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PURIFICATION OF HUMAN RED CELL GLUCOSE 6-PHOSPHATE DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY

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SUMMARY

Human glucose 6-phosphate dehydrogenase associated with NADPH was efficiently bound with agarose-bound NADP, whereas the enzyme associated with NADP was poorly bound with agarose-bound NADP. After the elimination of haemoglobin from haemolyzate by treatment with DEAE-cellulose, the enzyme was converted into the NADPH-bound form and was applied on an affinity column. The enzyme was specifically eluted from the column by NADP in the elution buffer. A homogeneous enzyme preparation was obtained in high yield.

INTRODUCTION

Since human glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49)* was first obtained in homogeneous form from red blood cells by multi-step column chromatographic purification[1], several alternative procedures have been reported, including (a) modification and simplification of the original chromatographic method[2,3] and (b) specific elution of the enzyme from an ion exchanger by its coenzyme, substrate or substrate analogue[4–6]. In the author's experience, most of these methods provide a homogeneous enzyme preparation from human red blood cells.

As affinity chromatography is the most specific and efficient method for enzyme purification, it is desirable to use it for the purification of glucose 6-phosphate dehydrogenase. Recently, methods for purifying the enzyme by using an affinity column of agarose-bound NADP have been reported[7,8]. However, the results were not satisfactory with respect to specificity and yield. This paper reports a specific affinity chromatographic method for the purification of glucose 6-phosphate dehydrogenase in which a homogeneous enzyme preparation was obtained in high yield.

MATERIALS AND METHODS

Agarose–NADP (agarose–adipic acid dihydrazide–NADP, 2.5 μmoles of

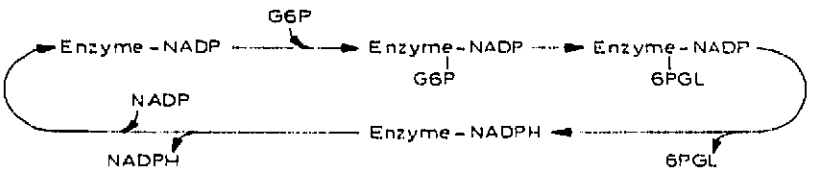
* Abbreviations: G6P = D-glucose 6-phosphate; 6PGL = 6-phosphogluconolactone.

NADP per millilitre of wet gel) was purchased from P.L. Biochemicals (Milwaukee, Wisc., U.S.A.) or was prepared in our laboratory by the method described by Lamed *et al.*[9]. Glucose 6-phosphate dehydrogenase was partially purified from out-dated human blood (ACD anticoagulant) by haemolysis and treatment with DEAE-cellulose as previously reported[1]. The haemoglobin-free enzyme solution thus obtained was treated with ammonium sulphate (40 g per 100 ml) and centrifuged. The enzyme precipitate from 1 l of blood was suspended in about 50 ml of 0.01 *M* Tris–Cl, pH 7.0, that was 1 m*M* in EDTA, 1 m*M* in 2-mercaptoethanol and 10 μM in NADP and dialyzed against 50 volumes of the same buffer overnight at 4°. The centrifuged supernatant liquid (80–100 ml), which contained about 500 units of the enzyme and about 1 g of protein, was used for subsequent experiments.

The glucose 6-phosphate dehydrogenase activity was assayed as previously reported[1]. One unit is defined as that amount of enzyme which catalyzes the reduction of 1 μmole of NADP per minute at 25° under the assay conditions. The activity of glutathione reductase and NADPH diaphorase was assayed by the method described by Beutler[10]. Protein was assayed by the method of Lowry *et al.*[11] with bovine serum albumin as the standard. Polyacrylamide disc electrophoresis was carried out as previously described[12].

RESULTS

Human glucose 6-phosphate dehydrogenase has one tightly bound NADP unit per active dimer, which consists of two identical subunits of molecular weight 55,000 (refs. 13 and 14). The bound NADP cannot be rapidly replaced with free NADP, unless the enzyme reaction takes place and the NADP-bound enzyme is regenerated through the following catalytic cycle:



Therefore, agarose–NADP may not effectively associate with the NADP-bound enzyme, but it may easily associate with the NADPH-bound enzyme*. In order to confirm this possibility, the following three small-scale experiments were carried out.

* Recently, it was reported that, in addition to tightly bound NADP, the enzyme can associate with another NADP per monomer[15]. This conclusion was derived from the titration of the enzyme with NADP, measuring the quenching of protein fluorescence. It is well known that, in contrast to the reduced nucleotides, NAD and NADP do not induce quenching of protein fluorescence, even if the coenzyme is bound to the protein. These experiments and conclusions cannot be considered valid.

