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PURIFICATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *ACER PSEUDOPLATANUS* L. USING IMMOBILISED PROCION RED HE-3B*

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SUMMARY

The effect of the support matrix on the capacity of immobilised Procion Red HE-3B for a higher plant $NADP^+$-dependent dehydrogenase is examined. A method is presented for the purification of 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44.) from *Acer pseudoplatanus* L. (sycamore) which includes chromatography on Procion Red–Sepharose. The enzyme was purified 380-fold with 40–50% recovery and a final specific activity of 80 units per mg protein. Preliminary evidence was obtained for the existence of isoenzymes of 6-phosphogluconate dehydrogenase in *Acer pseudoplatanus* L.

INTRODUCTION

The use of group-specific adsorbents for the purification of $NADP^+$-dependent dehydrogenases is now widespread[1]. Immobilised nucleotides such as $NADP^+$ and 2′,5′-ADP are often suitable as ligands[2–4], but their high cost and low capacities preclude their use in many instances. In addition, the covalent attachment of nucleotides to insoluble support materials requires complex and potentially hazardous synthetic procedures. In contrast, immobilised Cibacron Blue 3G-A (the triazine dye component of Blue Dextran) which binds many nucleotide-dependent enzymes, is easily prepared and relatively inexpensive and consequently has been used as a group-specific ligand for the purification of several kinases and dehydrogenases[5,6]. It has been suggested that the dye may interact with the nucleotide binding site of such proteins[7]. An additional advantage of Cibacron Blue 3G-A is its high operational capacity when compared with group-specific nucleotide adsorbents[8].

A related triazine dye, Procion Red HE-3B, which was first used in the purification of carboxypeptidase G from *Pseudomonas*[9], has more recently been shown to retard several dehydrogenases[8,10]. In particular, there is some evidence that this ligand exhibits a greater selectivity toward $NADP^+$-dependent enzymes than Ciba-

* Dedicated to Professor J. Porath on the occasion of his 60th birthday.

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cron Blue 3G-A[8]. We have investigated the use of immobilised Procion Red HE-3B for the purification of an $NADP^+$-dependent dehydrogenase from *Acer pseudoplatanus* L. While the enzymes of the pentose phosphate pathway from animal and microbial sources have been extensively purified and characterised, relatively little is known of the function and regulation of this process in higher plants. We report here a method for the isolation of 6-phosphogluconate dehydrogenase in high yield including chromatography on immobilised Procion Red HE-3B. This work forms part of a larger study of the regulation of carbohydrate oxidation in *Acer pseudoplatanus* L.[11–13].

MATERIALS AND METHODS

Materials

Cellulose and DEAE-cellulose (DE-52) were obtained from Whatman (Maidstone, Great Britain); Sepharose 6B, CL-6B, Sephadex G-200, Sephacryl S-200 and 2′,5′-ADP–Sepharose were from Pharmacia (G.B.) (London, Great Britain); Ultrogel AcA-54 was from LKB (Croydon, Great Britain); Indubiose Perles A6 was from Réactifs IBF (Clichy, France) and Unisphere P1000 from Hydron Labs. (New Brunswick, NY, U.S.A.). Procion Red HE-3B was a gift from ICI Organics Division (Manchester, Great Britain). 6-Phosphogluconate and $NADP^+$ were purchased from Boehringer (London) (London, Great Britain). Standard marker proteins for sodium dodecyl sulphate (SDS) electrophoresis were from BDH Chemicals Ltd. (Poole, Great Britain) and Minicon-A macrosolute concentrators and Matrex Gel Red A were obtained from Amicon (Woking, Great Britain). All other chemicals were purchased from BDH, and were of the highest purity available.

Culture of Acer pseudoplatanus L.

Non-photosynthetic cell suspension cultures of *Acer pseudoplatanus* L. were used throughout this work. The origin and maintenance in batch culture of this tissue were as described previously[11]. For extraction of 6-phosphogluconate dehydrogenase the cells were harvested at 10 days, since preliminary work established that maximum activity was present at this time.

Immobilisation of Procion Red HE-3B

Procion Red HE-3B derivatives of several support materials were prepared as described[6]. Immobilised ligand concentrations of all materials except cellulose, Unisphere P1000, Sephacryl S-200 and Sephadex G-200 were determined after hydrolysis in 50% (v/v) acetic acid at 100°C for 5 min by measurement of $A_{536\,nm}$ of the resulting solution. The remaining samples were suspended in 10% (v/v) glycerol and the ligand concentration determined directly by measurement at 536 nm against blanks containing an equivalent amount of matrix.

