

# Three Different Purification Protocols in Purification of G6PD from Sheep Brain Cortex

Cihangir ŞENGEZER\*, Nuriye Nuray ULUSU\*<sup>o</sup>

## Three Different Purification Protocols in Purification of G6PD from Sheep Brain Cortex

### Summary

The aim of this study was to determine the most efficient purification protocol for purification of glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) from sheep brain cortex. G6PD was purified by 2', 5'-ADP-Sepharose 4B affinity and DEAE Sepharose Fast Flow ion exchange chromatography columns in different orders. In all purification protocols, 105,000 x g supernatants were used after a homogenization step. In the first purification protocol, the supernatant was loaded onto an affinity column after an ultracentrifugation step, followed by ion exchange chromatography. In the second purification protocol, the supernatant was dialyzed after ultracentrifugation, then loaded onto an ion exchange column. The enzyme was eluted by linear KCl gradient. G6PD activity containing fractions were collected and dialyzed, then loaded onto an affinity chromatography column. The third protocol was similar to the first, except for addition of a dialysis step after ultracentrifugation. In the first protocol, the yield and the specific activity were found to be 68.33% and 51.25 U/mg protein, respectively. In the second purification protocol, G6PD was obtained with a yield of 24.89% and had a specific activity of 3.63 U/mg protein. In the third, the enzyme was obtained with a yield of 27.08% and had a specific activity of 7.8 U/mg protein. In this study, we compared three different protocols for purification of G6PD from sheep brain cortex.

**Key Words:** Glucose-6-phosphate dehydrogenase, purification, brain cortex.

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## Glukoz-6-Fosfat dehidrogenazın üç farklı yöntemle beyin korteksinden saflaştırılması

### Özet

Bu çalışmanın amacı glukoz-6-fosfat dehidrogenaz' ın (G6PD, EC 1.1.1.49) koyun beyin korteksinden en verimli saflaştırma metodunun saptanmasıdır. G6PD, 2',5'-ADP-Sepharose 4B afinite ve DEAE Sepharose Fast Flow iyon değiştirici kromatografilerini farklı sıralamaları kullanılarak saflaştırıldı. Bütün saflaştırma basamaklarında homojenizasyondan sonra 105,000 x g süpernatanı kullanıldı. Birinci saflaştırma protokolünde, ultrasantrifigasyondan sonra enzim afinite kromatografi kolonu ardından DEAE iyon değiştirici kromatografi kolonuna yüklendi. İkinci saflaştırma protokolünde, ultrasantrifigasyon basamağından sonra süpernatant diyaliz edilerek iyon değiştirici kromatografi kolonuna yüklendi. Enzim, KCl gradienti kullanılarak elüe edildi. G6PD aktivitesi içeren kesitler toplanıp diyaliz edildikten sonra afinite kromatografi kolonuna yüklendi. Üçüncü protokol birincisiyle benzer olup sadece ultrasantrifigasyondan sonra bir diyaliz basamağı içermektedir. Birinci protokolde, enzim %68.33 verimle ve 51.25 U/mg protein spesifik aktivite ile elde edildi. İkinci saflaştırma protokolünde, G6PD % 24.89 verimle ve 3.63 U/mg protein spesifik aktivitesi ile elde edildi. Üçüncü protokolde enzim % 27.08 verimle 7.8 U/mg protein spesifik aktivitesi ile elde edildi. Bu çalışmada farklı üç yöntemle G6PD koyun beyin korteksinden saflaştırılarak yöntemler karşılaştırıldı. **Anahtar Kelimeler:** Glukoz-6-fosfat dehidrogenaz, saflaştırma, beyin korteksi.

## INTRODUCTION

The pentose phosphate pathway is responsible for the generation of NADPH and de novo production of cellular ribose. Glucose-6-phosphate dehydrogenase (G6PD) is the first and regulating enzyme of this pathway (1). G6PD is as an antioxidant enzyme and its deficiency is associated

with neonatal jaundice, drug- or infection-mediated hemolytic crisis, favism and, less commonly, chronic non-spherocytic hemolytic anemia (2). One of the most important functions of G6PD is reduction of NADPH, which has many important roles in cell metabolism (3). G6PD activity

\* Hacettepe University, Faculty of Medicine, Department of Biochemistry Ankara, Turkey

