Journal of Chromatography, 398 (1987) 387–390
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 548

Note

Affinity chromatography of pig heart malate dehydrogenase on the Remazol Brilliant Blue R derivative of hydroxyethyl methacrylate gel*

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(Received March 10th, 1987)

Malate dehydrogenase (MDH; L-malate: NAD-oxidoreductase, E.C. 1.1.1.37) occurs in two forms (isoenzymes) identified as mitochondrial (m-MDH) and cytoplasmic (c-MDH) according to their localization in eukaryotic cells[1].

The purification of pig or beef heart MDH usually involves ammonium sulphate fractionation and several chromatographic steps[2–5]. For the separation of m- and c-MDH which differ in p*I* values (m-MDH is basic, c-MDH acidic), ion-exchange chromatography proved to be satisfactory. The purification of MDH was also achieved by dye-affinity chromatography on Cibacron Blue F3GA and Remazol Brilliant Blue R derivatives of polysaccharide gels (agarose, bead cellulose, dextran gel)[6–9].

This paper describes a simple purification of pig heart m-MDH by affinity chromatography on Spheron Blue R 1000, a Remazol Brilliant Blue R derivative of hydroxyethyl methacrylate gel (Spheron).

EXPERIMENTAL

Materials

Pig heart acetone powder was obtained from SPPCHO (Hlohovec, Czechoslovakia) and stored desiccated at 4°C. NADH and NAD were obtained from Boehringer (Mannheim, F.R.G.), oxalacetic acid from Reanal (Budapest, Hungary) and Remazol Brilliant Blue R from Hoechst (Frankfurt a.M., F.R.G.). Spheron 1000 (particle size 40–63 μm) and the other reagents (p.a. grade) were obtained from Lachema (Brno, Czechoslovakia).

Preparation of the Remazol Brilliant Blue R derivative of Spheron 1000

Spheron 1000 (10 g) was suspended in 0.1 *M* sodium bicarbonate and Remazol Brilliant Blue R (2.5 g) was added. The reaction mixture was heated to 50°C and kept for 2 h at this temperature under continuous stirring. The product was filtered off and washed with hot water (1 l), cold water (1 l), 2 *M* hydrochloric acid (200 ml) and water (*ca.* 2 l, to neutral pH). Then the gel was poured into a column (250 mm

* Presented in part as a poster communication at the *6th International Symposium on Bioaffinity Chromatography and Related Techniques, Prague, September 1–6, 1985.*

0021-9673/87/$03.50

× 15 mm) and washed with ethanol until the washings were colourless. Prior to drying under vacuum the product was washed with acetone (300 ml). The concentration of immobilized dye was *ca.* 0.2 mmol/g dry weight gel. By using various reactant ratios, products differing in the amount of dye bound were prepared.

Enzyme preparation

All procedures were carried out at 4–8°C. Pig heart acetone powder (50 g) was extracted with 500 ml of 0. *M* potassium phosphate buffer (pH 7.4) and the insoluble debris was removed by centrifugation at 5000 *g* for 10 min. The supernatant was brought to 50% saturation with solid ammonium sulphate and the precipitate was removed by centrifugation at 5000 *g* for 15 min. Most of the MDH activity was salted out from the supernatant at 80% saturation with ammonium sulphate. The resulting suspension was centrifuged at 5000 *g* for 15 min, the supernatant was discarded and the pellet was dissolved in a small amount of 0.01 *M* potassium phosphate buffer (pH 7.1). The solution was dialyzed against the same buffer. Prior to chromatography, a small amount of insoluble precipitate was removed by centrifugation at 5000 *g* for 10 min. An aliquot (10 ml) of the solution was applied to a column (140 mm × 14 mm) packed with Spheron Blue R 1000 and equilibrated with 0.01 *M* potassium phosphate buffer (pH 7.1). The column was then washed with 50 ml of the same buffer at a flow-rate of 70 ml/h. Bound m-MDH was specifically eluted with a solution of 1 m*M* NAD, 5 m*M* sodium sulphite in 0.01 *M* potassium phosphate buffer (pH 7.1) containing 0.5 *M* sodium chloride. Fractions of 6 ml were collected and assayed for MDH activity and protein concentration.

Enzyme analysis

The activity of malate dehydrogenase was determined spectrophotometrically in the presence of NADH and oxalacetate as substrates at 25°C[10]. The activity of aspartate aminotransferase (AST) was assayed using SeraPak:AST (Miles, Slough, U.K.)[11]. The protein concentration was calculated from the absorbance at 280 nm, while in specifically eluted fractions the protein content was determined by the Lowry method[12] as the absorbance at 280 nm was due to NAD/NADH. Assays were performed with a Perkin-Elmer 550 S recording spectrophotometer equipped with a thermostatically controlled sample compartment maintained at 25°C. Chromatofocusing was carried out on a Mono P column (200 mm × 5 mm) attached to a fast protein liquid chromatography (FPLC) system consisting of two pumps (P-500) and a gradient programmer (GP-250) from Pharmacia (Uppsala, Sweden). As starting and eluting buffers, 0.025 *M* Tris (pH 9.3) and Polybuffer 96 (90%)–Polybuffer 74 (10%) diluted 1:10 (pH 4.7) were used at a flow-rate of 60 ml/h.