Measurement of enzyme activity

6-Phosphogluconate dehydrogenase (E.C. 1.1.1.44.) was measured at 25°C by following the reduction of $NADP^+$ at 340 nm. The assay comprised 0.05 *M* Tris–HCl (pH 7.5) containing 15 m*M* $MgCl_2$, 2 m*M* 6-phosphogluconate and 0.16 m*M* $NADP^+$. The reaction was initiated by the addition of enzyme and was linear for 1–2 min. A unit of activity was defined as 1 μmole of $NADP^+$ reduced per min at 25°C and specific activity as units per mg protein. Enzyme protein was determined by the

Folin method[14] using bovine serum albumin as standard. Column fractions were monitored for protein by measurement of $A_{280\ nm}$.

Purification protocol

All operations were performed at 4°C.

(a) Preparation of cell-free extract. Cells (fresh weight 300–400 g) were harvested on a sintered glass funnel, washed successively with distilled water and 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA and suspended in one volume of the same buffer. Cells were disrupted by sonication and the homogenate centrifuged at 50,000 *g* for 30 min to remove particulate debris.

(b) $(NH_4)_2SO_4$ fractionation. The supernatant was stirred on an ice-bath and solid $(NH_4)_2SO_4$ added to give 50% $(NH_4)_2SO_4$ saturation. After 30 min the suspension was centrifuged at 50,000 *g* for 15 min. $(NH_4)_2SO_4$ was added to the supernatant to give 80% saturation and after 30 min the suspension was centrifuged for 15 min at 50,000 *g*. The pellet was dissolved in 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA–10% (v/v) glycerol and dialysed against the same buffer.

(c) DEAE-cellulose chromatography. The dialysed enzyme was applied to a column of DEAE-cellulose 52 (30 × 2.5 cm) equilibrated with 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA–10% (v/v) glycerol. Elution was performed by washing the column with 300 ml of the same buffer followed by a linear gradient of NaCl in buffer (0–0.3 *M* NaCl, total volume 500 ml). The flow-rate was 20–30 ml/h and fractions of *ca.* 7 ml were collected. Fractions containing 6-phosphogluconate dehydrogenase were combined and dialysed under vacuum against 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (v/v) glycerol.

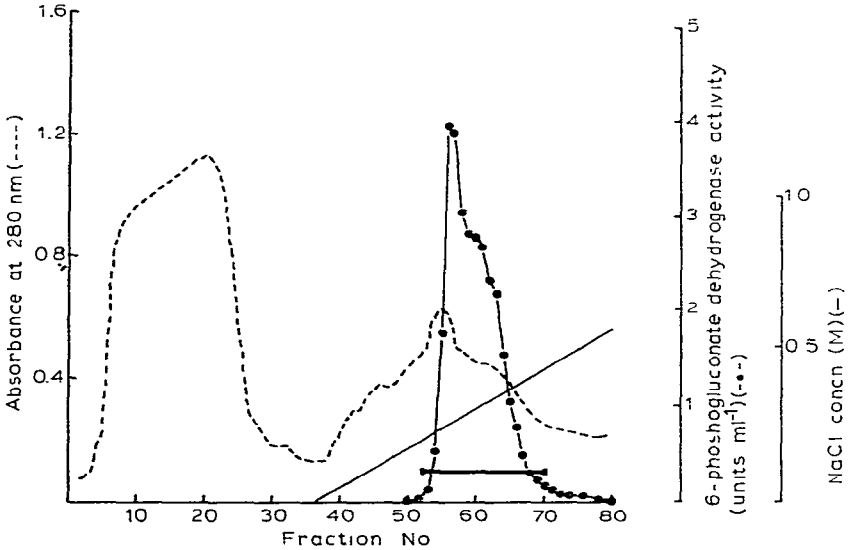

Fig. 1. Elution profile from Procion Red HE-3B–Sepharose 6B. Enzyme from step c (see Materials and methods) was applied at 10 ml/h to a column of Procion Red HE-3B–Sepharose 6B (25 × 1 cm) equilibrated with 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (v/v) glycerol, washed through with two or three column volumes of buffer, then developed with a salt gradient (0–1.0 *M* NaCl; total volume 200 ml). Fractions (4 ml) were assayed for 6-phosphogluconate dehydrogenase activity (●), absorbance at 280 nm (– – –) and conductivity (——). The horizontal bar represents those fractions which were pooled.

