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Purification and Characterization of Glucose-6-phosphate Dehydrogenase from Lake Van Fish (*Chalcalburnus tarichii* Pallas, 1811) Erythrocytes

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Glucose 6-phosphste dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) was purified with 2',5'-ADP Sepharose 4B affinity gel chromatography from Lake Van fish (*Chalcalburnus tarichii* pallas, 1811) erythrocytes and were investigated some characteristics and kinetics of the enzyme. Purification step of the G6PD were controlled with SDS-PAGE and molecular weight and submolecule was determined by gel filtration chromatography and SDS-PAGE. The activity of enzyme was measured by using Beutler's method. The purification procedure was composed of two steps: hemolysate preparation and 2',5'-ADP Sepharose 4B affinity gel chromatography. The purified enzyme, having the specific activity of 17, 38 EU/mg proteins, was purified 1,100-fold with a yield of 33, 54 %. Optimum pH, optimum temperature and stable pH of the G6PD were 8.5, 40ºC and 8.0, respectively. K_M and V_{max} values for $NADP^+$ and glucose 6-phosphate (G6-P) were also determined for the enzyme. For $NADP^+$, K_M and V_{max} value at optimum pH and 25ºC for the G6PD was 0.027 mM and 0.091 EU/mL, respectively. For G6-P, K_M and V_{max} value at optimum pH and 25ºC for the G6PD was 0.0439 mM and 0.013 EU/mL, respectively.

Key Words: Purification, Glucose 6-Phosphate Dehydrogenase, Lake Van Fish (*Chalcalburnus tarichii* Pallas, 1811), Erythrocyte.

INTRODUCTION

The cell has four major NADPH production systems corresponding to the activities of four cytoplasmic enzymes *i.e.*, glucose 6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6FGD) belonging to the pentose phosphate pathway while malic enzyme (ME) and NADPH depended isocytrate dehydrogenase (NADP-IDH)[1]. Glucose-6-phosphate dehydrogenase is the key enzyme, which catalyzes first step of pentose phosphate metabolic pathway)[2,3]. It is found in animal tissues, plants and microorganisms[4-6]. In animal tissues, the enzyme is localised in cytosol and mitochondria and in green plants in cytosol and chloroplast[7-9].

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G6PD was first isolated from human erythrocytes by Yoshida[10]. In following years, the enzyme was purified from ion-exchange materials by using the natural substrates *viz.*, G6-P and $NADP^+$. The affinity chromatography (2',5'-ADP Sepharose-4B) used first by De Flora and co-workers[11] is the common technique. Some modifications in this technique were made for Lake Van fish liver G6PD purification.

Unique source of NADPH in erythrocyte is a pentose phosphate metabolic pathway and synthesis of NADPH decreases in G6PD deficiency[12]. Major role of NADPH in erythrocyte is regeneration of reduced glutathione, which prevents haemoglobin denaturation, preserves the integrity of red blood cell membrane sulfhydryl groups and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells[13,14]. Decrease of G6PD results NADPH and reduced glutathione deficiency in erythrocyte and scarcity of reduced glutathione in erythrocyte causes early haemolysis in spleen[15].

There is no report about the purification and characterization of Lake Van fish erythrocytes G6PD enzyme. The aim of this study is to purify G6PD enzyme in *Chalcalburnus tarichii*, a fish species living in Lake Van (the pH is 9.5), which has a maximum depth of 450 m, by affinity chromatography to characterize and compare purified enzyme from *Chalcalburnus tarichii* with those the other living species.

EXPERIMENTAL

2',5'-ADP Sepharose-4B was purchased from Pharmacia. $NADP^+$, glucose-6-phosphate, protein assay reagent and chemicals for electrophoresis were purchased from Sigma Chem. Co. All other chemicals used for analytical grade were purchased from either Sigma or Merck.

