Purification and Kinetic Properties of Glucose-6-Phosphate Dehydrogenase from Goat (*Capra aegagrus hicrus*) Erythrocytes

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Summary: The aim of this study was to purify glucose-6-phosphate dehydrogenase (G6PD) from domestic goat (*Capra aegagrus hicrus*) erythrocytes and to investigate its some in vitro kinetic properties. Blood was taken on ACD-A solution and erythrocytes were purified, and then they were hemolysated. In the study DEAE-cellulose as anion and CM-Sephadex as cation exchange column chromatographies and ammonium sulphate precipitation and dialysis methods were used. G6PD was purified 45000 fold in a yield of 23 %. Molecular weight was found 210000 by Sephadex G-200. Four subunits of this enzyme were determined and their molecular weights were found 52000. K_m and V_{max} values for G6PD were determined as 3.34 x 10^{-4} M and 642 μm/min. In addition we found that levels of Mg^{2+} from 1 x 10^{-3} M to 6 x 10^{-3} M are increasing activity of G6PD and 10^{-4} M dehydroepiandrosterone is preventing whole activity of G6PD.

Introduction

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49; β-D-glucose-6-phosphatase; NADP oxidoreductase) is an enzyme that catalyzes the first step of the pentose phosphate pathway (PPP). The major roles of PPP are, (i) to convert glucose-6-phosohate (G6P) into ribose-5-phosphate which can be used for nucleotide biosynthesis; (ii) to produce NADPH, the major hydrogen donor in reductive biosynthesis which also provides production against oxidative stress; (iii) to serve as the route of entry of pentoses to the glycolytic pathway [1-3]. NADPH is necessary for the synthesis of fatty acids and other specific reductions [2-5].

Glucose-6-phosphate dehydrogenase is widely distributed and it has been previously isolated from microorganisms, plants and erythrocytes and various mammalian tissues [6-19].

In this work we studied glucose-6-phosphate dehydrogenase from goat erythrocytes and on its kinetic properties. We aimed to investigate in vitro properties of G6PD on goats erythrocytes physiological chemistry.

Results and Discussion

At all steps of purification of goat erythrocytes G6PD, the medium was supplemented with 2-mercaptoethanol as antioxidant [20, 21], EDTA was added for protection against proteases [2, 6], NADP was added to preserve the enzyme in its active form [2, 6, 13, 20, 21].

The elution profile of DEAE-cellulose (anion exchange) chromatography (step 3) was shown in Fig 1. At this step enzyme was purified 60 fold with 86 % yield. Ratio of 6PGD was decreased to 1.5 %.

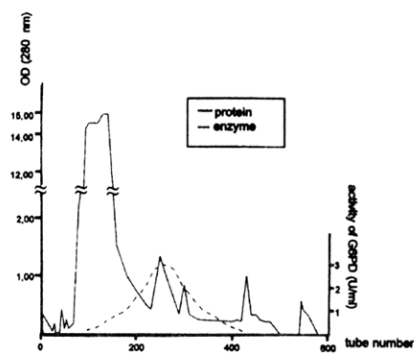

Fig. 1. DEAE- cellulose (anion exchange) chromatography (Step 3.) of G6PD and protein. Column size, 4x60 cm; eluent buffer, 50 mM phosphate buffer, pH 5.8, containing 2 μM NADP, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.3 M KCl. Fractions of 5 ml were collected.

Fig. 2 shows the elution profile of CM-sephadex (cation exchange) chromatography (step 4). At this step enzyme was became two separate fraction. Enzyme was purified 917 fold with 73 % yield.

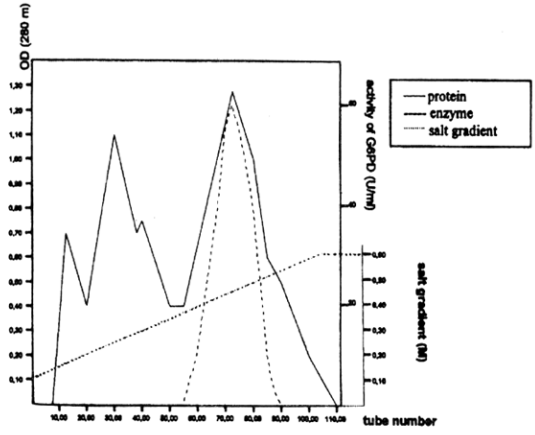

Fig. 2. CM-sephadex (cation exchange) chromatography (step 4). Column size, 4x60 cm; eluent buffer, 100 g CM-Sephadex was equilibrated with 5mMphosphate buffer, pH 5.8, containing 0.1 M KCl. At each column enzyme was became as two separate fractions. First fraction was in 50 ml and the second one was in 250 ml volume.