<sup>o</sup> Corresponding author e-mail: nnulusu@hacettepe.edu.tr

is also very important for protection of cells from mild oxidative stress and stimuli of hormones, growth factors, and nutrients (4,5). It was mentioned that oxidative stress has a crucial role in neurodegenerative diseases such as Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis (6), so deficiency of this enzyme may increase the risk of some diseases. This cytoplasmic enzyme also provides coenzymes and substrates for the other primary antioxidant enzymes (7). There are very few studies on G6PD metabolism in the brain. In one of the studies, the researchers studied the intensity of pentose phosphate metabolism of carbohydrates in various brain areas in normal and starved rat brains, then proposed that G6PD and 6-phosphogluconate dehydrogenase (6-PGD) are functionally and metabolically related to the glutathione system and during starvation, and they indirectly participate in the regulation of lipid peroxidation processes in the nervous tissues (8). In one of our previous studies (9), we established that G6PD level was markedly increased in diabetic rat brains compared to controls, although the activity level of 6-PGD did not change. G6PD is purified from various sources. In these studies, researchers obtained different purification folds, specific activities and yields in various tissues. It is hard to standardize and determine the most efficient purification protocol, because there are many variables that could easily affect the procedure, such as the type of chromatographic material and the characteristic properties of the tissue, which have a domino effect from the beginning to the end of the purification protocol. Property and concentration of the buffer are also key determinants that affect the enzyme purification.

Several researchers have purified G6PD using different purification protocols (10-12). However, there is no comparative study on purification of G6PD. There are only two studies about purification of G6PD from the brain (rabbit and rat). In those studies, G6PD was purified from the rabbit brain cortex using a single immunoaffinity chromatographic step and G6PD from the rat brain was purified 13,000-fold to a specific activity of 480 units/mg protein (13,14).

Considering the significance of G6PD in the brain, we preferred to purify G6PD from the sheep brain cortex using 2', 5'-ADP-Sepharose 4B affinity and DEAE Sepharose Fast Flow ion exchange chromatography columns in various

purification step orders in an effort to determine the most efficient purification method for this tissue.

## **MATERIALS and METHODS**

### **Tissue**

Sheep brain was obtained from a local slaughterhouse, kept in dry ice and processed within 2–3 h after death.

### **Chemicals**

Glucose-6-phosphate (G6P), nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>), 6-phosphogluconate (6-PGA), and DEAE Sepharose Fast Flow, Sephadex G-200, Tris [Tris (hydroxymethyl) aminomethane] were obtained from Sigma Chemical Co., MO, USA. 2', 5'-ADP-Sepharose 4B and Sephadex G-200 are from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA) is from British Drug Houses Ltd. Potassium phosphate and orthophosphoric acid are from BDH Chemical (Poole, UK).

All other chemicals were analytical grade and obtained from Sigma, USA.

### **Measurement of G6PD Activity**

G6PD activities were determined using a LKB Ultraspec-Plus (4054 UV/visible) spectrophotometer by monitoring NADPH production at 340 nm at 37°C (15). The assay mixture contained 10 mM MgCl<sub>2</sub>, 0.2 mM NADP<sup>+</sup> and 0.6 mM G6P in 100 mM Tris/HCl buffer, pH 8.0. Assays were carried out in duplicate and the activities were followed for 60 sec. The reaction was linear during this time period ( $\epsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$ ).

One unit (U) of activity is the amount of enzyme required to reduce one  $\mu\text{mol}$  of NADP<sup>+</sup> per min under the assay conditions. Specific activity is defined as units per mg of protein.

### **Measurement of 6-PGD Activity**

6-PGD activities were determined by a spectrophotometric method. The assay mixture contained 10 mM MgCl<sub>2</sub>, 0.2

mM NADP<sup>+</sup> and 0.6 mM 6-PGD in 100 mM Tris/HCl buffer, pH 8.0. Assays were carried out in duplicate and the activities were followed by monitoring NADPH production at 340 nm for 60 sec at 37°C. The reaction was linear during this time period ( $\epsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$ ) (16).

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

### Measurement of Protein Concentration

Protein concentrations were determined by the method of Bradford (17) using BSA as a standard. The absorbances of each fraction from column effluents were measured at 280 nm.