NADP-bound enzyme on the affinity column

The NADP-bound enzyme obtained as described under Materials and methods was dialyzed against 0.01 *M* Tris–Cl, pH 8.0, that was 10 m*M* in magnesium chloride for 3 h at 4°. The dialyzed enzyme solution was placed on an agarose–NADP column (0.5 cm, bed volume 1 ml) equilibrated with the dialysis buffer. As shown in Fig. 1, significant activity was detected in the effluent even during the sample application and less than 50% of the enzyme applied was adsorbed on the column. Approximately 37% of the enzyme activity was recovered from the column by specific elution with NADP.

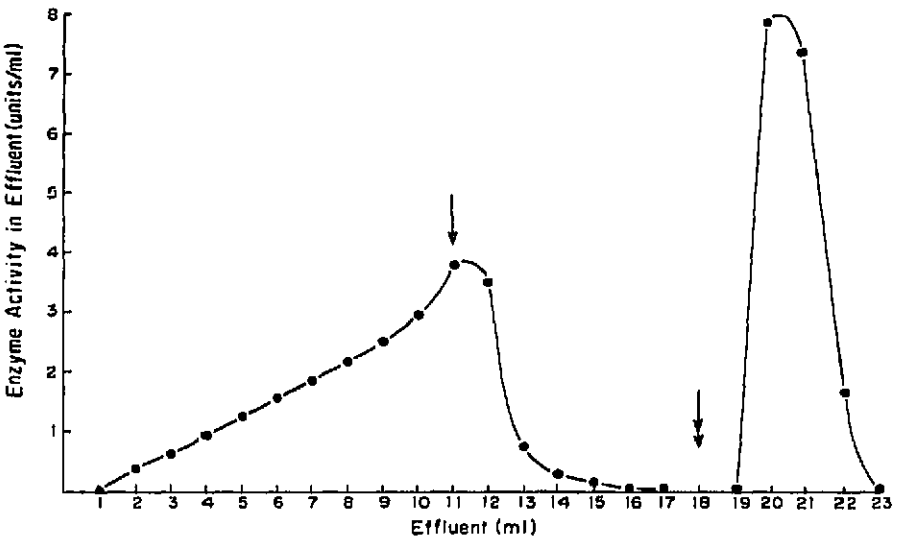


Fig. 1. Elution pattern of NADP-bound glucose 6-phosphate dehydrogenase from an agarose–NADP column. Partially purified NADP-bound enzyme (10 ml, 46 units) was placed on an agarose–NADP column (5 × 0.5 cm, bed volume 1 ml) equilibrated with 0.01 *M* Tris–Cl, pH 8.0, that was 10 m*M* in magnesium chloride. After the enzyme solution had been applied (at the single arrow), the column was washed continuously with the buffer. Then (at the double arrow) the column was eluted with the buffer that was 1 m*M* in NADP. Flow-rate, 0.4 ml/min. The chromatography was carried out at 25°.

NADP-bound enzyme on the affinity column

Magnesium chloride, NADP and glucose 6-phosphate were added to the enzyme solution to give final concentrations of 10 m*M*, 5 μM and 50 μM, respectively. The enzyme solution was dialyzed overnight against 0.01 *M* Tris–Cl, pH 7.0, that was 10 m*M* in magnesium chloride and 1 m*M* in 2-mercaptoethanol at 4°, changing the outside buffer three times during the dialysis. During the procedure, all NADP was reduced to NADPH by the enzyme and the NADPH-bound enzyme remained in the dialysis bag. The NADPH-bound enzyme was placed on an agarose–NADP column. Fig. 2 shows the elution profile of the enzyme. In contrast to the NADP-bound enzyme, the NADPH-bound enzyme was well adsorbed on the column; less than 10% of the total activity was not adsorbed during the sample application