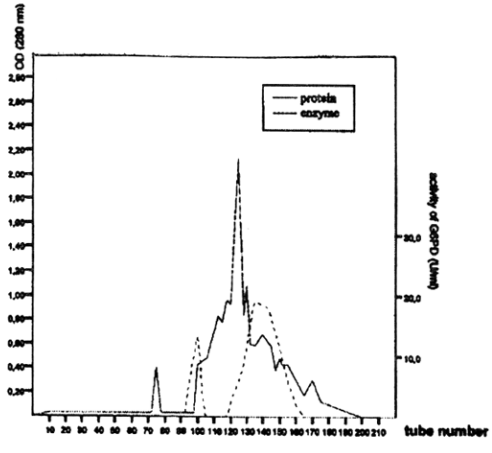

Fig. 3. Elution porfile of G6PD and protein from CM- Sephadex chromatography (Step 7). G6DP and protein activities were eluted with linear gradient of 0.1-0.6 M KCl prepared in 5 mM phosphate buffer, pH 5.8

Fig. 3 shows the elution profile of CM-sephadex with linear gradient of KCl (step 7). At this step enzyme was purified 10000 fold with 40 % yield.

Gel filtration and dialysis steps (step 9-10) were done 4 times. Fig. 4 shows the first and Fig. 5 shows the last. At this step enzyme was purified 44300 fold with 30 % yield.

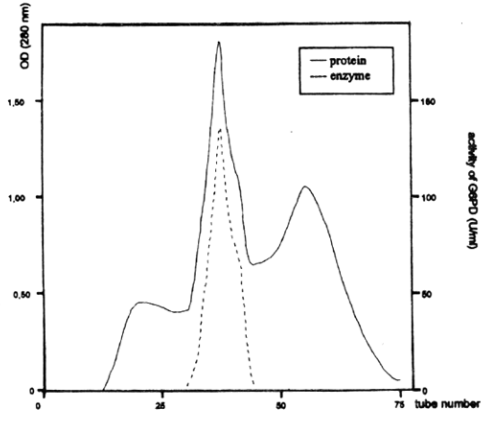

Fig. 4. Elution profile of G6PD and protein from Sephadex-G 200-120 column at first treatment (Step 9). Eluent buffer; 0.1 M acetate buffer, pH 6.0, containing 1mM EDTA, 10 μM $NADP^+$. Flow rate was 22 ml/h. Fractions of 2 ml were collected.

After all steps of purification, G6PD was purified 45000 fold with 23 % yield and specific activity of 135 U/mg protein. A summary of the purification steps is presented in Table-1.

By using washing value of the purified enzyme eluted from the Sephadex G-200 column, the molecular weight of G6PD was calculated as 210000

(Fig. 6). The purified enzyme gave a single band on cellulose acetate electrophoresis paper (Fig. 7).

For determining subunits of G6PD, purified enzyme was pretreated with guanidin hydroclorur and 2-mercaptoethanol then by using washing value which eluted as a single peak from the Sephadex G-200 column the molecular weight was calculated as 52000 from Fig. 6. We suggest that enzyme has got 4 subunits and their molecular weights are equal because we took a single peak from column.

Table 1. Purification steps of G6PD

Purification steps	Total volume (ml)	Total protein (mg)	Total G6PD (unit)	Total 6PGD (unit)	6PGD (G6PD+6PGD)	Specific activity of G6PD (U/mg)	Yield (%)	Fold
Step 2. Hemolization	24000	$5.00x10^6$	15000	5000	0.25	0.003	100	1
Step 3. DEAE-Cellulose column eluent	6x1700	$7.35x10^4$	13000	210	0.015	0.18	86	60
Step 4. Sephadex column eluent	4x300	$4.00x10^3$	11000	18	0.0016	2.75	73	917
Step 5. 30%-50% $(NH_4)_2SO_4$ fraction	100	$8.00x10^2$	7000	-	-	8.7	46	2900
Step 6 & 7. Dialysis and CM-Sephadex eluent	175	$2.00x10^2$	6000	-	-	30	40	10000
Step 8,9 &10. Dialysis and gel filtration	3x18	30	4500	-	-	133	30	44333
Step 11. 260-270 g/l $(NH_4)_2SO_4$ and dialysis	5	26	3500	-	-	135	23	45000

