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Rapid isolation of glucose-6-phosphate dehydrogenase from human erythrocytes by combined affinity and anion-exchange chromatography for biochemical characterization of variants

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ABSTRACT

A new method is presented for the purification of human glucose-6-phosphate dehydrogenase (G6PD) using affinity chromatography with 2'5'-ADP–Sepharose 4B followed by automated ion-exchange chromatography with DEAE 5PW. This rapid method allows a high recovery of enzyme activity from even a small amount of blood; the yield is about 90% after the first purification step and 70% at the end of the procedure. There is an excellent reproducibility of the kinetic parameters and optimal biochemical characterization of G6PD is achieved even for variants associated with severe enzyme deficiency (*e.g.* G6PD Mediterranean).

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49) is the key regulatory enzyme in the pentose phosphate pathway. The main physiological role of this enzyme in red blood cells is to provide NADPH, a compound required for numerous biosynthetic and detoxification reactions [1,2].

The human X-linked genetic locus encoding G6PD is highly polymorphic and is associated with more than 380 known biochemical variants. The two main difficulties in the study of these variants are the considerable weakness of the protein and its low concentration in mature erythrocytes. The purification methods currently used are based on long and complicated chromatographic procedures, the first of which, dating from the 1960s and 1970s [3–5], yield an only partly purified enzyme, starting from a large amount of blood. Other more recent methods [6–8], although having the advantage of requiring small volumes of blood and a single purification step, are not yet good enough for large-scale characterization studies.

Recent advances in the molecular characterization of G6PD variants [9–12] have shown that the biochemical heterogeneity of G6PD is often not associated with a comparable variability of the nucleotide sequence [13,14]. This makes a kinetic study essential to avoid possible ambiguities in correlating biochemical and molecular aspects.

This paper describes a purification method that is not only rapid, but requires only small volumes of blood for the complete characterization of variants.

EXPERIMENTAL

G6PD was purified by affinity chromatography from haemolysate using Bio-Rad glass col-

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umns (15 cm × 1.5 cm I.D. and 10 cm × 0.5 cm I.D.), packed with affinity gel. The second step consisted of ion-exchange chromatography with a Waters 650 advanced protein purification system (Millipore, Milford, MA, USA), combined with a Waters Protein Pak-glass DEAE 5PW (Nihon Waters, Tokyo, Japan) prepacked column (7.5 cm × 0.8 cm I.D.). The elution profile was monitored at 280 nm by a Waters UV Meter Lambda-Max 481 LC spectrophotometer.

The enzyme activity was determined at 340 nm with a Gilford 2400-S (Gilford Instruments Laboratory, Oberlin, OH, USA) spectrophotometer.

The affinity gel (2′5′-ADP–Sepharose 4B) was purchased from Pharmacia (Uppsala, Sweden). Glucose-6-phosphate (G6P; disodium salt), 6-phosphogluconate (6PG; trisodium salt), NADP and NADPH were supplied by Sigma (St. Louis, MO, USA) and Tris, EDTA and β-mercaptoethanol by Merck (Darmstadt, Germany). All other reagents were of the highest available commercial purity.

The following solutions were prepared: solution A, 0.1 *M* Tris–HCl, pH 7.6, containing 5 m*M* EDTA and 0.02% β-mercaptoethanol; solution B, 0.1 *M* potassium phosphate, pH 7.0; solution C, solution B containing 1 *M* sodium chloride and 0.02% sodium azide; solution D1, 5 m*M* potassium phosphate, pH 6.9, containing 1.25 m*M* EDTA and 1 m*M* aminocaproic acid; and solution D2, solution D1 containing 0.5 *M* potassium chloride.

Patients

All five G6PD-deficient patients studied in this work have been described as G6PD Mediterranean on the basis of severe enzyme deficiency detected in crude haemolysate and biochemical characterization in accordance with the WHO classification [15]. Twenty normal subjects with no enzyme deficiency were used as controls.