### Polyacrylamide Gel Electrophoresis (PAGE)

Discontinuous PAGE as 5% stacking gel and 10% resolving gel 0.75 mm x 70 mm x 100 mm was carried out under denaturing conditions. sodium dodecyl sulfate (SDS) / PAGE (10%) was carried out exactly as described by Laemli (18) and the silver staining method of Merrill (19) was used. Electrophoretic separation was carried out using the Mini Protean III system of Bio-Rad (USA) at room temperature at 120 V for 30 min.

### Sephadex G-200 Gel Filtration

The samples were applied to Sephadex G-200 gel filtration column according to the method of Andrews (20). Sephadex G-200 column (2.25 x 50 cm) was equilibrated with 50 mM potassium phosphate buffer containing 0.1 M KCl pH 7.5. The elution was done with the same buffer. Flow rate during application and elution was 10.2 mL/h and fraction volume was 1.7 mL at 10 min.

### Purification of G6PD from Sheep Kidney Cortex

We established three different purification procedures for sheep brain cortex G6PD. All the procedures were carried out at +4°C. In this study, we preferred to purify sheep

brain cortex G6PD by 2',5'-ADP-Sepharose 4B affinity and DEAE Sepharose Fast Flow ion exchange chromatography columns, using the columns in different orders during purification.

The white matter of the brain was removed and the grey matter of the cortex was minced with scissors after washing with 10 mM Tris/HCl buffer, pH 7.6, containing 1 mM 2-mercaptoethanol (2-ME) (buffer A) and homogenized using an IKA Ultra-turrax homogenizer with S18N-10G probe at 22,000 rpm approximately 2 min with 3 volumes of buffer A on ice. The homogenate was centrifuged at 105,000 x g for 60 min at 4°C. We used 105,000 x g supernatants after homogenization in all the purification protocols.

### First purification protocol:

The supernatant obtained from ultracentrifuge was loaded onto the 2', 5'-ADP-Sepharose 4B column (1.5 x 6.7 cm) equilibrated with buffer A (flow rate: 10.8 mL/h). The column was washed with the same buffer to remove all the non-specifically bound proteins and compounds. Then G6PD and 6-PGD were eluted with buffer A containing 0.1 mM NADP<sup>+</sup>. The active G6PD fractions were combined and loaded onto the DEAE Sepharose Fast Flow column (1.5 x 7.5 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.9 (buffer B). The flow rate was maintained at 10.2 mL/h and the column was washed with buffer B until the absorbance at 280 nm decreased to 0.002 O.D. G6PD and 6-PGD were separated by a linear gradient of KCl (0–300 mM) in buffer B. The first purification protocol is similar to our previous purification protocols in which G6PD was purified from sheep kidney cortex and bovine lens (10,21). A summary of the first purification protocol is presented in Table 1.

**Table 1.** A general presentation of the purification steps of the first protocol

Purification step	Volume (ml)	G-6-PD (U/ml)	Total G-6-PD (U/ml)	Protein mg/ml	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Homogenate	45,00	0,160	7,20	29,470	1326,15	0,0054	100,0	1,00
105 000 x g supernatant	32,00	0,200	6,40	3,420	109,44	0,0585	88,89	10,77
Eluent of 2', 5'-ADP Sepharose 4B	19,00	0,265	5,04	0,021	0,40	12,6191	69,93	2324,27
DEAE Sepharose Fast Flow	12,00	0,410	4,92	0,008	0,10	51,2500	68,33	9439,61

### Second purification protocol:

The second protocol consists of six steps: homogenization,

ultracentrifugation, dialysis, anion exchange chromatography, dialysis, and affinity chromatography. In this purification protocol, we dialyzed the supernatant for 6 h with buffer B after ultracentrifugation step. Then, the dialyzed sample was centrifuged at 10,400 x g for 20 min, and loaded onto the DEAE Sepharose Fast Flow ion exchange chromatography pre-equilibrated with buffer B. The column was washed with buffer B until the absorbance at 280 nm decreased to 0.029. The enzyme was eluted by KCl gradient (0–300 mM). G6PD activity containing fractions were collected and dialyzed with buffer A and centrifuged at 10,400 x g for 20 min then loaded onto the 2',5'-ADP-Sepharose 4B affinity chromatography column. The column was washed with buffer A until the absorbance of the eluent was sufficiently decreased at 280 nm. G6PD was then eluted with buffer A containing 0.1 mM NADP<sup>+</sup>.