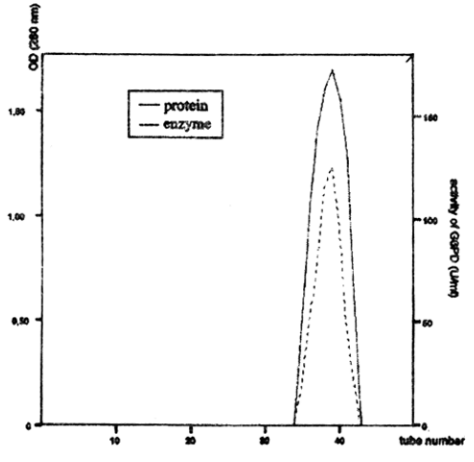

Fig.5. Elution profile of G6PD and protein from Sephadex-G 200-120 column at last treatment (Step 9). Eluent buffer; 0.1 M acetate buffer, pH 6.0, containing 1mM EDTA, 10 μM $NADP^+$. Flow rate was 22 ml/h. Fractions of 2 ml were collected.

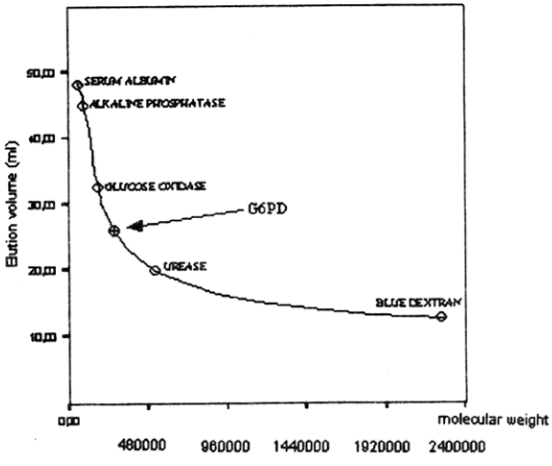

Fig. 6. Estimation of molecular weight of G6PD by gel filtration on Sephadex G-200. The protein standarts: bovine serum albumin, monomer 66 kD; alkaline phosphatase 80 kDa; glucose oxidase 154 kDa; urease 480 kDa; blue dextran 2000 kDa. The molecular weight of G6PD was found to be 210 kDa.

For calculating K_m and V_m values, amount of G6P was increased from 5 μl to 110 μl at buffer A. From these solutions activities were measured. From obtained data from this assay K_m and V_m were calculated and Michaelis-Menten (Fig. 8) and Lineaweaver-Burk (Fig. 9) curves were drawn. From these curves $-1/K_m$ and $-1/V_{max}$ were calculated as 3.2 x 10^3 M and 1.55 x 10^{-3} μM/min respectively. K_m and V_{max} were found 3.34 x 10^{-4} M and 642 μM/min respectively.

Fig. 7. The native cellulose acetate electrophoresis paper. The purified enzyme gave a single band.

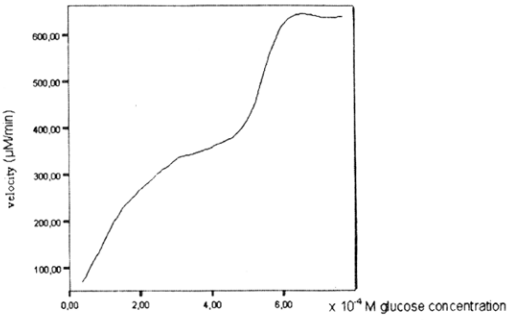

Fig. 8. Michaelis-Menten curve of G6PD.

Fig. 10 shows effect of different concentrations of Mg^{2+} on enzyme catalyze velocity. To determine this, Mg^{2+} was added at different concentrations to buffer A as $MgCl_2$ separately, V_x was taken as 100. the highest activity was taken as 100 and the others were calculated against this. From data we found that Mg^{2+} is increasing activity of G6PD.

