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Purification and Characterization of 6-Phosphogluconate Dehydrogenase from Japanese Quail (*Coturnix, coturnix japonica*) Erythrocytes

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ABSTRACT: In this study, the 6PGD enzyme from Japanese quail erythrocytes was purified with specific activity of 52.84 EU/mg and 69% yield of purification by 2', 5'- ADP Sepharose 4B affinity gel in a single chromatographic method. The purification folds of the enzyme were 4984 folds. The purified enzyme was checked using SDS polyacrylamide gel electrophoresis (SDS-PAGE) method; the result of gel showed a single band. The subunit molecular weight of the enzyme was calculated as 81 kDa by the SDS-PAGE method. The characterization studies of the 6PGD enzyme from erythrocytes of Japanese quail showed: the optimum ionic strength to be at 0.5 M Tris-HCl, optimum and stable pH values to be at 0.5 M Tris-HCl buffers pH 8.0. The optimal temperature for the enzyme activity was found at 60 ˚C. Finally, the K_M and V_{max} values for the 6PGD enzyme from Japanese quail's erythrocytes were calculated respectively for the 6PGA the K_M value found as 0.120 mM, V_{max} value as 0.191 EU/mL and for $NADP^+$ the K_M value as 0.017 mM and V_{max} value as 0.228 EU/mL.

Key words: Quail, 6PGD, erythrocytes, purification, characterization.

6-Fosfoglukonat Dehidrogenaz Enziminin Japon Bıldırcın (*Coturnix Coturnix Japonica*) Eritrositlerinden Saflaştırılması ve Karakterizasyonu

ÖZET: Bu çalışmada Japon bıldırcın eritrosit dokularından 6-fosfoglukonat dehidrogenaz enzimi (6PGD) 52,84 $EU.mg^{-1}$ spesifik aktiviteyle ve % 69 verimle 2', 5'-ADP Sepharose 4B afinite kromatografisi kullanılarak saflaştırıldı. Japon bıldırcın eritrositlerinden saflaştırılan 6PGD enzimi için saflaştırma katsayısı 4984 olarak bulundu. Enzim saflığının kontrolü için SDS-poliakrilamid jel elektroforezi (SDS-PAGE) yapıldı ve tek bant gözlendi. SDS-PAGE yöntemi kullanılarak enzimin alt birimlerinin molekül kutlesi 81 kDa olarak hesaplandı. Optimum iyonik şiddet 0,5 M Tris-HCl, optimum pH ve stabil pH 0,5 M Tris-HCl tamponu pH 8,0 olarak bulundu. Optimum sıcaklık 60 ºC olarak bulundu. Ayrica Japon bıldırcın 6PGD enziminin K_M ve V_{max} değerleri Lineweaver-Burk grafiklerinden sırasıyla 6PGA substratı için K_M değeri 0,120 mM, V_{max} değeri 0,191 EU/mL, $NADP^+$ substratı için K_M değeri 0,017 mM, V_{max} değerise 0,228 EU/mL olarak hesaplandı.

Anahtar kelimeler: Bıldırcın, 6PGD, eritrosit, saflaştırma, karakterizasyon.

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INTRODUCTION

6-Phosphogluconate Dehydrogenase (6PGD) enzyme commission number E.C 1.1.1.44 is the third enzyme of the pentose phosphate pathway. It is responsible for the conversion of 6-phosphogluconic acid 6PGA to D-ribulose 5-phosphate and CO_2 with existing of $NADP^+$ coenzyme (Beydemir et al. 2004). The enzyme is located in the cytoplasm of all living cells, including microorganisms, plants and animal cells (Toews et al., 1976).

Pentose phosphate pathway (PPP) (Figure 1), takes place in the cytoplasm of the cells since the enzymes in which catalyze the reactions of the PPP process are cytosolic enzymes. Here in PPP $NADP^+$ is used for dehydrogenation as the hydrogen atom acceptor. The reactions in this pathway are divided into two phases (irreversible oxidative phase, reversible non oxidative phase). First phase is the phase of dehydrogenation and decarboxylation of glucose 6-phosphate that yields a pentose sugar (ribulose 5-phosphate). Second phase is converting of ribulose 5-phosphate to glucose 6-phosphate by a series of reactions catalyzed by (trans ketolase and trans aldolase) enzymes (Murray et al., 2003).