Preparation of the hemolysate: Blood samples collected in EDTA were centrifuged (15 min, 2500 xg) and plasma was removed. The pack of red cells was washed three times with KCl (0.16 M) and hemolyzed with 5 volume of ice-cold water and then centrifuged (+ 4ºC, 10000 xg, for 0.5 h) to remove the ghosts and intact cells[11].

2',5'-ADP Sepharose-4B affinity chromatography: For 10 mL of bed volume, 2 g of dry 2',5'-ADP Sepharose 4B was washed several times in 400 mL of distilled water. With several washings, the impurities were removed and the gel conditioned. After removal of the air in the gel, it was resuspended in the buffer (0.1 M potassium-acetate + 0.1 M potassium-phosphate, pH 6.0) with a ratio of 25 % buffer and 75 % gel and was packed in a column (1 × 10 cm). After precipitation of the gel, it was equilibrated with the same buffer by means of a peristaltic pump (flow rate: 50 mL h^{-1}). The dialyzed enzyme solution obtained previously was loaded on the column and the flow rate was adjusted to 20 mL h^{-1}. Then, the column

was sequentially washed with 25 mL of 0.1 M potassium-acetate + 0.1 M potassium-phosphate, (pH 6.0) and 25 mL 0.1 M potassium-acetate + 0.1 M potassium-phosphate (pH 7.85). The washing with 0.1 M potassium chloride + 0.1 M potassium-phosphate, (pH 7.85) was continued until the final absorbance difference became 0.05. Finally, the enzyme was eluted with the solution of 80 mM potassium-phosphate + 80 mM potassium chloride + 0.5 mM $NADP^+$ + 10 mM EDTA (pH: 7.85). Then, enzyme solution dialyzed at 4ºC in distilled water and 50 mM potassium-acetate/5 mM potassium-phosphate buffer (pH 7.0) for 2 h with two changes of distilled water and buffer, respectively[11].

The enzyme activity was measured in final fractions and the activity-containing tubes were collected together. The protein was determined in the resultant solution. During all procedures, the temperature was kept at + 4ºC[11,16,17,18].

Activity determination: The enzymatic activity was measured by Beutler's method[19]. One enzyme unit was defined as the enzyme amount reducing 1 mmol $NADP^+$ per 1 min.

Protein determination: Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin being used as a standard[20].

SDS polyacrylamide gel electrophoresis (SDS-PAGE): The control of enzyme purity, using Laemmli's procedure, was carried out in 3 and 8 % acrylamide concentrations for stacking and running gel, respectively. To the gel solution was added 10 % SDS. The gel was stabilized in the solution containing 50 % propanol + 10 % TCA + 40 % distilled water for 0.5 h. The staining was made for *ca.* 2 h in the solution of 0.1 % Coommassie Brillant Blue R-250 + 50 % methanol + 10 % acetic acid. Finally, the washing was carried out in the solution of 50 % methanol + 10 % acetic acid + 40 % distilled water until protein bands were cleared[21].

Optimal pH determination: For the optimal pH determination, the enzyme activity was measured in 0.1 M *tris*-HCl and phosphate buffers within the pH of 7.2 to 8.9 and of 4.9 to 8.0, respectively.

Stable pH determination: For this purpose, the enzyme activity was determined in 0.1 M *tris*-HCl buffer in pH of 7.0, 8.0 and 9.0 and in 0.1 M phosphate buffer in pH of 5.0, 6.0, 7.0 and 8.0. In each experiment, the equal volumes of buffer and enzyme solutions were mixed and stored refrigerated (+ 4ºC). Activity determinations were made every 8 h for 24 h.

Effect of temperature on G6PD activity: The enzyme activity was measured between 20 and 90ºC at optimal pH for this purpose.

Molecular weight determination

Sephadex G-200 gel filtration chromatography: The molecular weight of the enzyme was determined on the basis of Andrew's method[15]. The enzyme-containing tube was first determined. The void volume was

observed with Blue Dextran 2000. Horse heart cytochrome C (12400), bovine erythrocyte carbonic anhydrase (29000), bovine serum albumin (66000), yeast alcohol dehydrogenase (150000) and sweet potato β-amylase (200000) were used as standards (Sigma: MW-GF-200).