(d) Procion Red HE-3B–Sepharose chromatography. The dialysed and concentrated extract was applied to a 25 × 1 cm column of Procion Red HE-3B–Sepharose 6B equilibrated with 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (v/v) glycerol. Unretarded protein was removed by washing with two or three column volumes of buffer. Enzyme was eluted in a linear gradient of NaCl in buffer (0–1.0 *M* NaCl, total volume 200 ml). Approximately 4-ml fractions were collected at 10 ml/h. Fig. 1 shows a typical result. Fractions containing enzyme were combined and dialysed under vacuum as described above.

(e) 2′,5′-ADP–Sepharose chromatography. The dialysate was applied to a 15 × 1 cm column of 2′,5′-ADP–Sepharose 4B equilibrated with 0.05 *M* Tris–HCl (pH 7.5)–1 m*M* EDTA–10% (V/V) glycerol at 10 ml/h. The column was washed with three volumes of buffer and developed with a linear gradient of $NADP^+$ in buffer (0–1.0 m*M* $NADP^+$, total volume 200 ml). Fractions containing 6-phosphogluconate dehydrogenase were pooled and concentrated by vacuum dialysis against column buffer. Further concentration was achieved using a Minicon-A macrosolute concentrator.

Gel electrophoresis

Gel electrophoresis was performed in 7.5% gels and Tris–glycine buffer (pH 8.5)[15] at 2–3 mA per gel and 4°C for 1–2 h. Protein was detected by incubation in 0.25% (w/v) Coomassie brilliant blue in methanol–water–acetic acid (5:5:1, v/v/v) followed by destaining in several washes of methanol–water–acetic acid (3:35:2, v/v/v). Enzyme activity was located by incubating gels in the dark at 37°C in 0.1 *M* Tris–HCl (pH 7.5) containing 0.4 mg/ml phenazine methosulphate, 0.4 mg/ml nitro blue tetrazolium, 0.5 m*M* $NADP^+$, 1.4 m*M* 6-phosphogluconate and 2.5 m*M* $MgCl_2$. Bands corresponding to the position of the enzyme were fixed in water–ethanol–acetic acid–glycerol (2:1:1:1, v/v).

Sodium dodecyl sulphate polyacrylamide electrophoresis was as described[16]. Calibration was achieved by running in parallel a standard mixture of proteins in the mol. wt. range 14,300–71,500. Gels were stained for protein as described above.

RESULTS AND DISCUSSION

Effect of the matrix on ligand capacity

It has been shown that the choice of support material for a ligand may dramatically affect its capacity as an adsorbent in affinity chromatography[17]. We therefore first examined the suitability of several insoluble matrices as supports for Procion Red HE-3B and their influence on the interaction between bound dye and 6-phosphogluconate dehydrogenase (Table I). All gels were covalently coupled to Procion Red HE-3B under similar conditions (see Materials and methods) and most of the prepared adsorbents contained 2–3 μmole dye per ml swollen gel. The capacities of the adsorbents for 6-phosphogluconate dehydrogenase were determined by frontal analysis chromatography[18] as described in the legend of Table I. Attachment of the dye to a support matrix apparently significantly affected its performance as a ligand, since a 200-fold variation was measured between the capacities of the adsorbents tested for enzyme. The results confirm a previous study of interactions between Cibacron Blue 3G-A adsorbents and human serum albumin[17] in that cellulose, Ultrogel

TABLE I

CAPACITIES OF IMMOBILISED PROCION RED HE-3B DERIVATIVES FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE

The capacities of Procion Red HE-3B derivatives were determined by frontal analysis chromatography[18] at 4°C using micro-columns (diameter 6 mm) containing 1 ml adsorbent equilibrated in 0.05 *M* Tris–HCl (pH 8.0)–1 m*M* EDTA–10% glycerol. 6-Phosphogluconate dehydrogenase, partially purified from a cell-free extract from *Acer pseudoplatanus* L. by $(NH_4)_2SO_4$ fractionation (see Materials and methods), and dialysed against column buffer was applied at 2 ml/h until the enzyme activity and $A_{280\ nm}$ of the eluate and applied sample were identical. Unretarded protein was eluted with buffer, then bound enzyme was desorbed by a linear gradient of NaCl (0–1.0 *M* NaCl; total volume 20 ml). The frontal elution profile allowed calculation of the amount of enzyme retarded by the column after correction for binding to a column of unsubstituted support under identical conditions.