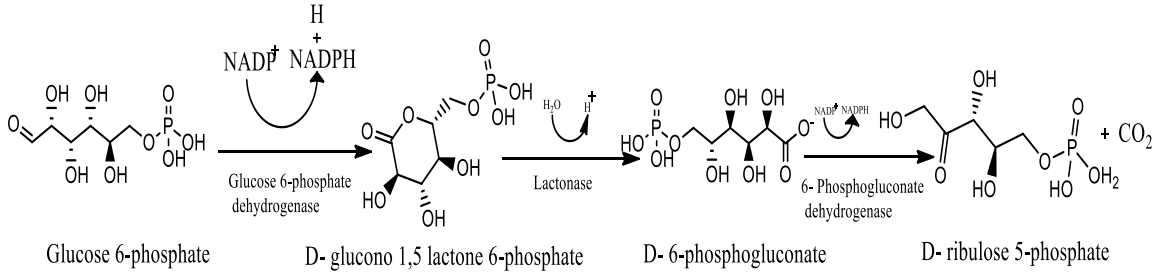

Figure 1: 6PGD enzyme catalyzing reaction

In the enzyme's reaction, NADPH is also produced. It has the function of protecting cells against oxidizing agents by producing the reduced glutathione GSH (Bianchi et al. 2001; Nelson and Cox 2005). Besides that, NADPH plays a role in producing of many biomolecules as fatty acids, steroids and some amino acids (Human et al. 1983; Srivastava and Beutler 1970). Due to its functions 6PGD could be called as an antioxidant enzyme. In cases enzyme's deficiency, the production levels of NADPH would be less than normal. In some cases no NADPH productions would be observed at all. This condition causes an increase of the erythrocyte's sensitivity towards the activity of the hemolytic oxidative agents and leads to damaging of the cells. The enzyme's kinetic properties, its role in the pentose phosphate pathway, regulations of the enzyme, genetics and the enzyme's role for the production of fatty acids had been studied for long time (Zera et al., 2014). The 6PGD enzyme from the prokaryotic and eukaryotic microorganisms sequenced, the result showed it had 468 amino acids. The arrangement of the protein amino acids is almost similar in the prokaryotic and eukaryotic microorganisms, reversibly the bovine 6PGD that comprises of 466 amino acids shows the central region on the protein to be alike of those of bacterial enzyme but different at the protein C- and N- terminals (Reizer et al. 1991). The sequences of the amino acids for 6PGD enzymes from about 40 various sources had been reported containing pig (Harbitz et al., 1990), Bacillus subtilis (Fujita and Fujita, 1987), sheep (Carnes and Walker, 1983), human (Kwok et al., 1996) and Escherichia coli (Nasoff et al. 1984). The three-dimensional enzyme structure for sheep's liver enzyme is the only one that was determined at a resolution of 2.5 A° (Adams et al., 1983). The sequencing results for the 6PGD enzyme from 37 various sources showed that the highest important regions of the enzyme's structure like the active site, in which the enzymes functions of binding to the substrates and coenzymes are happen, is located in conserved regions in the enzyme's

structure. In addition some other regions of the enzymes structures from all different species showed a big similarity in their locations (Goulielmos et al., 2004). The aim of the study was purification and characterization of 6- phosphogluconate dehydrogenase enzyme from quail erythrocyte.

EXPERIMENTALS AND METHODS

Chemicals

The chemicals used for conducting all the experiments throughout this study were all analytical graded from Sigma, Merck, Fishcer Scientific, ChemSolute bio and Applichem and they are listed as follows:

From Sigma: ammonium sulfate, magnesium chloride, sodium chloride, sodium bicarbonate, sodium hydroxide, potassium phosphate, potassium chloride, EDTA ethylene diamine tetra acetic acid, Tris (trihydroxy methyl amino methane), isopropanol, β-mercaptoethanol, acrylamide, TEMED (N, N, N, N tetramethylethylenediamine), silver nitrate, hydrochloric acid, phosphoric acid, glycerine, ethanol, methanol, acetic acid, bovine serum albumin, 6PGA (6-phosphogluconate monosodium salt), 2', 5'-ADP Sepharose 4B. From Merck: potassium acetate, bromothymol blue, sodium acetate, potassium hydroxide, glycine amino acid and trichloroacetic acid. From Fishcer Scientific: SDS (sodium dodecyl sulfate), Commasie Brilliant Blue G-250, Brilliant Blue R-250 electrophoresis grade. From the Applichem: $NADP^+$ and from the ChemSolute Bio: ammonium persulfate.