Preparation of haemolysate

A 2.5-ml aliquot of whole blood was obtained from normal subjects (B+) and 15 ml from G6PD-deficient patients. The blood was collected in acid–citrate–dextrose and stored at 4°C for not more than fifteen days [16].

Leukocytes and platelets were removed by filtration through mixed α-cellulose and microcrystalline cellulose in a 1:1 ratio, according to Beutler *et al.* [17].

Erythrocytes were washed three times with five volumes of ice-cold 0.154 *M* sodium chloride solution using a centrifuge for 15 min at 1000 *g* at room temperature.

Haemolysis was carried out by adding nine volumes of 1:10 solution A to the packed red cells and storing the mixture at 4°C for 30 min. The stroma were removed from this haemolysate by ultracentrifugation at 5000 *g* for 10 min at 4°C and then collecting the supernatant.

Purification by affinity chromatography

Haemolysate (1:10) was diluted with 0.3 volumes of solution A containing 10 m*M* magnesium chloride.

The enzymes from subjects with G6PD deficiency were purified in glass columns measuring 15 cm × 1.5 cm I.D. and that of normal subjects in columns of 10 cm × 0.5 cm I.D. (3 and 0.3 ml of bed volume, respectively). The columns were packed with 2′5′-ADP–Sepharose affinity gel.

2′5′-ADP–Sepharose 4B gel was activated using solution B, as recommended by the manufacturer, and then equilibrated with solution A to adjust the pH to 7.6.

Haemolysate (1:10) was applied to the stationary phase with a peristaltic pump, after which the flow-rate was increased using moderate pressure with a syringe. The gel was then washed with solution A to remove all the non-specifically bound contaminating compounds, including haemoglobin (at least 10 and 30 ml, respectively, for the two types of column). After the elution of G6PD, the gel could be recycled eight to ten times, if previously washed with 50 ml of solution C and stored in this buffer at 4°C.

Purification by anion-exchange chromatography

To obtain high-resolution chromatograms from this automated procedure performed with the Waters 650 apparatus, the sample eluted in the previous step had to be purified through 0.22-μm filters. For the same reason, all solvents used were prepared with doubly distilled water, passed through 0.22-μm filters and then degassed by the apparatus itself.

DEAE 5PW prepacked gel was equilibrated in the isocratic mode at a flow-rate of 0.5 ml/min for at least 2 h using solution D1; the filtered sample was loaded into a loop of suitable volume (5 or 7.5 ml) and maintained at a low temperature in a thermostated bath.

Elution was then carried out using a linear gradient of potassium chloride from 130 to 260 m*M* in 30 min (from 26 to 52% of solution D2) at a flow-rate of 0.5 ml/min. The gradient was followed by elution for 10 min at a flow-rate of 1.0 ml/min using 100% solution D2 to remove any proteic residues still bound to the gel. The gel was then washed with doubly distilled water at a flow-rate of 1.0 ml/min for about 1 h. A constant operating pressure of about 6 bar was maintained during the experiment.

Determination of protein concentration

The protein concentration was evaluated by the method of Lowry *et al.* [18] using a bovine serum albumin solution as standard.

Assay methods

G6PD and 6-phosphogluconate dehydrogenase activities, the kinetic parameters for the substrates, the percentage use of the substrate analogues and the optimum pH were determined using the standard procedure proposed by the WHO [15]. Heat stability tests were performed according to the recommendations of the Internal Committee for Standardization in Haematology [19].

Polyacrylamide gel electrophoresis (PAGE) was performed as previously described by De Flora *et al.* [20] and Bonsignore *et al.* [21].

RESULTS

Purification by affinity chromatography

This step allows the separation of G6PD and all NADP-binding proteins from the haemolysate on the basis of their affinity for the 2′5′-ADP ligand. To exclusively elute G6PD, 5 ml of 0.4 m*M* NADP in solution A were used for normal subjects (Fig. 1) and 7.5 ml of the same solution for the G6PD-deficient subjects. These volumes were suitable for the complete recovery of the enzyme.