SDS-PAGE: The subunit determination was made by SDS-PAGE[21]. Rabbit myosin (205000), *E. coli* β-galactosidase (116000), rabbit phosphorylase B (97400), bovine albumin (66000), chicken ovalbumin (45000) and bovine carbonic anhydrase (29000) were used as standards (Sigma: MW-SDS-200).

Kinetic studies: For K_M and V_{max} evaluation, Lineweaver-Burk curves were used[22], which were obtained in five different concentrations of $NADP^+$ (0.0008, 0.0016, 0.0024, 0.004 and 0.008 mM) and in the constant concentration of G6-P (0.6 mM). The same experiments were done for G6-P (in five different concentrations of G6-P: 0.0048, 0.012, 0.0192, 0.024 and 0.036 mM and in fixed $NADP^+$ concentration (0.2 mM). All kinetic studies were performed at 25ºC and in optimal pH (0.1 M *tris*-HCl, pH 8.0).

RESULTS AND DISCUSSION

As shown in Table-1, specific activity was calculated for hemolyzate and purified enzyme solution as 17.38 EU/mg protein, a yield of 33.54 % and a purification coefficient of 1100 fold.

TABLE-1
PURIFICATION SCHEME OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM LAKE VAN FISH ERYTHROCYTES

Purification step	Activity (U/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Hemolyzate	2.19	40	138	5520	87.6	0.0158	100	1
2',5'-ADP Sepharose 4B affinity chromatography	2.26	13	0.13	1.69	29.38	17.38	33.54	1.100

Fig. 1 exhibits the SDS-PAGE made for the purity and molecular weight of the enzyme. For the standard proteins and G6PD, R_f values were calculated and R_f -log MW graph (Fig. 2) was obtained according to Laemmli procedure, showing a molecular weight of 67999 Da for G6PD. The molecular weight of the enzyme was also determined by gel filtration chromatography. K_{av}-log MW graph was obtained (Fig. 3), which showed a molecular weight of 138000 Da for G6PD.

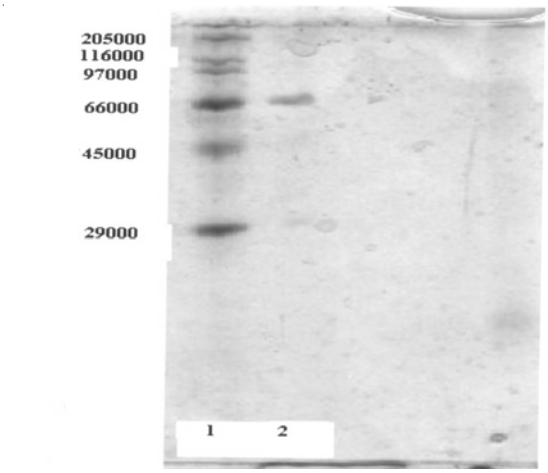

Fig. 1. S DS-PAGE bands of G6PD (Lane 1: standard proteins, Line 2: like van fish fish G6PD erythrocyte

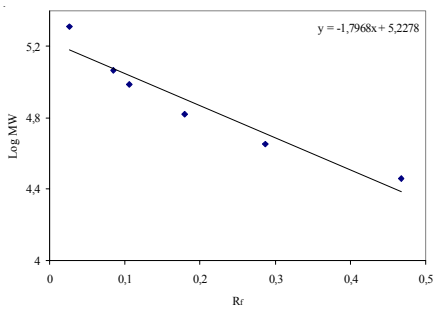

Fig. 2. Standard R_f-log MW graph of G6PD using SDS-PAGE. (Standards: rabbit myosin (205000), *E. coli* β-galactosidase (116000), rabbit phosphorylase B (97400), bovine albumin (66000), chicken ovalbumin (45000) and bovine carbonic anhydrase (29,000)). R_f: 0.22