Sample collection and preparing the hemolysate

Blood samples of the Japanese quail were obtained using decapitation method from the agriculture faculty's research farm of the Bingol University. Blood samples were kept at 4°C and transferred to the laboratory within EDTA containing tubes. In order to distinguish the erythrocyte from the plasma, blood samples were filtered to remove any impurities and equally distributed into eppendorf tubes then centrifuged at 2500 rpm at 4°C for 15 minutes. The plasma and leukocytes were removed using a dropper then the precipitated erythrocytes washed for three times by 0.16 M KCl solution and each time the solution centrifuged for 15 minutes at 2500 rpm at 4°C. Later in order to obtain the hemolysate, the RBCs were hemolyzed by using 1:5 volume of ice water, the hemolysate solution was mixed well to ensure the cell membrane decomposition. Then, for the removal of the cell debris in the hemolysate, the blood was centrifuged for 30 minutes at 4°C using a speed of 10 000 g. After the centrifugation completed, the supernatant was isolated from the precipitate and used for further enzyme purification processes that include the quantitative protein determinations and loading the hemolysate to the 2', 5'-ADP Sepharose 4B affinity gel chromatography column (Ninfali et al., 1990).

2', 5'-ADP Sepharose 4B affinity gel chromatography

For preparing the affinity gel, 2 g of solid 2', 5'-ADP Sepharose 4B were poured into a 10 mL (1×10 cm) volume tube, the substance washed repeatedly using 400 mL of distilled water (D.W) for removing any foreign materials in the gel if existed; during the washing process, the gel swelled up. The air bubbles removed inside the swelled gel by water trompe using a balancing homogenizing buffer (0.1 M potassium acetate/ 0.1 M potassium phosphate pH 6.0). By the end of this process, the suspension of the gel was accomplished in 1×10 cm closed column system where the gel packed. In the next step, the packed gel was washed using a balancing buffer and the flow rate controlled by a peristatic pump. The washing process done by using a setting of 20 mL. $Hour^{-1}$ of the peristatic pump (Ninfali et al. 1990). After the column was homogenized with the (pH 7.3) balancing buffer, the column would be ready for using. The prepared hemolysate from previous step loaded into the affinity column then washed repeatedly and respectively with 25 mL volume portions of 0.1 M potassium acetate/ 0.1 M potassium

phosphate (pH 7.3). Then 25 mL of 0.1 M KCl / 0.1 M potassium phosphate pH 7.85 the flow rate set up fixed at 20 mL/hour by using the peristatic pump and temperature kept at 4℃. After removing all of the unwanted debris and proteins, elution buffer containing 80 mM potassium phosphate/ 80 mM KCl + 5 mM $NADP^+$ and 10 mM EDTA pH 7.3, used to elute out the attached 6PGD enzyme from the gel in the column. The eluate should be collected in fractions using labeled Eppendorf tubes, and each fraction should be tested for the enzyme's activity (Ninfali et al., 1990; Morelli et al., 1978).

Measurement of 6PGD enzyme activity:

The enzyme's activity measured spectrophotometrically at 25℃ using Beutler's method. In this method the absorbance of reduction of $NADP^+$ to NADPH was measured depending on time at 340 nm.

Qualitative determination of proteins

The eluted fractions from the affinity column were tested for existence of proteins qualitatively. The absorbances of fractions were measured against the elution buffer as blank at 280 nm which is the maximum wavelength (λmax) for tryptophan, phenyl alanine and tyrosin (Segel, 1968).

Quantitative determination of protein

The Bradford assay protocol was used for quantitative determination of proteins, BSA was used as a standard (Bradford, 1976).