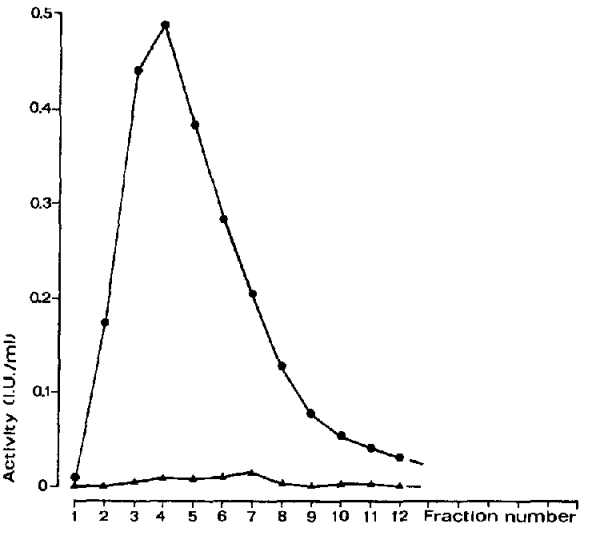

Fig. 1. Elution profile (activity *versus* time) of normal erythrocyte G6PD on a 2′,5′-ADP–Sepharose 4B affinity column. Eluent, 0.4 m*M* NADP in solution A; flow-rate, 1.0 ml/min; volume of fractions, 0.5 ml. (●) G6PD activity; (▲) 6PGD activity.

A good yield of pure enzyme was obtained in 15–20 min starting from the haemolysate, which could also be used for Km G6P and optimum pH studies and to determine the percentage use of galactose-6-phosphate and 2-deoxyglucose-6-phosphate [2,19]. These assays required a saturating concentration of NADP and were therefore not influenced by its presence in the eluate; furthermore, as is shown later, they were not altered by contaminating enzymatic activities. To complete the characterization, NADP and traces of other proteins were removed by anion-exchange chromatography.

Purification by anion-exchange chromatography

Excellent recovery of enzyme activity was achieved using the described linear gradient. The G6PD elution was at a maximum between 220 and 230 m*M* potassium chloride. The eluent was collected in volumes not exceeding 0.5–1.0 ml for normal G6PD subjects and not exceeding 300 μl for the G6PD Mediterranean variant.

The length of this procedure, including sample preparation, was about 40 min, starting from the eluate obtained from the affinity chromatography step.

The complete elution profile is shown in Fig. 2.