### **Third purification protocol:**

The third protocol was similar to the first except for the addition of a dialysis step after ultracentrifugation. We performed dialysis in buffer A. Then the dialyzed sample was centrifuged at 10,400 x g for 20 min. All the other purification steps were similar to the first purification protocol.

## **RESULTS**

### **Purification of G6PD from Sheep Brain Cortex**

In this study, three rapid procedures have been improved to purify G6PD from the sheep brain cortex. In all procedures, 105,000 x g supernatants, which were run for 1 h, were used after homogenization. We used the same chromatographic columns (2',5'-ADP-Sepharose 4B affinity chromatography and DEAE Sepharose Fast Flow ion exchange chromatography) which were equal in size. The orders of the purification steps (affinity and ion exchange chromatography) were changed to obtain the most efficient result. We used the same buffers for the same columns; 2',5'-ADP-Sepharose 4B affinity chromatography column was equilibrated with buffer A and the enzyme was eluted from the column with 10 mM Tris/HCl pH 7.6 containing 1 mM 2-ME and 0.1 mM NADP<sup>+</sup>. DEAE Sepharose Fast Flow ion exchange chromatography column was equilibrated with 10 mM potassium phosphate buffer, pH

6.9, and the enzyme was eluted from column with linear gradient of 0–300 mM KCl prepared in 10 mM potassium phosphate buffer, pH 6.9. We used same flow rate in both of the columns (10.2 mL/h).

We attempted to standardize every variable, including dialysis buffers, and dialysis buffer was selected according to chromatography column. For example, if affinity chromatography was to be used after dialysis, we chose buffer A for dialysis.

In the first purification protocol, the affinity column was washed with buffer A until the absorbance at 280 nm decreased to 0.019 O.D. Only a small amount of 6-PGD bound to the affinity column. The elution profile of 2',5'-ADP-Sepharose 4B affinity chromatography is shown in Figure 1. Since 6-PGD was bound to the affinity gel, the fractions containing G6PD and 6-PGD were coeluted. The activity containing fractions were collected and loaded onto the ion exchange column. The ion exchange column was washed with buffer B until the absorbance decreased to 0.002 O.D. at 280 nm. Figure 2 shows the elution profile of DEAE Sepharose Fast Flow ion exchange chromatography. G6PD and 6-PGD were both bound to the ion-exchange column and were well separated from each other by a linear gradient of KCl of 0-300 mM. A summary of the first purification protocol is presented in Table 1.

In the second purification protocol, we changed the order of ion exchange and affinity chromatography. After ultracentrifugation at 105,000 x g for 60 min, we dialyzed throughout for 6 h in potassium phosphate buffer, pH 6.9. The dialyzed supernatant was centrifuged at 10,400 x g for 20 min to get rid of the denatured proteins. Then, the obtained supernatant was loaded onto the DEAE Sepharose Fast Flow anion exchange chromatography column. We eluted the enzyme by KCl gradient (0-300 mM) (Fig. 3). G6PD activity containing fractions were collected and dialyzed with buffer A, then loaded onto the 2',5'-ADP-Sepharose 4B affinity chromatography column. The absorbance was measured until O.D. decreased to 0.021, at 280 nm, and G6PD was eluted with buffer A containing 0.1 mM NADP<sup>+</sup> (Fig. 4). A summary of the second purification protocol is given in Table 2.

In the last purification protocol, only an additional dialysis step (after ultracentrifugation) was added to the first purification procedure. Like the other purification protocols, the last protocol was also carried out at +4°C. The dialyzed sample was loaded onto the affinity column. All the other purification steps were the same as with the first protocol. The results are shown in Figure 5. G6PD activity containing fractions were combined and loaded onto the ion exchange column. G6PD was eluted with potassium phosphate buffer containing 300 mM KCl gradient. The results are shown in Figure 6. A summary of the third purification protocol is given in Table 3.