SDS-PAGE

The eluted 6PGD enzyme from the affinity column checked for determining of its purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis. 0.1% SDS containing 3% and 10% of acrylamide and bisacrylamide respectively were used in stacking gel and separation gels. The molecular weight of the 6PGD enzyme purified was determined by SDS-PAGE (Laemmli, 1970).

Determination of the optimum pH

The pH value which the enzyme has its maximum activity at is assigned as the optimum pH value for the 6PGD enzyme activity. Different buffers with different ranges of pH values used. First is TrisHCl buffer, 0.5 M pH ranges of 7.0 - 9.0. Second buffer is potassium phosphate 1 M, pH ranges from 5.5 - 7.5 and 8.0.

Determination of the stable pH

Two buffers 0.5 M Tris-HCl and 1 M potassium phosphate were used for determining the stable pH value; the Tris-HCl buffer pH ranged from 7.0 – 9.0 and pH range of potassium phosphate buffer was from 5.5 to 8.0.

Determination of the optimum ionic strength

For determining the effect of ionic strength on the 6PGD enzyme, different series concentrations of Tris-HCl buffer (10 mM -1000 mM) used in order to determine the maximum activity of the 6PGD enzyme.

Determining the optimum temperature

In order to determine the optimum temperature for the maximum enzyme's activity, the enzyme assayed using 0.5 M, Tris-HCl buffer pH 8.0 at different ranges of temperatures (0 ℃ to 70 ℃). The temperature at which the enzyme gave its maximum activity assigned as the optimum temperature for the enzyme.

Kinetic studies

For determining the K_M and V_{max} values of the substrate and coenzyme (6PGA and $NADP^+$) of the 6PGD enzyme, a method contained of using stable concentrations of $NADP^+$ with 8 different concentrations of 6PGA (100 mM, 200 mM, 300 mM, 400 mM and 500 mM) for finding the 6PGAs (K_M and V_{max}), and likewise, for $NADP^+$ coenzyme, stable concentrations of 6PGA used in the enzyme's assay with 8 different concentration of the $NADP^+$ (20mM, 40 mM, 66 mM, 75mM, 100 mM) coenzyme solution for finding the $NADP^+$s (K_M and V_{max}) values. The data collected from these measurements used for plotting of the Lineweaver-Burk graphs for both $NADP^+$ and 6PGA in which the values for K_M and V_{max} were found by calculations in order to indicate the affinity of the 6PGD enzyme towards the two substrate and coenzyme.

RESULTS AND DISCUSSIONS

In this study, 6PGD enzyme was purified and characterized in quail erythrocytes. (Coturnix coturnix japonica) erythrocytes for the first time. Different methods were used for the purification of 6PGD enzyme throughout the literature of enzymology like DEAE cellulose; CM-cellulose ions exchange chromatography (Villet and Dalziel, 1969), DEAE Sephadex, hydroxy apatite (Silverberg and Dalziel, 1973), $NADP^+$ Sepharose, $NADP^+$ agarose (Betts and Mayer, 1975), matrix gel-A column chromatography (Somers et al. 1991), DEAE Sephadex A-50 ion-exchange chromatography (Demir et al., 2003), 2', 5'-ADP-sepharose 4B affinity gel (Altıkat et al., 2002; Akyüz et al., 2003; Bayındır et al., 2018; Akkoyun et al., 2018). The method used for the purification of the enzyme was 2', 5'-ADP Sepharose 4B affinity gel chromatography, which is a very powerful, reliable, easy, economic and a single step purification method that gives very good purification yields with an ability to purify bulk amounts of the enzymes (Temel et al., 2017a,b; Temel and Kocyigit, 2017). These advantages of the method are some of the characteristics of it which make it by comparison to other purification methods to purify different enzymes (Temel et al., 2018; Temel and Bayındır, 2019).

The SDS-PAGE experiment was conducted for checking of the purity and the determining of molecular weights of the purified enzyme that eluted from the affinity column, the results of the test showed a single band protein on the gel for two of the eluates that had the maximum activity of the 6PGD in them. This proved the existence of only one type of protein in the eluate fraction which is the 6PGD enzyme from the Japanese quail erythrocytes (Figure 2).