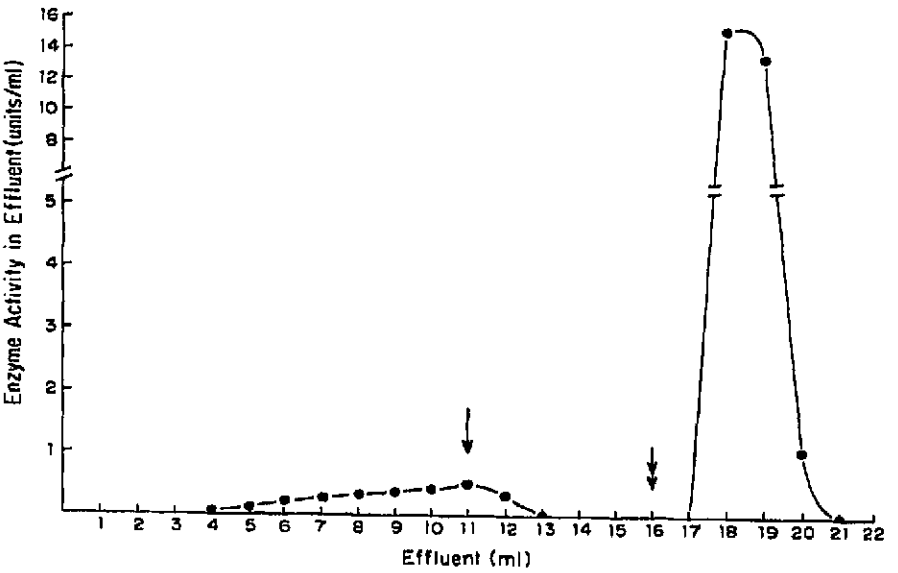


Fig. 2. Elution pattern of NADPH-bound glucose 6-phosphate dehydrogenase from an agarose–NADP column. Partially purified NADPH-bound enzyme (10 ml, 41 units) was placed on an agarose–NADP column, washed with the buffer, and eluted with the buffer containing NADP as described in Fig. 1.

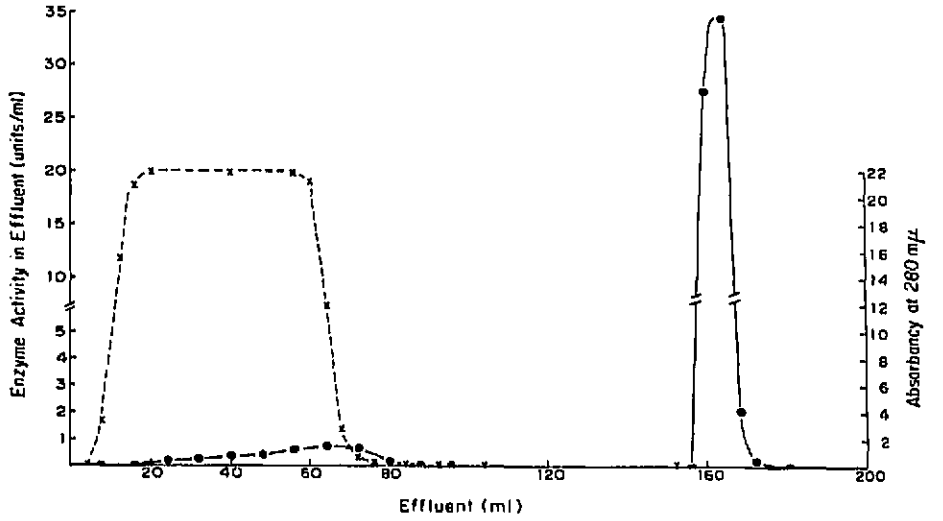


Fig. 3. Elution pattern of NADPH-bound glucose 6-phosphate dehydrogenase from a preparative agarose–NADP column. ●—●, Enzyme activity; ×–·–·–×, absorbance at 280 nm. Partially purified NADPH-bound enzyme (60 ml, 280 units) was applied on an agarose–NADP column (15 × 0.8 cm, bed volume 8 ml). After the enzyme solution had been applied, the column was washed with 100 ml of the Tris–Cl buffer, and the enzyme was eluted from the column. Flow-rate, 1 ml/min.

and washing. About 80% of the enzyme placed on the column was specifically eluted from the column by the buffer containing NADP.

Preparation of glucose 6-phosphate dehydrogenase by affinity chromatography

The dialyzed NADPH-bound enzyme solution (50–70 ml, total enzyme activity 240–300 units, specific activity 0.5 unit/mg) was applied on an agarose–NADP column (15 × 0.8 cm, bed volume 8 ml). A typical elution profile is shown in Fig. 3. Most of the impurity protein, as monitored by the absorbance at 280 nm, was eluted during the sample application and washing, and only a small amount of the enzyme was eluted during these processes. Over 80% of the enzyme applied on the column was recovered by the specific elution. The enzyme preparation thus obtained had a specific activity of 150–160 units/mg. As homogeneous human glucose 6-phosphate dehydrogenase has a specific activity of about 180 (ref. 1), the purity of the enzyme preparation was 80–90%. Polyacrylamide gel electrophoresis indicated one major protein band that had enzyme activity (Fig. 4).