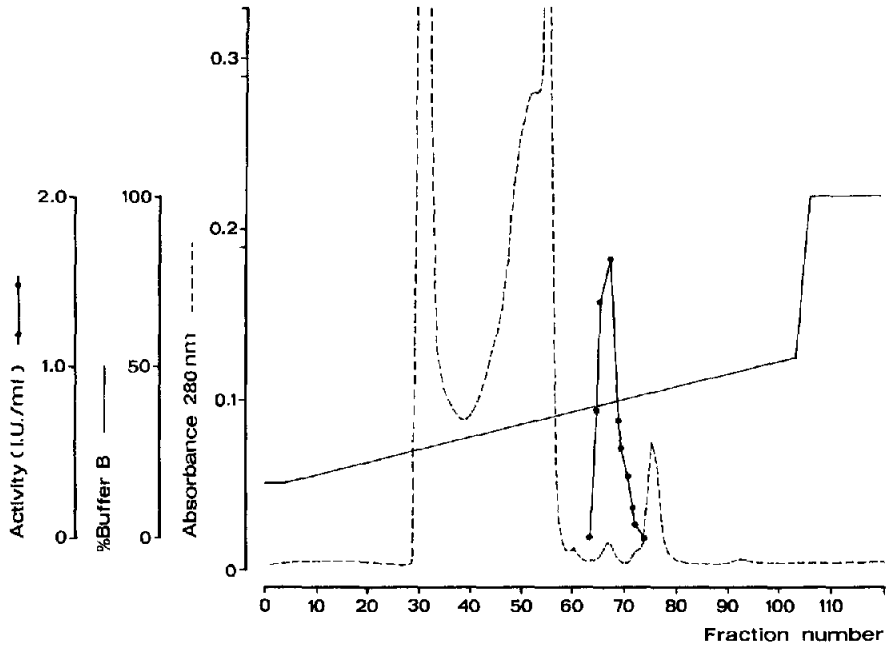

Fig. 2. Separation of normal erythrocyte G6PD on a Protein Pak-glass DEAE 5PW column. Buffer A, solution D1; buffer B, solution D2; flow-rate, 0.5 ml/min; volume of fractions, 150 μl. (●) G6PD activity.

With this method, NADP and residual traces of haemoglobin and other proteins are completely separated from G6PD, allowing the use of these fractions for the evaluation of all other kinetic parameters (percentage use of deamino-NADP, Km NADP and Ki NADPH), without the need for dialysis.

The concentrated fractions eluted in this step could be stored at 4°C for several days without significant loss of activity.

Tables I and II give the purification data and yield of the method for normal subjects and those carrying the G6PD Mediterranean variant. The overall results showed a 60 000-fold increase of G6PD-specific activity after the last purification step.

The yield of enzymatic activity achieved for normal subjects was estimated at about 70% at the end of the procedure, with peaks of over 90% in the intermediate step. There was a slightly lower yield, attributable to the greater lability of the enzyme, for subjects with G6PD deficiency.

Purity criteria

The final preparation of G6PD satisfied the following homogeneity standards.

(1) The specific activity in normal subjects at the end of the procedure was about 170 I.U./mg of protein, in accordance with the values reported by other workers who used more purification steps [3–6].

(2) PAGE at different pH values revealed only one or two bands if the gel was stained with silver staining, corresponding to the tetramers and dimers of the enzyme [22] (Fig. 3).

(3) Sodium dodecyl sulphate (SDS) PAGE showed G6PD in addition to with a minor band, which could correspond to the protein FX that binds nicotinamidic nucleotides; this has been described by Morelli and De Flora [23] but has still not been identified in erythrocytic dehydrogenases. The impurity was easily removed from the enzymatic preparation by gel permeation chromatography (Fig. 4).

(4) The absence of the following enzymatic activities was confirmed: 6-phosphogluconate dehydrogenase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glutathione reductase, triosephosphate isomerase [19], malate dehydrogenase, isocitrate dehydrogenase and glutamate dehydrogenase [24].

TABLE I

PURIFICATION OF G6PD FROM NORMAL SUBJECTS (G6PD B+)

Mean data from twenty different experiments are shown.

Purification step	Total volume (ml)	Total proteins (mg)	Total units (I.U.)	Specific activity (I.U./mg of protein)	Accumulative purification[a]	Yield (%)
Haemolysate	8	546.7	1.425	0.0026	1	100
2′5′-ADP–Sepharose 4B	5	–[b]	1.311	–	–	92
DEAE 5PW	1	0.006	1.012	168.7	64 884	71

[a] Ratio of specific activity, taking that of haemolysate as 1.
[b] Proteins were not detectable at this level. See text for details.

TABLE II

PURIFICATION OF G6PD FROM B+-DEFICIENT SUBJECTS (G6PD MEDITERRANEAN)

Mean data from five different experiments are shown.