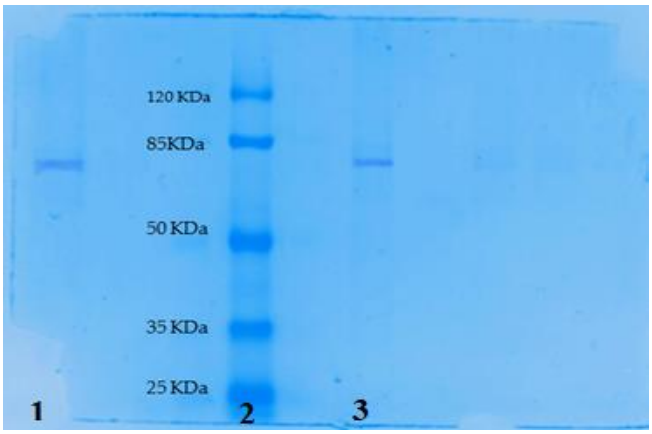

Figure 2. The SDS-PAGE photograph for the 6PGD Japanese quail's erythrocytes enzyme: the 120 kDa protein is β-galactosidase from *E. coli*, 85 kDa protein is bovine serum albumin from bovine plasma, 50 kDa protein is the egg white ovalbomin, the 35 kDa protein is carbonic anhydrase protein from human erythrocytes and 29 kDa standard protein is CA from cattle's erythrocytes in line 2. The 81 kDa protein in lane 1 and 3 is the 6-phosphogluconate dehydrogenase enzyme protein from Japanese quail's erythrocytes.

The quality and efficiency of the purification method was calculated using the Bradford's method (Bradford 1976). The amount of proteins, volumes and activities in the hemolysate and purified enzyme samples were used for calculation of the purification and the resulted purified enzyme solution. The purification result shows 69% yields of purification, 4984 fold purification of the proteins, and the specific activity of 52.84 EU. mg^{-1} for the purified 6PGD enzyme (Table 1).

Table 1. The information about purification of the 6PGD Japanese quail's erythrocytes enzyme using 2', 5'-ADP Sepharose 4B affinity gel chromatography

Sample	Volume (mL)	Protein ($mg.mL^{-1}$)	Total protein (mg)	Activity ($EU.mL^{-1}$)	Total activity (EU)	Specific activity ($EU.mg^{-1}$)	Yield %	Purification folds
Hemolysate	25	8.097	202.425	0.086	2.15	0.0106	100	1
2', 5'-ADP Sepharose 4B affinity column	5	0.00562	0.02812	0.297	1.485	52.84	69	4984

6PGD enzyme had been investigated in many different sources and checked for its kinetic properties and characterized for its functions. The enzyme had been purified from sheep's liver (Villet and Dalziel, 1972), parsley's leaves (Demir et al., 2003), chicken's liver (Erat, 2005), rat's small intestine (Ceyhan et al., 2005), Van's cat erythrocytes (Kiliç, 2007), human's erythrocytes (Özabacigil, 2005), human cerebral (Weisz et al., 1985), rabbit mammary glands (Betts and Mayer, 1975) and rat heart and lung tissues (Adem, 2010). The enzyme's molecular weight was determined by SDS-PAGE method described previously. The molecular weight of the subunit of the purified enzyme found to be ~81 kDa. The molecular weights of 6PGD enzymes from different sources in literature had been 59.5 kDa from the rat's erythrocytes (Beydemir et al., 2004), 52.6 kDa from Van's cat erythrocyte (Kiliç, 2007), 52 kDa from rat's small intestine (Ceyhan et al., 2005), *E. coli* bacteria had 53 kDa 6PGD (Wang and Zhang, 2009), 118.34 kDa from rainbow trout by gel filtration chromatography (Taranci, 2011) and 24.1 kDa from parsley (Demir et al., 2003). By looking through above molecular weights from different sources, we find that the molecular weight of 6PGD enzyme from Japanese quail's is different from all the mentioned sources.