**Table 2.** A general summary of the purification steps of the second protocol

Purification (steps)	Volume (ml)	Activity (U/ml)	Total Activity (Units)	Protein mg/ml	Total protein (mg)	Specific Activity (U/mg protein)	Yield (%)	Purification (fold)
Homogenate	60,00	0,060	3,62	61,81	3708,60	0,00098	100,00	1,00
105 000 x g supernatant	44,00	0,080	3,54	7,43	326,92	0,01082	97,78	11,09
Dialyate	45,00	0,076	3,44	7,13	320,85	0,01071	95,00	10,98
DEAE Sepharose								
Fast Flow	7,80	0,157	1,22	0,062	0,48	2,52871	33,80	2592,03
Dialyate	7,00	0,145	1,01	0,059	0,41	2,45288	28,00	2514,3
Eluent of 2', 5'-ADP-Sepharose 4B	8,00	0,113	0,90	0,031	0,25	3,63097	24,89	3721,9

**Table 3.** A general summary of the purification steps of the third protocol

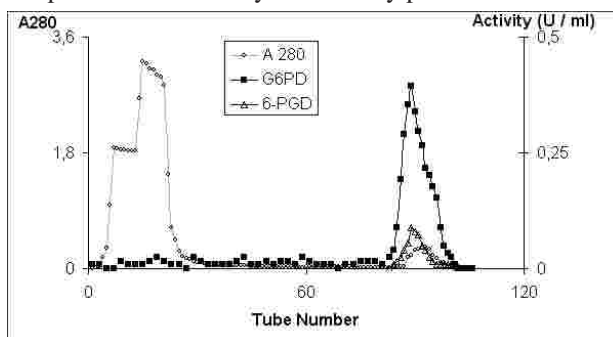
Purification (steps)	Volume (ml)	Activity (U/ml)	Total Activity (Units)	Protein mg/ml	Total protein (mg)	Specific Activity (U/mg protein)	Yield (%)	Purification (fold)
Homogenate	30,00	0,072	2,16	46,03	1380,9	0,00156	100,00	1,00
105 000 x g supernatant	19,00	0,093	1,77	7,4	140,6	0,01257	81,81	8,03
Dialyate	17,00	0,097	1,65	1,58	26,8	0,06159	76,34	39,4
Eluent of 2', 5'-ADP-Sepharose 4B	8,00	0,157	1,26	0,05	0,38	3,34043	58,15	2135,6
DEAE Sepharose Fast Flow	5,00	0,117	0,59	0,02	0,08	7,80000	27,08	4986,6

### The Control of G6PD Purity by SDS/PAGE and Sephadex G-200 Gel Filtration Method

We have controlled the purity of our enzymes using two different methods. First, we used SDS/PAGE electrophoresis. The purified enzyme gave a single band on SDS polyacrylamide slab gel electrophoresis. The gel was stained with silver staining. 15 µl (0.007 mg/mL) of the purified enzyme was applied on the gel (Fig. 7). The purified enzyme gave a single protein band on SDS/PAGE.

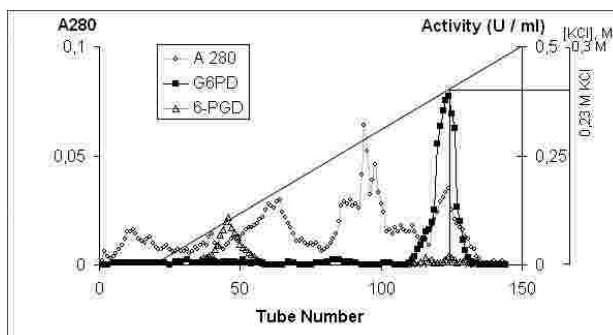
In the second method, the enzyme was loaded onto the Sephadex G-200 gel filtration column. With this method, various proteins are separated on the basis of their molecular weight. The elution profile is shown in Figure 8. It is seen from the elution profile that no other enzyme or protein contamination was present, and a single peak was produced

from the Sephadex G-200 column. Using these two methods, we proved that our enzyme is totally pure.



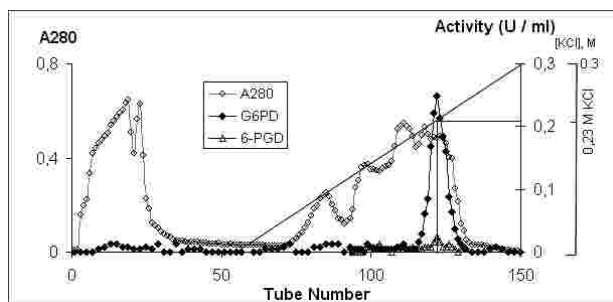
**Figure 1.** Affinity chromatography of G6PD and 6-PGD on 2', 5'-ADP-Sepharose 4B.