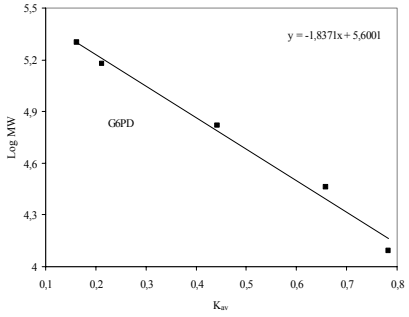

Fig. 3. Standard K_{av}-log MW graph of G6PD using gel filtration. (Standards: horse heart cytochrome C (12400), bovine erythrocyte carbonic anhydrase (29000), bovine serum albumin (66000), yeast alcohol dehydrogenase (150000) and sweet potato β-amylase (200000). K_{av}: 0.25

Kinetic parameters as optimum pH, optimum temperature, K_M and V_{max} were calculated from graphics on purified GLPD enzyme. As shown in Fig. 4, optimum pH value was found by means of activity-pH graphs and optimum pH was 8.5. The stable pH of the enzyme was 8.0 in *tris*-HCl (Fig. 6), optimum temperature value was found at optimum pH and was 40ºC as shown in Fig. 5. As shown in Figs. 7 and 8, K_M and V_{max} values at optimum pH and 25ºC were determined by means of Lineweaver-Burk graphics using 1/V-1/[S] values. For $NADP^+$, K_M and V_{max} values at

optimum pH and 25ºC was 0.027 mM and 0.091 EU/mL, respectively (Fig. 7). For G6PD, K_M and V_{max} values at optimum pH and 25ºC was 0.0439 mM and 0.013 EU/mL, respectively (Fig. 8).

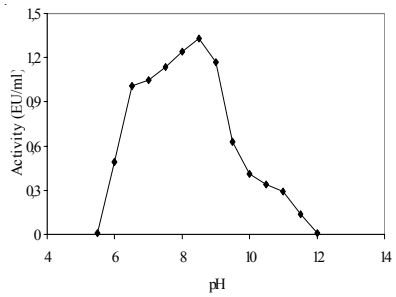

Fig. 4. Activity-pH graph of G6PD

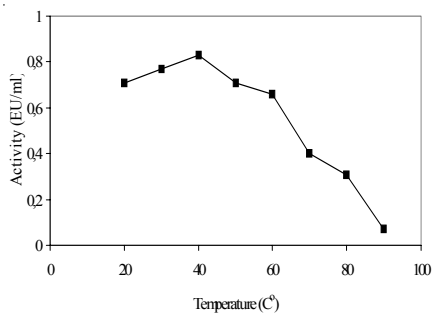

Fig. 5. The effect of the temperature on G6PD

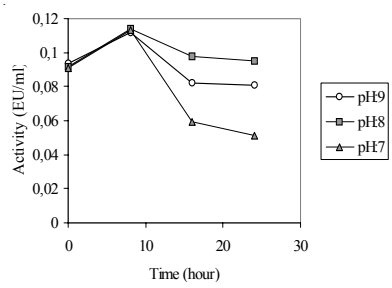

Fig. 6. Stable pH graphs of G6PD in 1 M *tris*-HCl buffer

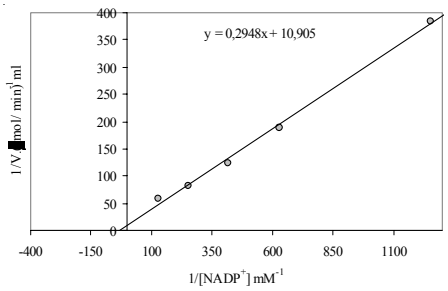

Fig. 7. Lineweaver-Burk graph with 5 different $NADP^+$ concentrations and with constant G6-P concentration