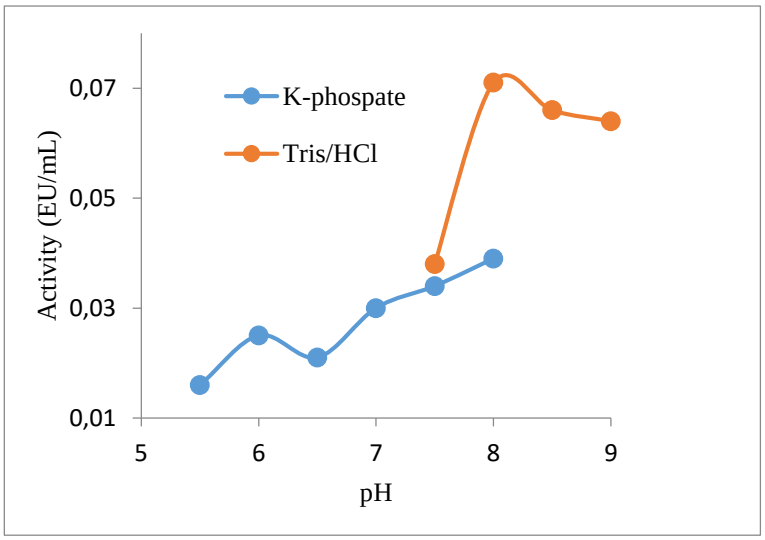

Figure 3. The optimum pH value test of the 6PGD enzyme activity from japanese quail erythrocytes

The optimal pH value test for the quail's 6PGD enzyme activity was performed using two different buffer solutions 0.5 M Tris-HCl buffer and 1 M potassium phosphate buffer as described before. The results showed that the optimum pH value for the enzyme's activity was at pH 8.0, in Tris-HCl buffer (Figure 3).

The effect of ionic strength on the enzyme activity was tested as described previously. The optimum ionic strength was determined at pH 8.0, in the 500 mM Tris-HCl, buffer (Figure 4).

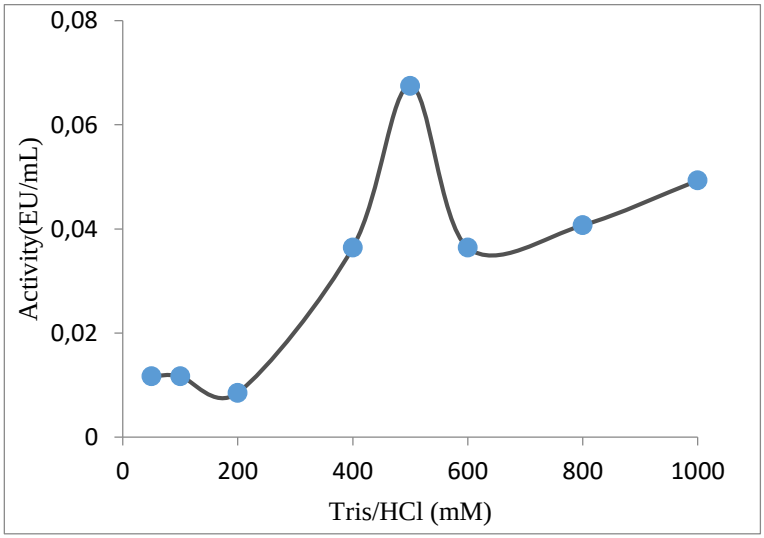

Figure 4. The optimum ionic strength effect on 6PGD Japanese quail's erythrocyte enzyme

The optimal temperature test for the enzyme's activity conducted as described in before, an increase in the activity of the enzyme was observed due to the elevating temperatures. The activity increased until 60°C where the enzyme had its maximum activity. However, the enzyme lost its activity when the temperature elevated more than 60°C due to the denaturation of the enzyme's protein structure by the effect of heat (Figure 5).