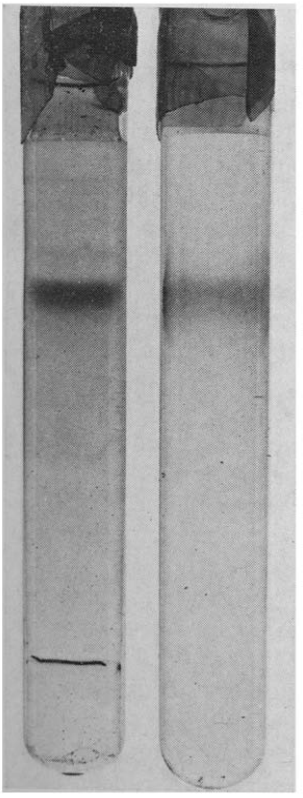


Fig. 4. Polyacrylamide gel electrophoresis of the enzyme purified through an agarose–NADP column. Electrophoresis was carried out in a 7.5% acrylamide gel with Tris–glycine, pH 8.9. The right-hand gel was stained by its enzymatic activity as previously described[16]. The left-hand gel was stained with amido black dye. Faint impurity protein bands can be seen in the left-hand gel. A piece of copper wire was inserted to mark the position of the front dye. The R_F value of the major protein band (left-hand gel) and that of the enzyme (right-hand gel) was 0.26.

The activity of two other NADP-dependent enzymes, NADPH diaphorase and glutathione reductase, was assayed before and after the affinity chromatography. Glutathione reductase was absent, but NADPH diaphorase was present in the enzyme solution applied on the column and in the purified enzyme preparation. The activity ratio between NADPH diaphorase and glucose 6-phosphate dehydrogenase was 0.014 in the enzyme solution applied on the column and 0.00015 in the purified enzyme. The impurities, including a small amount of NADPH diaphorase, were easily removed from the enzyme preparation by gel filtration. The enzyme solution from the affinity chromatography was concentrated to about 1.5 ml by vacuum dialysis. The concentrated solution was applied on a Sephadex G-200 column (50 × 1.2 cm) equilibrated with 0.02 *M* disodium hydrogen orthophosphate–potassium dihydrogen orthophosphate solution, pH 7.0, that was 1 m*M* in EDTA, 1 m*M* in 2-mercaptoethanol and 10 μM in NADP, and eluted with the same buffer. Homogeneous glucose 6-phosphate dehydrogenase with a specific activity of 180 units, free from NADPH diaphorase, was obtained from the haemolyzate with an overall yield of about 60%.

DISCUSSION

This work has demonstrated that the NADPH-bound enzyme is adsorbed more efficiently than the NADP-bound enzyme on agarose–NADP. Glucose 6-phosphate dehydrogenase, which has no bound NADP or NADPH, is presumably efficiently bound with agarose–NADP. The enzyme, free from NADP and NADPH, can be prepared by treatment of the enzyme with acidic ammonium sulphate[17] or with *p*-chloromercuribenzoate[18]. However, the nucleotide-free enzyme, particularly mutant human enzymes (A^- variant and Mediterranean variant) are labile[19]. A greater portion of the enzyme is expected to be inactivated during the purification procedure using such an enzyme. Therefore, the use of the NADPH-bound enzyme is the best practical method for the purification of the enzyme by affinity chromatography.

We tried to purify the A^- variant enzyme, which is associated with red cell enzyme deficiency and instability[20], by the specific elution methods using a substrate analogue[5] or using NADP[6]. In contrast to the normal enzyme, a large amount of the variant enzyme was not eluted from a carboxymethyl-Sephadex column. A similar unsatisfactory result for the purification of the A^- variant enzyme has been reported by other investigators[21]. The A^- variant enzyme was successfully purified by affinity chromatography as described in this paper.

Comparing the amount of NADP in the agarose adsorbent (2.5 μmoles per millilitre of wet gel), the adsorbable amount of glucose 6-phosphate is very low. Theoretically, 1 ml of the agarose–NADP gel could adsorb 275 mg (or 49,500 units) of the enzyme. However, only about 40 units of the enzyme were adsorbed, which implies that only 0.1% of the total NADP bound on agarose is available for association with the enzyme. Such low efficiency has often been observed on association of agarose-bound nucleotide with several other dehydrogenases[22,23]. A particular steric orientation of the nucleotide residues on the gel is presumably required for adsorption of the enzyme.

The agarose–NADPH, which was produced by treatment of the gel with glucose 6-phosphate dehydrogenase and glucose 6-phosphate, cannot associate efficiently with the enzyme. Therefore, the enzyme solution should be extensively

dialyzed prior to application on the adsorbent, as described above. Alternatively, the enzyme solution should be passed through a small anion-exchange column (Dowex 1-X8, 5 × 0.7 cm) in order to eliminate completely glucose 6-phosphate from the enzyme solution.

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