The optimum pH of the enzyme activity was examined. V_x was taken as 100 and activities were measured. As shown in Fig. 11 velocity versus pH curve has maximum value at pH 8.0. At pH 10.0 $Mg(OH)_2$ was precipiated so activity couldn't measured.

Inhibitor effect of dehydroepiandrosterone was also determined V_x was taken as 100 and activity was measured. At 10^{-4} M concentration of dehydroepiandrosterone enzyme was inhibited all.

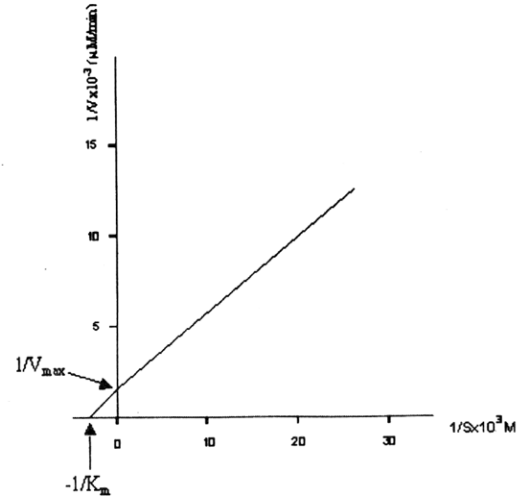

Fig. 9. Lineaweaver-Burk curve. From this curve K_m and V_{max} were found 3.34 x 10^{-4} M and 642 µM/min respectively

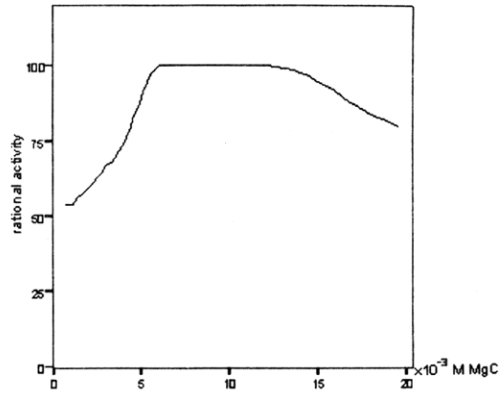

Fig. 10. Effect of different concentrations of Mg^{2+} on enzyme catalyze velocity

The activity of G6PD of erythrocytes increase at different diseases [22] and decrease by old age. Lots of studies were designed to purify this enzyme from different tissues of various animals [6-19]. Purification of enzyme from erythroctes takes long time because of high volume of blood which must used. For this reason dextrose and like factors can be used as nutritious.

In this study G6PD was purified 45000 fold, similarly Watanable *et al* purified this enzyme

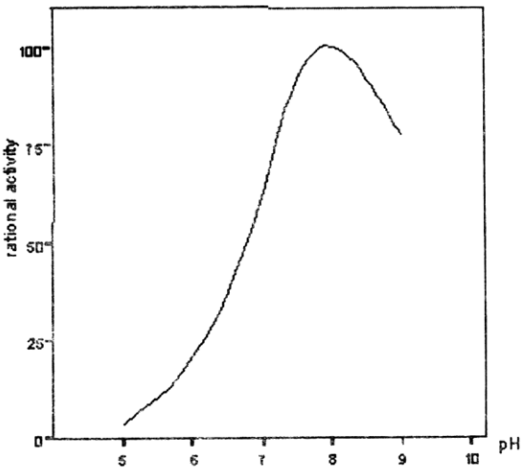

Fig. 11 Effect of different values of pH on activity of enzyme

258000 fold [9], Cohen *et al* 72000 fold [14], Özer *et al* 2000 fold [23] and Ulusu et al 19700 fold [24], this enzyme was purified at different fold in different studies.

At step 4 of purification, enzyme was became as two separate fractions from CM-sephadex column, similarly with study of Malcolm *et al* [25]. This can be by reason of a part of enzyme which divorce from enzyme, maybe a subunit.

Molecular weight and number of subunits of G6PD depend on species of animal and tissue. Various values were found at human erythrocytes [9, 14]. Watanable et al. found that molecular weight of G6PD was 210000 and number of subunits were 4 and molecular weights of each subunits were 53000 [9], Cohen et al found that molecular weight of G6PD was 258000 and number of subunits were 6 and molecular weights of each subunits were 43000 [14] similarly we found that molecular weight of G6PD was 210000 and number of subunits were 4 and molecular weights of each subunits were 52000.