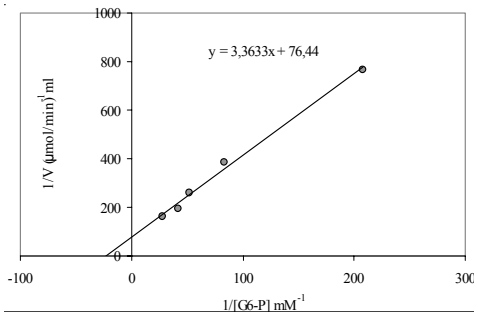

Fig. 8. Lineweaver-Burk graph with 5 different G6-P concentrations and with constant $NADP^+$ concentration

The importance of G6PD in metabolism is well known for many years. In its deficiency, many medical problems emerge[12]. We think that the easier purification methods of the enzyme make it possible that investigations on the subject can be easily done.

The enzyme has been eluted from ion exchange material by using one of its ligands. De Flora *et al.*[7] used first 2',5'-ADP Sepharose-4B for this purpose. The others were also used three consecutive steps for purification: DEAE Sephadex, Phosphocellulose (P11) and affinity chromatography on 2',5'-ADP Sepharose-4B[11]. The three steps take a long time and thus result in a decline in enzyme activity during the procedure. For this reason, we omitted the first two steps. After the homogenate was made, the sample was directly applied to 2',5'-ADP sepharose-4B column. Moreover, we used 0.5 mM $NADP^+$ instead of 0.2 mM $NADP^+$ used by Ninfali *et al.*[11] for elution, resulting in more concentrated enzyme elution. In the present study the enzyme was purified from 40 mL of hemolyzate within 5 or 6 h we were able to obtain 1100-fold purification with a yield of 33.54%. The specific activity and purification degree were similar to those of previous studies[2,3,7,11,22,23]. In addition, the advantage of the purification method suggested in the study is that it is a less time-consuming and of low cost.

Another most important advantage of this modified method is that the 6-phosphogluconate dehydrogenase (6PGD) enzyme, the second step enzyme of pentose phosphate pathway, has been able to be removed during purification, which was confirmed by the fact that the purified enzyme solution did not contain 6PGD activity.

Optimal pH of G6PD has been determined as 8.5 using 0.1 M *tris*-HCl (Fig. 4). The pH determined was similar to that in the previous studies[2,16,22,23]. The stable pH of the enzyme was 8.0 in 0.1 M *tris*-HCl (Fig. 6). The enzyme was seen to show the highest activity at 40ºC (Fig. 5).

Fig. 1 shows the SDS-PAGE made for the purity and molecular weight determination of the enzyme. A high purity for the enzyme has been obtained. For the standard proteins and G6PD, R_f values were calculated and R_f-log MW graph (Fig. 2) was obtained according to Laemmli procedure[20], showing a molecular weight of 67999 Da for G6PD. G6PDs of different origin have similar molecular weight as follows: 62 kDa and 69 kDa from bovine lens[22] and in rat cortex and liver[23] in dog liver[24] and in human placenta[25] by SDS-PAGE, respectively. The molecular weight of the enzyme was also determined by gel filtration. A K_{av}-log MW graph was obtained (Fig. 3), which showed a molecular weight of 138300 Da for G6PD. The two graphs did not show similar molecular weights, suggesting the enzyme may be a dimer in an active state. The molecular weights of G6PD enzyme subunits of different origin are between 50-67 kDa[3]. The values obtained from Van Fish erythrocytes G6PD are similar to these results.

The Lineweaver-Burk graphs were shown in Figs. 7 and 8, which were constructed for $NADP^+$ and G6-P. K_M of 0.0439 mM and V_{max} of 0.013 EU/mL were obtained for $NADP^+$ and 0.027 mM and 0.091 EU/mL for G6-P. These K_M values are very similar to those obtained in rat cortex and liver, in dog liver and in human placenta[23-25]. The K_M for $NADP^+$ is lower than that for G6-P, suggesting the lower affinity of G6PD to G6-P when compared with $NADP^+$.