Column size, 1.5 \_ 6.7 ml; column were equilibrated and washed with, 10 mM Tris/HCl buffer pH 7.6 containing 1 mM 2-ME and EDTA. Enzyme elution buffer: same as the washing buffer containing 0.1 mM NADP+; flow rate, 8, 4 ml/h. Fractions of 1.40 ml were collected.



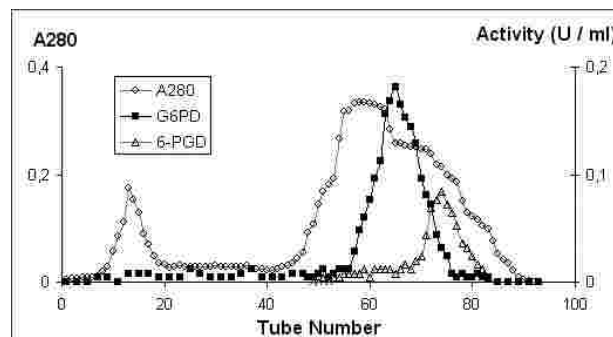
**Figure 2.** Elution profile of G6PD from DEAE Sepharose Fast Flow.

Column size, 1.5 \_ 7.5 cm. G6PD was eluted with by a linear gradient of KCl (0 – 300 mM) in 10 mM potassium phosphate buffer, pH 6.9 at a flow rate of 8, 4 ml/h.

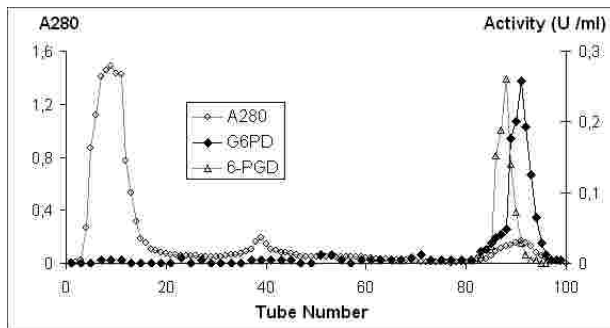


**Figure 3.** Elution profile of G6PD from DEAE Sepharose Fast Flow.

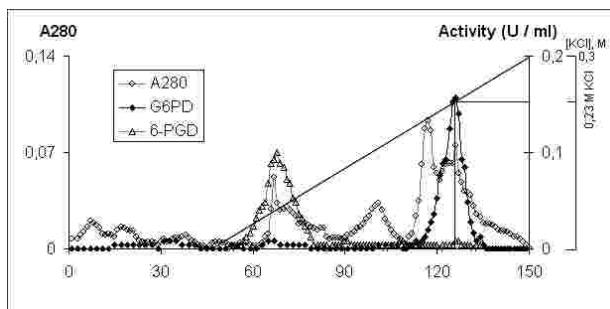
G6PD was eluted with by a linear gradient of KCl (0–300 mM) in 10 mM potassium phosphate buffer, pH 6.9.



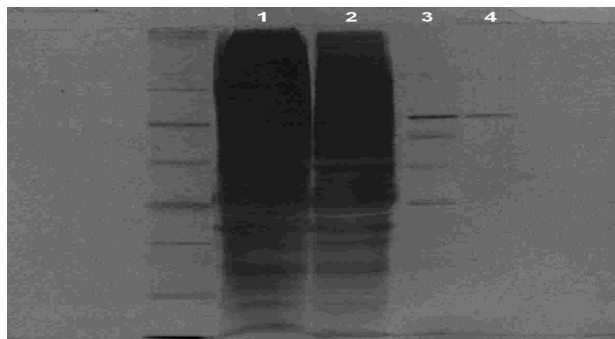
**Figure 4.** Affinity chromatography of G6PD and 6-PGD on 2', 5'-ADP-Sepharose 4B.



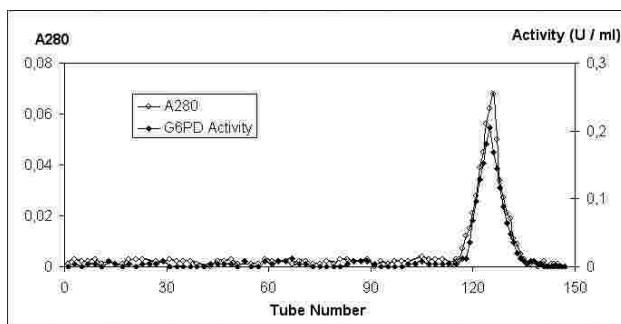
**Figure 5 :** Affinity chromatography of G6PD and 6-PGD on 2', 5'-ADP-Sepharose 4B.