Support matrix	*Ligand concentration (μmole/ml swollen gel)*	*Capacity for 6-phosphogluconate dehydrogenase (units of enzyme activity bound/μmole immobilised Procion Red HE-3B)*
Cellulose	2.0	0.1
Ultrogel AcA-54	0.6	0.3
Unisphere P1000	2.9	0.4
Sephadex G-200	2.0	23.1
Sephacryl S-200	1.8	8.5
Sepharose 6B	2.0	22.1
Sepharose CL-6B	2.8	5.8
Indubiose Perles A6	0.2	26.0
Matrex Gel Red A	2.8	22.1

and Unisphere apparently restrict access of protein to the ligand. The high capacities of the substituted dextrans and agaroses suggest that these matrices present the ligand in a more accessible manner. Cross-linked dextran (Sephadex G-200) and the un-cross-linked 6% agaroses (Sepharose 6B and Indubiose Perles A6) had the highest molar capacities for 6-phosphogluconate dehydrogenase. Inclusion of polyacrylamide bridges (Sephacryl S-200) or cross-linkage of the agarose (Sepharose CL-6B) decreased ligand effectiveness relative to the corresponding un-cross-linked matrices. In contrast, the molar capacity of Matrex Gel Red A (Amicon; Procion Red HE-3B attached to a cross-linked 4% agarose) was similar to that of our Sepharose 6B adsorbent. Consideration of respective ligand concentrations, capacities for enzyme and flow properties of the adsorbents indicated that agarose (Sepharose 6B) or cross-linked agarose (Matrex Gel Red A) were the preferred supports for Procion Red HE-3B in the purification of 6-phosphogluconate dehydrogenase from *Acer pseudoplatanus* L. The inferior flow properties of Sephadex G-200 make this matrix unsuitable for large scale preparative work. Nevertheless, the ease with which triazine dyes can be coupled to cross-linked dextrans[19] apparently without the damage to the bead structure which is associated with cyanogen bromide activation[20], allows the possibility of their application in molecular sieving combined with affinity chromatography ("affinity gel filtration"[21]) using the dye either directly as a ligand or indirectly as a spacer for other affinity ligands[22].

Chromatography of 6-phosphogluconate dehydrogenase on Procion Red HE-3B–Sepharose 6B.

Cibacron Blue 3G-A is widely used as a general group-specific ligand in the purification of nucleotide-dependent enzymes[5]. Displacement of adsorbed enzyme from immobilised Cibacron Blue is frequently achieved by "biospecific" elution with appropriate coenzyme or substrate at significantly lower ionic strenghts than those at which salt solutions are effective. This has been taken as an indication that the immobilised dye interacts directly with the nucleotide-binding site of proteins[7,22]. While more recent information shows that "biospecific" elution need not necessarily imply any such specificity in the adsorptive mechanism (*cf.*, ref. 6). X-ray crystallographic studies have demonstrated marked similarities between the binding of NAD and Cibacron Blue 3G-A to liver alcohol dehydrogenase[23].

The demonstration that Procion Red HE-3B also retards dehydrogenases[8], acts as an inhibitor in free solution[19] and exhibits some selectivity toward NADP-dependent enzymes[8], indicated that elution of 6-phosphogluconate dehydrogenase by coenzyme might be successful. However, in this instance we were unable to displace this enzyme from Procion Red HE-3B–Sepharose 6B using $NADP^+$ (0–10 m*M*). Furthermore, 6-phosphogluconate, citrate and pyrophosphate (the latter two being competitive inhibitors with respect to both coenzyme and substrate for the mammalian enzyme[24]) could not elute the *Acer pseudoplatanus* L. enzyme at ionic strengths below that at which sodium chloride is effective (*cf.*, ref. 25). The failure of substrate (as opposed to coenzyme) and inhibitors to elute the enzyme confirms recent observations of the chromatographic behaviour of *Bacillus stearthermophilus* 6-phosphogluconate dehydrogenase[19] and bovine liver NADPH-dependent 5′,10′-methylene tetrahydrofolate dehydrogenase[10] on immobilised Procion Red HE-3B. Rather than supporting the idea that the ligand Procion Red HE-3B binds at the dinucleotide fold for the above enzymes, these data seem to us to suggest non-involvement of the substrate binding site. Since presaturation with nucleotide ($NADP^+$) dramatically alters the subsequent chromatographic behaviour of at least one of the above enzymes[19], we might conclude that the dinucleotide fold is occupied by the dye when the enzyme binds to these columns. However, the very effective salt-mediated desorption of these columns (reminiscent of the behaviour of immobilised nucleotides) seems to be inconsistent with this hypothesis because very few kinetically determined binding constants (*e.g.*, k_M for $NADP^+$) are salt-dependent. The view expressed by Edwards and Woody[26], that these dyes do not assume a single unique conformation on different enzymes possessing the dinucleotide fold, seems to be consistent with our data.