RESULTS AND DISCUSSION

Spheron Blue R 1000, a Remazol Brilliant Blue R derivative of a macroporous hydroxyethyl methacrylate gel, was prepared by coupling the dye directly to hydroxyl groups of Spheron in an aqueous alkaline medium at 50°C. For further experiments, Spheron Blue R 1000 containing *ca.* 0.2 mmol of immobilized dye per g gel was used. The capacity of the sorbent was *ca.* 10 mg of pure m-MDH per g dry weight gel. Spheron Blue R 1000 is reusable, no decrease in binding capacity being observed after ten chromatographic cycles.

TABLE I

PURIFICATION OF THE MITOCHONDRIAL MALATE DEHYDROGENASE FROM PIG HEART

For conditions see Experimental.

	Protein (mg)	*Total activity (U)*	*Specific activity (U/mg)*	*Purification*	*Total yield (%)*
Crude supernatant	587	28 754	49	–	(100)
Ammonium sulphate fractionation (50–80% saturation)	280	23 290	83	1.7	81
Affinity chromatography (Spheron Blue R 1000), NAD eluate	24	13 802	575	11.7	48*

* MDH in the supernatant from ammonium sulphate fractionation consisted of about 70% mitochondrial isoenzyme and 30% c-MDH. The NAD eluate from the Spheron Blue R 1000 column contained only m-MDH.

The purification of m-MDH involves two main steps: an ammonium sulphate fractionation and a dye-affinity chromatography on Spheron Blue R 1000. The first step resulted in a nearly two-fold purification of MDH and a considerable reduction of the sample volume (see Table I). Dye-affinity chromatography on Spheron Blue R 1000 proved to be a convenient method for the separation of MDH isoenzymes and for purification of m-MDH. Unlike affinity sorbents with bound Cibacron Blue F3GA, Spheron Blue R 1000 exhibited a rather high affinity for pig heart mitochondrial MDH but did not bind either c-MDH or contaminating enzymes (AST, lactate dehydrogenase LDH) under the described conditions. Bound m-MDH was specifically eluted with 1 m*M* NAD, 5 m*M* sodium sulphite in 0.01 *M* potassium phosphate buffer (pH 7.1) containing 0.5 *M* sodium chloride[13]. An approximately seven-fold increase in specific activity of m-MDH was achieved with a yield of 79–87% of

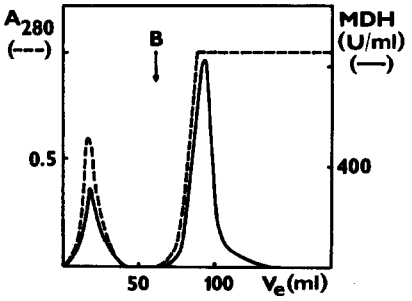

Fig. 1. Affinity chromatography of malate dehydrogenase. A solution of crude MDH (10 ml, total protein 275 mg, MDH activity 22750 U) in 0.01 *M* potassium phosphate buffer (pH 7.1) was applied to the Spheron Blue R 1000 column (140 mm × 14 mm). Elution was performed at 6°C and at a flow-rate of 70 ml/h. Contaminating proteins and c-MDH were eluted with 0.01 *M* potassium phosphate buffer (pH 7.1) (buffer A), m-MDH with the same buffer containing 1 mM NAD, 5 m*M* sodium sulphite and 0.5 *M* sodium chloride (buffer B).

mitochondrial isoenzyme. The application of NAD–sodium sulphite resulted in a complete elution of m-MDH (Table I, Fig. 1). The elution with 1 m*M* NADH, 0.5 *M* sodium chloride in 0.01 *M* potassium phosphate buffer (pH 7.1) provided practically the same results, but NAD–sodium sulphite was preferred because of the considerably lower cost of NAD, which would be important in a large-scale purification. The use of a buffer with high ionic strength only (0.5 *M* sodium chloride in 0.01 *M* potassium phosphate, pH 7.1) resulted in a slow and incomplete elution of m-MDH, the recovery being less than 65%.

The purity of the eluted enzyme was tested by chromatofocusing on a Mono P HR 5/20 column. Approximate values of the isoelectric points of m-MDH (8.4) and c-MDH (5.0) were estimated. The purified m-MDH was practically free of main contaminating activities; the AST/MDH activity ratio was $1:10^4$ and the LDH/MDH ratio was $1:10^3$. Thus, the m-MDH preparation obtained in this one-step dye-affinity chromatography procedure is sufficiently pure to be used in an assay for AST activity.

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