.; Rosemeyer, M. A. *Eur. J.Biochem.* **1974**, *42*, 213–223.

25. Ninfali, P.; Orsenigo, T.; Barociani, S. R. *Prep. Biochem. Biotechnol.* **1990**, *20*, 297–309.

26. Akyüz, M.; Erat, M.; Çiftçi, M.; Gümüştekin, K.; Bakan, N. *J. Enzyme Inhib. Med. Chem.* **2013,** *19*, 361–365.

27. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–251.

28. Beutler, E. *Red Cell Metabolism*; Grune & Stratton: New York, NY, USA, 1984.

29. Laemmli, D. K. *Nature* **1970**, *227*, 680–683.

30. Rosemeyer, M. N. *Cell. Biochem. Func.* **1987**, *5*, 79–95.