The applications of decreasing (*cf.*, ref. 19) temperature, aprotic solvents and dyes as desorption methods have not been widely studied. A solution of Procion Red HE-3B at 4 μM eluted 6-phosphogluconate dehydrogenase, but subsequent difficulty in dissociating the dye–protein complex render this effect of limited preparative use. In the present instance we found desorption was achieved most effectively by an increase in ionic strength. Recoveries of 80–100 % were routinely obtained using a linear 0–1.0 *M* NaCl gradient. At the purely practical level, data which were collected from the behaviour of at least ten different enzymes on 60 different immobilised dyes[27], suggest that elution methods might be selected according to the following order:

$$\text{salt} \approx \text{cofactor} \approx \text{pH} \approx \text{inhibitors} > \text{substrate}$$

TABLE II
PURIFICATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *ACER PSEUDOPLATANUS* L.

Approximately 400 g (wet weight) of cells were used. Data refer to a single experiment. Similar results were obtained in five separate experiments.

Step	*Total volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Purification (n-fold)*	*Yield (%)*
Crude extract	685	1192	253	0.21	1.0	100
$(NH_4)_2SO_4$ fractionation	43	206	211	1.02	4.9	83
DEAE-cellulose	77	66	162	2.45	11.7	64
Procion Red–Sepharose	44	16.1	131	8.14	38.8	52
2′,5′-ADP–Sepharose	9.5	1.32	105	79.5	379	41

Purification of 6-phosphogluconate dehydrogenase from Acer pseudoplatanus L.

The result of a large-scale purification of the enzyme, which included chromatography on Procion Red HE-3B–Sepharose 6B, is shown in Table II. Several contaminating proteins co-eluted with 6-phosphogluconate dehydrogenase from the latter adsorbent, but could be removed by subsequent chromatography on 2′,5′-ADP–Sepharose 4B. Omission on the Procion Red step did not yield a pure product.

A final specific activity of 80 units per mg protein (pH 7.5, 25°C) was achieved with 40–50% recovery, representing a 380-fold purification relative to the crude cell-free homogenate. Gel electrophoresis of the purified enzyme indicated that the preparation was free of detectable non-enzymic protein and revealed the existence of two

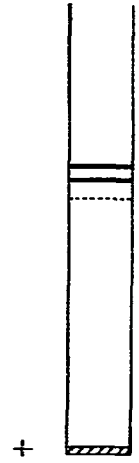

Fig. 2. Distribution of 6-phosphogluconate dehydrogenase after polyacrylamide gel electrophoresis. Purified 6-phosphogluconate dehydrogenase was applied to 7.5% polyacrylamide gels at pH 8.5 and subjected to electrophoresis at 2–3 mA per gel at 4°C for 1–2 h. The figure shows the pattern obtained by subsequent staining for either protein or 6-phosphogluconate dehydrogenase activity.

major species of 6-phosphogluconate dehydrogenase. These were estimated to occur in roughly equal amounts, as determined using either protein or enzyme-specific staining, with a minor component of higher mobility comprising less than 5% of the total protein (Fig. 2). In the presence of sodium dodecyl sulphate, two proteins with molecular weights of 45,000 and 49,000 were found in equal amounts. The existence in higher plants of isoenzymes of several reductive pentose phosphate enzymes is well documented [28–30]. Studies with both leaf[31] and non-photosynthetic tissues such as root [32,33] and endosperm[34] have shown that separate cytosolic and plastid forms of 6-phosphogluconate dehydrogenase occur. While further characterisation of the species identified by electrophoresis in the present work has not yet been performed, it is likely that the major proteins correspond to cytosolic and plastid forms of 6-phosphogluconate dehydrogenase in *Acer pseudoplatanus* L. The identity of the minor component also remains to be determined.

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