The observation of different specific activities for G6PD from different sources is not uncommon [10, 13, 26]. Even from the same tissue, specific activities ranging from 35.5 to 730 U/mg protein and different association forms, from mono-mer to hexamer, have been reported [6, 13]. As written above we found specific activity 135 U/mg and form was hexamer. This difference can be depend on species of animal, tissue, store and assay conditions.

The aggregation state and the specific activity of G6PD depend on several factors such as enzyme, NADP, salt, Mg^{2+} (or Mn^{2+}) concentrations and pH [6, 7, 20, 21, 27, 28, 29]. In this study at in-vitro conditions for activation energy of enzyme optimum pH was found as 8.0. This is in good agreement with the published results [2, 6, 26, 30]. High concentration of Mg^{2+} decrease the activation also.

In this study K_m was found as 3.34×10^{-4} M. Watanable et al. and Cohen et al. were found 1×10^{-4} M and 3.9×10^{-5} M respectively [9, 14]. These are in good agreement with our results. According to these data while purification degree was increasing K_m value is decreasing that is affinity to substrate is rising.

Michaelis-Menten curve was seem allosteric, we found that 10^{-4} M concentration of dehydro-epiandrosterone which is a steroid was preventing whole activity of G6PD.

Experimental

DEAE-cellulose, glucose-6-phosphate (G6 P), 6-phosphogluconate (6-PGA), Tris [Tris (hydroxymethyl) ammomethane], ethylenediamine tetraacetic acid (EDTA), $NADP^+$ were obtained from Sigma, USA. Sephadex G-200 was from Pharmacia-LKB, Sweden. All other chemicals were standart products of Sigma, USA.

Assay of G6PD

Enzyme activities were determined spectrophotometrically by monitoring the NADPH production at 340 nm and at 37 ^{0}C [31, 32]. The assay mixture contained 70 mM $MgCl_2$, 2 mM $NADP^+$ and 7 mM G6P in 0.125 M Tris/HCl buffer, pH 8.0 (buffer A). Assays were carried out in dublicate and the activities were followd for 40 seconds. The reaction was linear during this time period. In the kinetic studies, the asseys were performed in buffer A. For calculating K_m and V_m, amount of G6P was increased from 5 μl to 110 μl at buffer A.

One unit of enzyme activity was defined as the amount of the enzyme which converts one µmol of NADP to NADPH per min under the assay

conditions. Specific activity was defined as units per mg of protein.

Assay of 6-PGD

Activities were measured by 7 mM 6-PGA as substrate in the assay mixture given above for G6PD measurement [22, 33-34].

Since 6-PGD also catalyzes the production of NADPH, in the earlier steps of the purification, both G6PD and 6PGD activities were measured as a sum and the initial velocities of G6PD were calculated by subtracting the 6PGD activities.

Protein assay

Protein concentrations in fractions was estimated by measuring the absorbance at 280 nm and 260 nm in the combined samples at each purification step, protein content was determined by the method of Layne using bovine serum albumin as standart [35].

Purification of goat erythrocyte G6PD

Step 1. Taking blood

From 1-2 years old domestic goats 35 liters blood were taken on 5.25 liters ACD-A solution (22 g Na-citrate, 8 g citric acid, 24 g dextrose, 1000 ml nonpirogenize distilled water) and put to –5 ^{0}C.

Step 2. Purification of erythrocytes and hemolization

Blood was centrifuged at 4000 xg in 50 ml plastic tubes and supernatant was removed with vacum pomp. Precipitate was washed three times with isotonic solution (V:V) containing 0.15 mol KCl and 1 mM EDTA. At every time of washing supernatant and other cells on erythrocytes were removed by vacum pomp. At the end 15 liters pure erythrocytes were obtained. A buffer which contains 50 ml toluen and 1 ml 2-mercaptoethanol per liter was added on erythrocytes and shaked for 5 minutes to hemolization. Hemolizate was centrifuged at 5000 xg, supernatant was taken and its volume was found 24 liters. Protein and enzyme activities were measured from supernatant.