Purification step	Total volume (ml)	Total proteins (mg)	Total units (I.U.)	Specific activity (I.U./mg of protein)	Accumulative purification[a]	Yield (%)
Haemolysate	48	3170	0.01165	3.6×10^{-6}	1	100
2′5′-ADP–Sepharose 4B	7.5	–[b]	0.0094	–	–	81
DEAE 5PW	0.45	0.039	0.0073	0.188	52 222	63

[a] Ratio of specific activity, taking that of haemolysate as 1.
[b] Proteins not detectable at this level. See text for details.

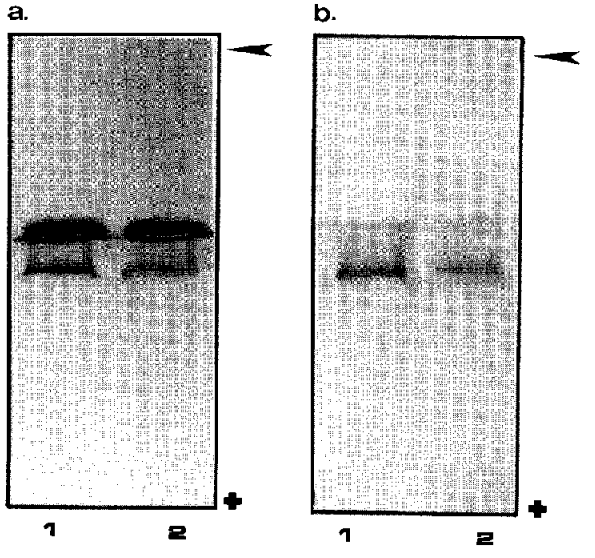

Fig. 3. Native PAGE of purified G6PD. Acrylamide concentration, 7.5%; running buffers, (a) Tris–HCl, 0.01 *M*, pH 7.3; (b) Tris–HCl, 0.01 *M*, pH 8.8.

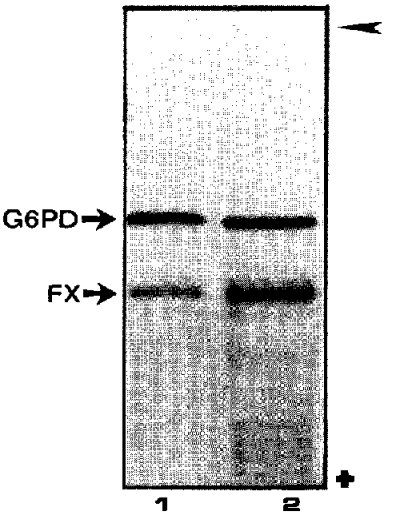

Fig. 4. SDS-PAGE of purified G6PD. Acrylamide concentration, 10%; running buffer, Tris–glycine, pH 8.3.

DISCUSSION

The purification procedure described here is relatively simple and rapid, and its reliability and yield are good. Furthermore, the blood volumes required are small, yet a complete biochemical characterization of G6PD can be obtained, even for variants associated with severe activity deficiency (3–5% of levels in normal subjects). This method, unlike those described previously, allows the characterization of the enzyme in a single day, starting from the withdrawal of blood, thus reducing to a minimum the decrease in activity caused by long methods and dialysis. The high calculated yield (90–95%) after the affinity chromatography step means that this eluate can be used for some kinetic and activity studies, even when starting from a small amount of cellular lysates. In this way, for example, it is possible to assay G6PD in lysates obtained from less than $2 \cdot 10^6$ erythroid progenitors in culture, in various stages of maturation (data not shown).

The main advantages of the automated anion-exchange chromatography are attributable to the simple washing procedures, particularly of the column, and to the complete removal of not only NADP used as the eluent in the first step, but also the other contaminating proteins. Apart from the obvious practical benefits, this method offers the possibility of the precise and reliable determinations of Km NADP and Ki NADPH parameters; until now the lack of such data has greatly handicapped the complete characterization of many G6PD-deficient variants. The accurate determination of these values assumes even greater importance in the light of recent progress in the study of the NADP -NADPH binding site at the primary structure level [12], which is now being investigated in this laboratory.

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