Herein we describe purification of G6PD enzyme from Lake Van fish (*Chalcalburnus tarichii* pallas, 1811) erythrocytes and investigation of its kinetic properties such as, optimum pH, optimum temperature, stable pH, molecular weight, K_M and V_{max}.

REFERENCES

1. J.B. Barroso, J. Peragón, L. García-Salguero, M. Higuera and J. Lupiáñez, *J. Biochem. Cell Biol.*, **31**, 277 (1999).
2. M. Ciftci, A. Ciltas and O. Erdogan, *Vet. Med-Czech.*, **49**, 327 (2004).
3. D.S. Shreve and H.R. Levy, *Biochem. Biochem. Biophys. Res. Com.*,**78**, 1369 (1977).
4. F.K. Gleason, *Arc. Biochem. Biophys.*, **334**, 277 (1996).
5. E.B. Kurutas and I. Tuncer, *Turk. J. Vet. Anim. Sci.*, **24**, 511 (2000).
6. Y. Nemoto and T. Sasakuma, *Plant Sci.*, **158**, 53 (2000).
7. H.R. Levy, in ed.: A. Meister, Glucose-6-phosphate Dehydrogenase, Advan. Enzymol., 48, John Wiley and Sons, New York, p. 97 (1979).
8. C. Schnarrenberger, A. Oeser and N.E. Tolbert, *Arch. Biochem. Biophys.*, **154**, 438 (1973).
9. O. Ocheretina, I. Haferkamp and H. Tellioglu, *Genetics*, **258**, 147 (2000).
10. A. Yoshida, *J. Biol. Chem.*, **241**, 4966 (1966).
11. P. Ninfali, I. Orsenigo, L. Barociani and S. Rapa, *Prep. Biochem.*, **20**, 297 (1990).
12. A. Telefoncu and F. Zihnioglu, *Tr. J. Med. Sci.*, **14**, 57 (1989).
13. J. Deutsch, in eds.: H.U. Bergmeyer and J. Bergmeyer, Glucose-6-phosphate Dehydrogenase, Methods of Enzymatic analysis, Verlagsgerellschaff, VCH, pp. 190-196 (1990).
14. B.B. Weksler, A. Moore and J. Tepler, in eds.: T.E. Andreoli, C.C.J. Carpenter, F. Plum and L.H. Smith Jr., Hematology, Cecil Essentials of Medicine, WB Saunders Company, Philadelphia, edn. 2, pp. 341-363 (1990).
15. P. Andrews, *Biochem.*, **96**, 595 (1965).
16. I. Mohammed. M.L Tuews and W.R. A. Carpar, *J. Biol. Chem.*, **251**, 2255 (1976).
17. A. Morelli, U. Benatti, G.F. Gaetani and A. De Flora, *Proc. Natl. Acad. Sci.*, **75**, 1979 (1978).
19. E. Beutler, Red Cell Metabolism Mannual of Biochemical Methods, Academic Press London, pp. 68-70 (1971).
20. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
21. D.K. Laemmli, *Nature*, **227**, 680 (1970).
22. I.E. Segel, Enzyme Kinetics, Wiley, Toronto (1975).
23. N.N. Ulusu, M.S. Kus, N.L. Acan and E.F. Tezcan, *Int. J. Biochem. Cell Biol.*, **31**, 787 (1999).
24. F.J. Corpas, L. Garcia-Salquero, J. Peragon and J.A. Lupianez, *Life Sci.*, **56**: 179 (1995).
25. C. Bilgi, I, Unsal. N, Ozer, N. Erbil and M.K. Karaca, *Tr. J. Med. Sci.*, **24**, 291 (1995).
26. O. Nazmi, Y. Aksoy and I.H Ogus, *Int. J. Biochem. Cell Biol.*, **33**, 221 (2001).

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