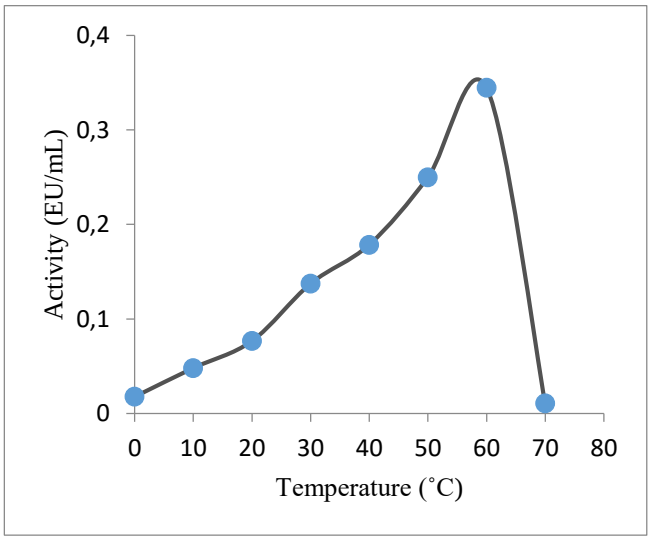

Figure 5. The effect of elevating temperatures on the 6PGD Japanese quail's enzyme activity.

The enzyme's stability tested against time and storage conditions in acidic/basic mediums as described before. The samples were assayed twice a day. The most suitable buffer solution that mostly kept the activity of the 6PGD enzyme of Japanese quail was 0.5 M Tris-HCl pH 8.0 buffer. The enzyme

maintained its activity for highest levels along seven days of measurements in the buffer. However, the most suitable pH value of the stored 6PGD enzyme in the potassium phosphate buffer was at pH 7.5 (Figure 6a,b).

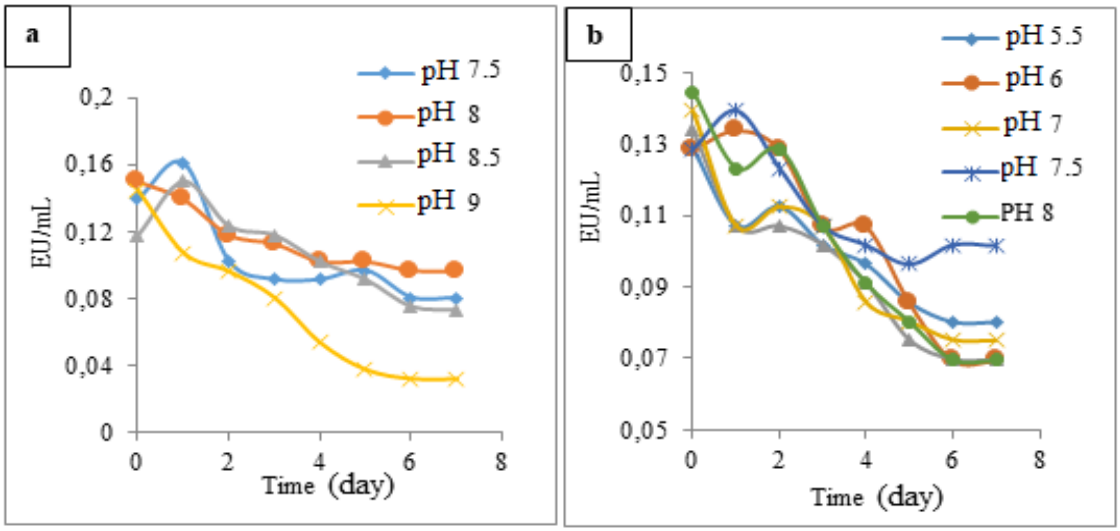

Figure 6a. The stability of the 6PGD Japanese quail's erythrocyte enzyme in Tris-HCl buffer over seven days time. **b.** The stability of the 6PGD Japanese quail's erythrocytes enzyme in potassium phosphate buffer over seven days time