**Figure 6 :** Elution profile of G6PD from DEAE Sepharose Fast Flow. Column size, 1.5 x 7.5 cm. G6PD was eluted with by a linear gradient of KCl (0 – 300 mM) in 10 mM potassium phosphate buffer, pH 6.9.



**Figure 7 :** SDS/PAGE of sheep brain cortex G6PD. Silver staining (SDS/PAGE): lane 1: homogenate; lane 2: 105 000 x g supernatant; lane 3: 2', 5'-ADP-Sepharose 4B eluate; lane 4: DEAE Sepharose ion exchange eluate.



**Figure 8 :** The control of G6PD purity by Sephadex G-200 gel filtration. G6PD was eluted using 50 mM potassium phosphate containing 0.1 M KCl pH 7.5 and fraction volume was 1.7 mL fractions. Flow rate was 10.2 mL/h during application and elution.

**DISCUSSION**

Affinity and ion exchange chromatography are widely used in many laboratories for the purification of numerous enzymes and proteins. We have reported herein three different purification protocols for determination of the most efficient purification protocol for G6PD from the sheep brain cortex. These purification protocols are very convenient, inexpensive and rapid methods for obtaining homogeneous preparations for this enzyme. We tried to standardize many parts of the protocols to demonstrate and emphasize the importance of the orders of column chromatographies. We obtained different yields, specific activities and folds in these purification protocols. In all enzyme purification protocols, the speed is very important. The fastest purification protocol was the first, followed by the third and consequently by the time-consuming second protocol. The best results were obtained in the first purification protocol in many ways. It was shown to be the simplest, cheapest and the fastest. The first purification protocol has many advantages -the enzyme sample is ready for kinetic studies. Most researchers use the affinity chromatography as a final purification step. Elutions of the enzyme are generally done by their substrates or inhibitors from the affinity chromatography. However, the enzyme sample must not contain its substrate or inhibitor, so researchers must add a Sephadex G-25 column chromatography or dialysis step to their purification protocol. With this additional property, we obtained the highest purification fold (9439.61), highest yield (68.33%) and highest specific activity (51.25 U/mg protein), as seen in Table 1.

The dialysis step is an important purification step in many purification protocols. This step usually follows the ultracentrifugation step. It is known that supernatants contain both enzymes and their substrates. The dialysis step must be added to the purification protocol if substrates of the enzyme have an interaction with chromatography column materials. Salts and various ions may also affect enzyme interactions with chromatography column material (for example ion exchange chromatography). However, in the presented study, exception of the dialysis step positively affected the recovery of the enzyme. This may be operative and effective in other enzyme purification protocols. In the second protocol, we obtained the enzyme with lower

yields (24.89%). This may be due to the additional two dialysis steps added to the protocol. Meanwhile, this protocol has a disadvantage because the purified enzyme contains its substrate (NADP<sup>+</sup>) at the end of the purification; thus, this may add a calculation step when studying the kinetic properties of this enzyme. The third and the last protocol is the same as the first protocol with an additional dialysis step. We added this step to get rid of the natural substrates that may affect the binding of G6PD to the affinity column (Substrates may compete with the enzyme for binding to the 2', 5'-ADP-Sepharose 4B affinity column). In all column chromatographies, G6PD activities were measured in all test tubes on loading and washing the columns. In our previous studies, we purified G6PD from various tissues by using same chromatography material (10,21,22). We can say that by using the first purification protocol, we purified the enzyme with a good yield and fold in a short time. We have also shown that in this short and simple protocol, we obtained an absolutely pure enzyme by SDS/PAGE electrophoresis and Sephadex G-200 gel filtration.

## CONCLUSION

G6PD has a very important role in protection from oxidative stress. Many diseases are associated with oxidative stress, and G6PD-deficient individuals face difficulty in coping with oxidative stress. Thus, a number of further studies are required for understanding the properties and roles of G6PD. Therefore, it is very important to investigate new purification protocols for G6PD in different tissues. These additional studies will undoubtedly enrich our comprehension and improve our strategies for new purification protocols for G6PD or other enzymes from various tissues.

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