On 24 liters supernatant 12 liters of 5 mM phosphate buffer, pH 6.5 (buffer B) was added and total volume was found 36 liters. To this sample 55 mg $NADP^{+}$, 21 ml 2-mercaptoethanol and 13.4 g EDTA were added for having 2μM NADPH, 0.1 % 2-mercaptoethanol (V:V) and 1mM EDTA at medium.

Step 3. DEAE- cellulose (anion exchange) chromatography

This step was done by the method of Yoshida [13]. 300 g DEAE-cellulose was equilibrated with buffer B for 4 hours. And then loaded onto 4x60 cm columns (6 units) and equilibrated with the same buffer. To each columns 6 liters of the sample which obtained from step 2 was loaded. After loading of sample column was washed with buffer B containing 1.5 mg NADP, 372. 24 mg EDTA and 1 mol 2-mercaptethanol for having 2 μM $NADP^{+}$, 1mM EDTA and 0.1 % 2-mercaptoethanol (V:V) per liter, until no protein can determined at elution which come out from column. Thus proteins and enzymes which can't bind to column were removed. And then enzymes and proteins which binds to column were eluted with 50 mM phosphate buffer, pH 5.8, containing 2 μM NADP, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.3 M KCl (buffer C). The elutions coming out from columns were collected by fractional collector separately 5 ml each. Thus from 6 columns 10200 ml active elutions obtained.

Step 4. CM-Sephadex (cation exchange) chromatography

This step was done by the method which explained by Cohen and Resenmeyer [36]. 100 g CM-Sephadex was equilibrated with 5mM phosphate buffer, pH 5.8, containing 0.1 M KCl (buffer D) for overnight. Then loaded onto 4x60 cm columns (4 units). At the same time 10200 ml active solution obtained from step 3 was equilibrated to pH 5.8 with 0.5 M acetic acid. Then to each column 2550 samples were loaded. After loading columns were washed buffer D containing 2 μM NADP and 1 mM EDTA until no absobance determined at 280 nm. Thus proteins can't bind to gel were removed. Then columns were eluted with 50 mM phosphate buffer, pH 7.0, containing 2 μM NADP, 1 mM EDTA (buffer E). Flow rate was 0.4 ml/min. Each 5 ml elutions collected separately. At each column enzyme was became as two separate fractions. First fraction was in 50 ml and the second one was in 250 ml volume. Active parts were putted together. From 4 columns 1200 ml active solutions obtained.

Step 5. Ammonium sulphate precipitation

Firstly the 30 % ammonium sulphate precipitated from 1200 ml active solution which

conditions. Specific activity was defined as units per mg of protein.

Assay of 6-PGD

Activities were measured by 7 mM 6-PGA as substrate in the assay mixture given above for G6PD measurement [22, 33-34].

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Step 4. CM-Sephadex (cation exchange) chromatography

This step was done by the method which explained by Cohen and Resenmeyer [36]. 100 g CM-Sephadex was equilibrated with 5mM phosphate buffer, pH 5.8, containing 0.1 M KCl (buffer D) for overnight. Then loaded onto 4x60 cm columns (4 units). At the same time 10200 ml active solution obtained from step 3 was equilibrated to pH 5.8 with 0.5 M acetic acid. Then to each column 2550 samples were loaded. After loading columns were washed buffer D containing 2 μM NADP and 1 mM EDTA until no absobance determined at 280 nm. Thus proteins can't bind to gel were removed. Then columns were eluted with 50 mM phosphate buffer, pH 7.0, containing 2 μM NADP, 1 mM EDTA (buffer E). Flow rate was 0.4 ml/min. Each 5 ml elutions collected separately. At each column enzyme was became as two separate fractions. First fraction was in 50 ml and the second one was in 250 ml volume. Active parts were putted together. From 4 columns 1200 ml active solutions obtained.

Step 5. Ammonium sulphate precipitation

Firstly the 30 % ammonium sulphate precipitated from 1200 ml active solution which

obtained from step 4. 176 g $(NH_4)_2SO_4$ was added per liter and waited overnight. Then centrifuged at 5000 xg and supernatant removed. Precipitate was dissolved in 50mM phosphate buffer, pH 7.0, and activity was determined at elution separately. And founded that enzymes are at supernatant. This time the 50 % ammonium sulphate precipitated from supernatant. For this 127 g $(NH_4)_2SO_4$ was added on 1 lt of 30 % ammonium sulphate [37]. Then activity was determined at precipitate and supernatant. Activity was found at precipitate. So that suprenatant was thrown. Precipitate was dissolved with 100 ml buffer E.