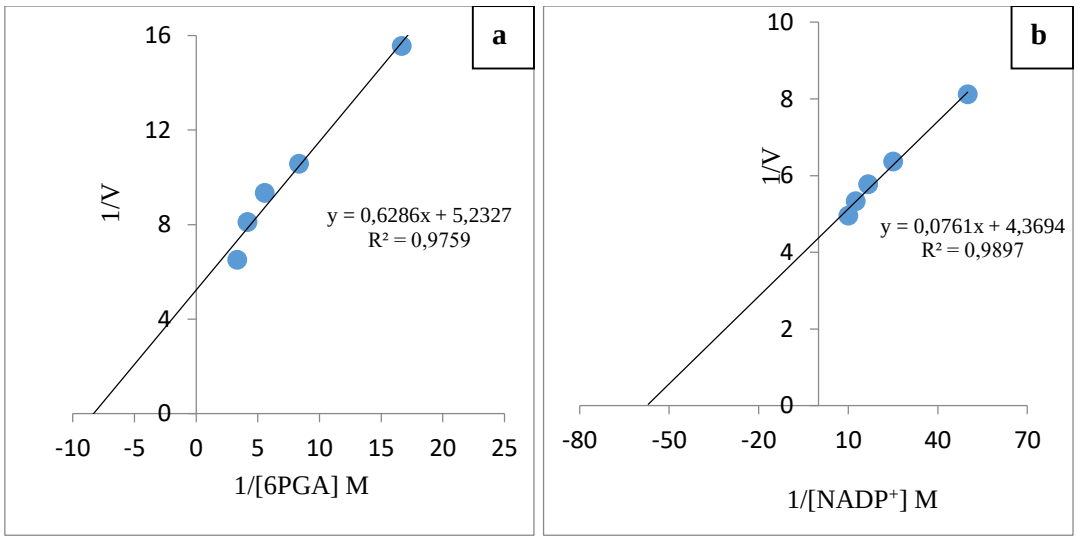

Figure 9a. The Lineweaver-Burk reciprocal plot for the 6PGA substrate: different concentrations of the 6PGA substrate for 6PGD quail's erythrocytes enzyme used in order to find the maximum velocity of the enzyme and the Michaelis- Menten constant for the substrate **b**. The Lineweaver-Burk reciprocal plot for $NADP^+$ coenzyme: different concentrations of $NADP^+$ coenzyme for 6PGD Japanese quail's erythrocytes enzyme used in order to find the maximum velocity of the enzyme and the Michaelis - Menten constant for the coenzyme.

For finding the K_M and V_{max} values for the enzyme substrates and coenzyme (6PGA and $NADP^+$), at the stable concentration of the $NADP^+$ the enzyme activity was checked by using of eight different concentration of 6PGA, the collected data from the measurements used for plotting the Lineweaver-Burk graph between 1/V vs [1/6PGA] mM, and the values of K_M and V_{max} were founded for 6PGA as K_M 0.120 mM and V_{max} 0.1911 EU. mL^{-1}. Then by using constant concentration of the 6PGA and

different concentrations of $NADP^+$ the enzyme activity was checked as well, and the collected data from the measurements used to plot the Lineweaver-Burk graph between 1/V vs [1/$NADP^+$] mM then the K_M and V_{max} values calculated from the graph for $NADP^+$ as K_M 0.0174 mM and V_{max} 0.2288 $EU.mL^{-1}$. By comparing the K_M values for both of the 6PGA and $NADP^+$ we can see that the K_M value of the $NADP^+$ is smaller than the K_M value for 6PGA, which means $NADP^+$ is more favorable than 6PGA for the catalytic reactions of 6PGD enzyme of Japanese quail erythrocytes (Figure 9a,b).

CONCLUSION

The 6-phosphogluconate dehydrogenase (6PGD) enzyme from the erythrocytes of Japanese quail (Coturnix coturnix japonica) was purified at the end of this study. The enzyme is the third enzyme of the pentose phosphate pathway (PPP) and it is considered as a secondary anti-oxidant enzyme for its role in producing the necessary NADPH that is required for removing the oxidant effects of the free radicals in the cells. The enzyme purification method was single step purification by the 2', 5'-ADP Sepharose 4B affinity gel chromatography and the purified 6PGD enzyme at the end of the process was checked for its purity using the sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) method. The result was observing purely a single band on the gel which indicated that the enzyme had well-being purified and there was only one protein in the purified solution.

The purified 6PGD enzyme of the Japanese quail erythrocytes was characterized for determining its behaviors like the stable pH value, optimum pH, optimum temperature value, optimum ionic strength, K_M and V_{max} values for the natural enzyme's substrate and its coenzyme as well as its molecular weight.

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