Step 6. Dialysis

The 100 ml sample which obtained from step 5 was dialysed against 50 mM phosphate buffer, pH 7.0, containing 5 μM NADP · (buffer F) at dialysator for 24 hours. Dialysis buffer was changed 4 times.

Step 7. CM- Sephadex chromatography

Dialysate was centrifuged at 5000 xg for 45 minutes. Elution was done containing 0.1 M KCl. Then by the method which described at step 4, elution and CM-Sephadex gele were loaded to K16 column together and washed with buffer D until the absorbance of the effluent was reading at 280 nm. Then by gradient mixer, from 0.1 M KCl through to 0.6 M KCl salt gradient was treated to column by using 5 mM phosphate buffer, pH 5.8, containing 0.1 M KCl, 10 μM $NADP^+$ and 5 mM phosphate buffer, pH 5.8, containing 0.6 M KCl, 10 μM $NADP^+$ Tubes containg activity were combined.

Step 8. Dialysis

The sample was dialysed against 0.1 M acetate buffer, pH 6.0, containing 1mM EDTA, 10 μM $NADP^+$ (buffer G). Dialysis buffer was changed 6 times.

Step 9-10. Gel filtration and dialysis

Dialysate was evaporated to remain 6 ml at vacum evaporator. Then centrifuged at 5000 xg for 45 minutes, supernatant was taken and divided into three equal volumes and loaded onto K16 column which containing 4.58 g Sephadex G-200-120 equilibrated with buffer G for 3 hours as 2 ml samples separately [38]. By using peristaltik pomp column was eluted with buffer G and the flow rate was 22 ml/h. Fractions were obtained as 2 ml elutions.

Tubes containing activity were combined and dialysed against buffer G and evaporated to remain 2 ml at vacum evaporator. Then loaded onto same column. This assay was done three times more. All dialysates were combined and concentrated to remain 5 ml at evaporator.

Step 11. Ammonium sulphate precipitation

Buffer G containing 600 mg ammonium sulphate per liter was added on 5 ml enzyme which obtained from step 10 by drops. This operation continues until precipitation has been seen. Precipitate has waited at room temperature for 3 hours and centrifuged at cold conditions. Supernatant was thrown. Precipitate was dissolved with 5 ml buffer G. Then dialysed against 250 ml buffer G twice. This dialysate contains purified G6PD.

Protein electrophoresis

Treatment was done with buffer H, pH 8.6, ionic force 0.005. 1 ml of enzyme solution which obtained from step 11 was concentrated 10 times with evaporator. This concentrate was loaded onto cellulose acetate electrophoresis paper equilibrated with buffer H under 200 volt for 60 minutes [20, 25, 38, 39, 40, 41]. Then acetates were waited at Ponceau-S dye (0.2 g dye dissolved in 3 % tricloracetic acid) for 5 minutes [42]. Then paper was washed in three vessel containing 100 ml 5 % acetic acid respectively for 5 minutes. Celulose acetates were put between filter papers to dry, after drying waited in 30 % dimethyl sulphoxyde to become transparent for 5 minutes and spreaded on a slide and waited in Pasteur owen for 5 minutes. Then examined at densiometer.

Statistical analyses

The kinetic data were analyzed and kinetic constants were calculated by SPSS.

Determination of subunits of G6PD

0.5 ml purified enzyme was mixed with 1 ml 6 M guanidyne hydroclorur containing 1 mM EDTA and incubated at 42 ^{0}C for 45 minutes. 1 ml of this solution was taken and the same procedure of protein assay was done to this part.

Determination of Mg^{2+} effect

To determine effect of Mg^{2+} on G6PD, Mg^{2+} was added to blank and sample tubes as MgCl. The activity was measured separately.

Determination of pH effect

To determine effect of pH, a tris buffer was prepared as buffer A at different pH's as 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9 and 10. The activity was measured separately.

Determination of inhibitors

Effect of dehydroepiandrosterone on G6PD was determined. From buffer A and 10^{-3} M dehydroepiandrosterone solution a mixture was prepared which's concentration was 10^{-4} M and then activity was measured.

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